Journal of Immunology Research

New Insights for Immune-Based Diagnosis and Therapy for Infectious Diseases

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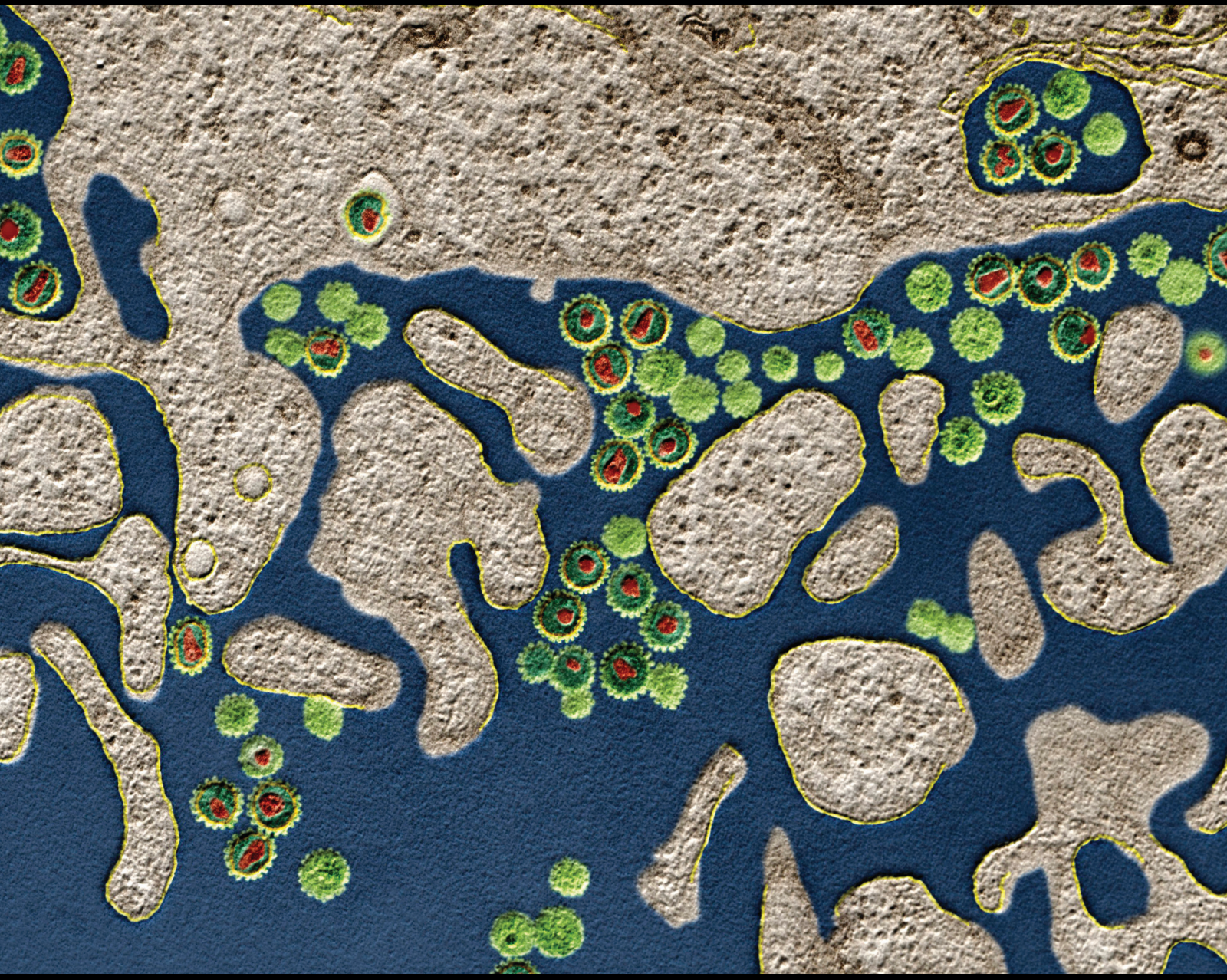

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Editorial Board

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Editorial

New Insights for Immune-Based Diagnosis and Therapy for Infectious Diseases

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The role of the immune system in infections has been extensively exploited in order to develop vaccinal approaches as well as diagnostic and therapeutic tools. For example, in the diagnostic field, research investigating specific humoral immune responses has long been used to develop novel diagnostic tools. In fact, seropositivity is considered an important key factor for the determination of an occurring infection. Recently, in addition to the classical investigation on the presence or absence of antibodies directed against specific antigens, the titer of specific antibodies, defined as index, or the composition of antibody responses by immune-based assays, is gaining importance as a prognostic marker for certain infectious diseases (e.g., during the course of JC virus infection) or as a noninvasive means to stratify patients according to their disease status after infection with *H. pylori* as described by L. Formichella et al. in this open special issue. In addition, it is not just specific antibodies that are important for diagnosis, but the evaluation of T-cell immunity could also play a central role for routine laboratory diagnostics and for the management of the patient, as described by G. Freer et al., in the course of human cytomegalovirus (HCMV) infection. However, other proteins not associated with adaptive immune response can be used as indicators of infection, as an example, delta procalcitonin in critically ill patients, as described by D. Trásy et al.

On the other hand, in the immunotherapeutic field, the idea of engineering the immune system has always been an attractive concept in order to improve and exploit the specificity and functional characteristics of immune cells and molecules, with a primary focus on antibodies. In fact, starting from the basic concept that the immune response is the key element to resolve an infection, a therapeutic and a prophylactic strategy for infectious diseases is usually centered on immune-based approaches. In particular, prophylactic strategies are principally focused on the stimulation of a specific immune response against the pathogen, that is, the active immunization. Alternatively, immunotherapeutic strategies are based on the concept of a passive immunization. In this case, immunoglobulins obtained from sera of immune individuals or by the generation of antigen-specific monoclonal antibodies (mAbs) are administered to protect a susceptible or infected host. The concept of mAb administration to resolve an infection was originally proposed by Paul Ehrlich when he was referring to mAbs as the "magic bullets." In this regard, several anti-infective mAbs are approaching the clinics in the next few years and many more are currently under development. Furthermore, Y. Xu et al. describe the protection of the mother and her baby during passive immunoprophylaxis using animal models of antibody transport.

Moreover, antibodies and in particular mAbs, thanks to their high specificity, can be very useful in the early diagnosis

of infectious diseases. As an example, G. A. Kirchenbaum and T. M. Ross describe the generation of the first mAbs recognizing ferret immunoglobulins. It has been established that the domestic ferret is an ideal animal model to study several pathogens that cause infections in humans, especially respiratory diseases caused by viruses such as the respiratory syncytial virus (RSV) and influenza virus. The availability of such specific reagents is thus pivotal to study the host-pathogen interaction as well as the immune response to these infections.

In the last decades, increasingly sophisticated techniques have made it possible to analyze the antibody repertoire in depth, most notably in the context of certain infections for which it is important to understand which antibodies confer the key determinants for protection, such as in the case of human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus infections. As the knowledge of the field continues to evolve, it is becoming evident that not only the binding properties of antibodies are important for understanding the signatures of an effective immune response but also the extraneutralizing properties of antibodies, such as the Fc-effector functions, which go under the name of system serology.

In this open special issue, all these aspects are covered by seven review articles and nine research papers discussing how we can exploit and utilize the immune system to understand new host-pathogen relationships as well as for the development of novel prophylactic, therapeutic, and diagnostic tools. We hope that the readers of this open special issue will appreciate the interesting findings and the reviewed concepts of the field discussed in the papers published in it.

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Research Article

Validation of a Novel Immunoline Assay for Patient Stratification according to Virulence of the Infecting *Helicobacter pylori* Strain and Eradication Status

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Helicobacter pylori infection shows a worldwide prevalence of around 50%. However, only a minority of infected individuals develop clinical symptoms or diseases. The presence of *H. pylori* virulence factors, such as CagA and VacA, has been associated with disease development, but assessment of virulence factor presence requires gastric biopsies. Here, we evaluate the *H. pylori recom*Line test for risk stratification of infected patients by comparing the test score and immune recognition of type I or type II strains defined by the virulence factors CagA, VacA, GroEL, UreA, HcpC, and gGT with patient's disease status according to histology. Moreover, the immune responses of eradicated individuals from two different populations were analysed. Their immune response frequencies and intensities against all antigens except CagA declined below the detection limit. CagA was particularly long lasting in both independent populations. An isolated CagA band often represents past eradication with a likelihood of 88.7%. In addition, a high *recom*Line score was significantly associated with high-grade gastritis, atrophy, intestinal metaplasia, and gastric cancer. Thus, the *recom*Line is a sensitive and specific noninvasive test for detecting serum responses against *H. pylori* in actively infected and eradicated individuals. Moreover, it allows stratifying patients according to their disease state.

1. Introduction

H. pylori is a common and widespread bacterial pathogen that infects more than half of the world's population [1]. *H. pylori* colonizes the human stomach and always leads to the development of an active gastritis [2], which in the majority of cases remains asymptomatic. Chronic gastritis may, however, lead to the development of several gastrointestinal diseases such as chronic atrophic gastritis [3], duodenal and gastric ulcers [4], in 1-2% to the

development of gastric cancer [5, 6], and lymphoma [7, 8]. Especially, patients with the so-called corpus dominant severe gastritis are at risk for gastric cancer, as severe gastric inflammation can lead to atrophy, metaplasia, dysplasia, and finally, gastric cancer [9]. More than 90% of all gastric cancer cases are associated with a chronic *H. pylori* infection [10] and lead to more than 700,000 stomach cancer-related deaths per year worldwide [11]. Thus, gastric cancer remains the third leading cause of cancer-related mortality [12]. Studies have shown that eradication therapy of *H. pylori* can prevent gastric cancer development [13, 14]. Due to the high number of infected individuals, it is necessary to identify patients at increased risk for gastroduodenal disease in order to subject them to preventive eradication therapy. A number of methods for the detection of *H. pylori* are currently available: histological analysis, microbial culture, and urease breath test are most commonly used. However, the first two diagnostic methods require upper gastrointestinal endoscopy in order to obtain biopsy specimens for testing. None of the aforementioned methods is able to predict the outcome of an *H. pylori* infection in terms of malignant or benign outcome. The line assay system (Helicobacter *recom*Line) analyses immunoglobulin G (IgG) antibody responses to six *H. pylori* antigens (CagA, VacA, GroEL, UreA, HcpC, and gGT), some of which are already known to be linked to a higher risk of disease, as for instance the antigens CagA, VacA, and GroEL [15–17]. The *recom*Line differentiates the more virulent type I *H. pylori* immune response (CagA and/or VacA positive) and the less pathogenic type II immune response (CagA/VacA negative). As the severity of inflammation, and thus immune response, correlates with the risk for gastric cancer [9], the *recom*Line may have predictive properties, which we aimed at evaluating in the present study. We validated the performance of the *recom*Line test compared to histology as a gold standard, with particular focus on the correlation between the *recom*Line test results and chronicity/activity of inflammation as well as most severe lesions according to histology.

As eradication of *H. pylori* has become quite common, tests were developed to confirm treatment success. The Maastricht guidelines consider the urea breath test adequate to confirm successful treatment four weeks after eradication therapy [18]. Antibody responses towards antigens usually sustain for a long period after eradication therapy, as previously shown [19–22]. These studies suggest that serology was not applicable for eradication control. However, while some immune responses to certain antigens persist for decades (especially CagA), antibody titres against other antigens decrease within a shorter period, also depending on the Ig-class tested [23]. This phenomenon could also be utilized as readout to confirm treatment success by analysing the decline of antibody responses, as shown before [24]. Wang et al. conclude from their study that it would be the reasonable and even perhaps preferred method of monitoring *H. pylori* infections [25]. In order to get a better insight into posttreatment IgG immune responses, we analysed sera of a subgroup of patients that had undergone documented eradication therapy by applying the *recom*Line assay.

2. Methods

2.1. Study Populations and Histology. The analysis was based on a study population recruited between October 2010 and February 2012 by two gastroenterological practitioners in Munich and Bayreuth (Germany). Patients receiving active immunosuppressive therapy, showing coagulation defects, or suffering from malignant diseases were excluded from the study. Serum samples and gastric biopsies of the antrum and corpus were obtained from patients with upper abdominal complaints who underwent gastroscopy after informed consent. The serum samples were stored at −20°C until testing for IgG antibodies against *H. pylori*. For all participants included, age, gender, medical history, and histology status were recorded. *H. pylori* status was defined via histology performed at the Institute of Pathology, Klinikum Bayreuth, or the Institute of Pathology at the Technische Universität München. Therefore, biopsies were fixed in 4% neutral buffered formalin, dehydrated in a series of increasing alcohols and xylene, embedded in paraffin, serially sectioned, deparaffinized, and stained. All biopsies were stained routinely with H&E and immunohistochemical detection of for *H. pylori* at the Institute of Pathology, Klinikum Bayreuth, using Roche monoclonal antibody clone SP48 rabbit-anti-human 790-1014 on Ventana Benchmark Ultra, Strassbourg, France. Pathological evaluation of tissue samples was done according to the updated Sydney System [26]. The chronicity of inflammation was scored by the mucosal infiltration with lymphocytes and plasma cells. The activity of inflammation was scored based on the number of neutrophilic granulocytes within the tunica propria. Both parameters are indicated as mild, moderate, and severe. Apart from chronicity and activity of inflammation, other pathological findings such as atrophy, metaplasia, dysplasia, ulcer, and carcinoma were recorded. For statistical analysis, these were grouped as most severe lesions. No gastric lymphomas were detected in the present study cohort.

Eradicated cases were defined either through histology (Ex-*H. pylori* gastritis) or by documentation of the attending gastroenterologist. The time since eradication was either defined by a known date of eradication (submitted by the gastroenterologist) or determined according to the last date of *H. pylori* positivity in histology, which was followed by eradication therapy and resulted in an Ex-*H. pylori* gastritis in histology or a negative histological result, confirming the success of eradication. In addition, a Dutch cohort was tested prospectively with the *recom*Line not knowing current *H. pylori* status. The study was approved by the ethics committee of the Technische Universität München (May 20, 2009).

2.2. The recomLine Serologic Assay System. The *recom*Line is based on six recombinant antigens: CagA, VacA, GroEL, UreA, HcpC, and gGT, which are immobilized on a nitrocellulose. On every test strip, a reaction control, antibody control, and cutoff control band are included. For test quantification, the scanner OpticPro S28 (Plustek, Korea) and *recom*Scan software (Mikrogen, Germany) were used according to the manufacturer's instructions. The developed test strips were digitalized by scanning, and the band patterns

were assessed via a defined algorithm. The band intensities were determined in more than 750 grey levels and are given in arbitrary units (AU). The recomLine antigens CagA, VacA, and GroEL were scored with two points each, and the other antigens with one point each, adding up to a maximum score of nine points. Results with one point were categorized as borderline and two or more points were considered positive. Representative images of five *recom*Line tests are shown in Supplementary Figure 1 available online at https://doi.org/10.1155/2017/8394593. Detailed information and description on the development of the *recom*Line has been published earlier [27].

2.3. Statistical Analysis. Statistical analysis was performed as described before [27]. The performance of the *recom*Line was validated against histological assessment as gold standard calculating the sensitivity and specificity. The immune response frequency was calculated comparing the ratio between patients positive for a certain antigen and the total number of individuals tested positive. The test results for *H. pylori* positive and negative patients analysed with the *recom*Line were validated against histology results. Differences in the study population were assessed using Pearson's χ^2 test (c^2) or the nonparametric Kruskal-Wallis test (H). Mean single values were compared using the two-tailed t-test with 95% CIs (t). Significances in mean band intensity were calculated using the one-way ANOVA (F). The p values were depicted in the figure legends as follows: letters indicating the test used, number/numbers in parentheses indicating the degree(s) of freedom, and followed by the defined statistics value and the significance level. Differences were rated as significant at $p < 0.05$.

3. Results

3.1. Patient Population and Performance of recomLine Assay. The German cohort of 1291 patients is characterized as follows: 447 (34.6%) patients were *H. pylori* negative and 409 (31.7%) patients were *H. pylori* positive according to histology. The negative population was included to determine the basic test performance values (sensitivity and specificity) [27]. As this manuscript focuses on the serological response of the *H. pylori* positive and eradicated patients, more detailed information on the *H. pylori* negative patients are not presented. 435 (33.7%) patients had previously undergone *H. pylori* eradication treatment. The cumulative (past and present) prevalence of *H. pylori* in the study population was 65.4% and increased with age. *H. pylori*-eradicated patients were significantly ($p < 0.01$) older than *H. pylori* negative patients. The mean age was 53.6 ± 16.2 ranging from 18 to 89 years. In total, more female patients (708, 54.8%) were included in the study. Detailed information on the study populations analysed can be found in the Supplementary Table 1. The performance values previously determined for the *recom*Line assay [27] were confirmed in this extended population, as shown in Supplementary Table 2.

3.2. Eradicated Patients Frequently Show Only a CagA Antibody Response. In order to assess (i) how serologic responses to individual antigens changes over time and (ii) if the serological response to individual antigens might have the capacity to differentiate *H. pylori* infection pre- or posttreatment, we investigated the immune response patterns of a subgroup of individuals that had received antibiotic treatment in the past.

Of all the individuals categorised as eradicated, detailed information on eradication was known for 64.6% (281/435). In total, 74.7% (210/281) of individuals with documented eradication were *recom*Line positive. Moreover, a negative correlation between the time since eradication and a positive test result was observed (Figure 1(a)). The overall *recom*Line positivity rates decreased from 79.0% at 0–5 years after eradication therapy to 61.8% 12–17 years after therapy. This negative correlation could also be observed analysing the mean *recom*Line score (Figure 1(b)).

Besides the decrease of the total number of positive cases, we also analysed the decrease of serologic response on a single antigen level. Figure 1(c) shows the relationship between the time since eradication therapy and the serological response to the antigens on the *recom*Line compared to individuals with an active ongoing *H. pylori* infection. Noteworthy, the antibody response against all antigens decreased over time except for the CagA response that was particularly long lasting (Supplementary Figure 2). This can be explained by the mean band intensities as shown in Figure 1(d). A decrease in mean serological signal was shown for all antigens tested. However, a significant decrease was demonstrated for CagA ($p < 0.01$), VacA ($p < 0.01$), GroEL ($p < 0.01$), HcpC ($p < 0.01$), and gGT ($p < 0.01$). The mean CagA seroresponse intensity decreased by 43.3% (117/271 AU) over time but compared to the other antigens still showed a significantly higher ($p < 0.01$) mean band intensity above cutoff in the group of 12–17 years after eradication as indicated in Figure 1(d). The signals for the other antigens decrease to or below detection limit six years after eradication, which explains the high isolated CagA positivity rates (only positive for CagA) for this group. Concerning the relative positivity of CagA, we observed that CagA was significantly ($p < 0.01$) more often positive in eradicated patients (86.3%) than in *H. pylori* positive patients with 69.2% positivity (Supplementary Figure 2). Moreover, 43.3% (91/210) of all *recom*Line positive cases in the eradicated cohort showed an isolated CagA seroresponse, compared to 5.5% (22/400) in the group with an ongoing *H. pylori* infection. Comparing these two groups, the probability that a patient showing an isolated CagA band had been treated in the past is 88.7% $((0.433/(0.433 + 0.055)) * 100)$. In order to substantiate this finding, we analysed a Dutch cohort in which the *H. pylori* status was blinded. We initially observed that in this cohort, the mean *recom*Line score was significantly ($t(595) = 13.0$, $p < 0.001$) lower (2.5 ± 2.3) compared to the score of positive cases in the German cohort (4.8 ± 1.9). Upon unblinding, it was confirmed that 84.6% (193/228) of the individuals tested turned out to be *H. pylor*i negative by histology. Analysing the individuals with documented eradication ($n = 65$) in more detail, the antigen frequency in this population showed the same pattern as observed in the German population, defined by

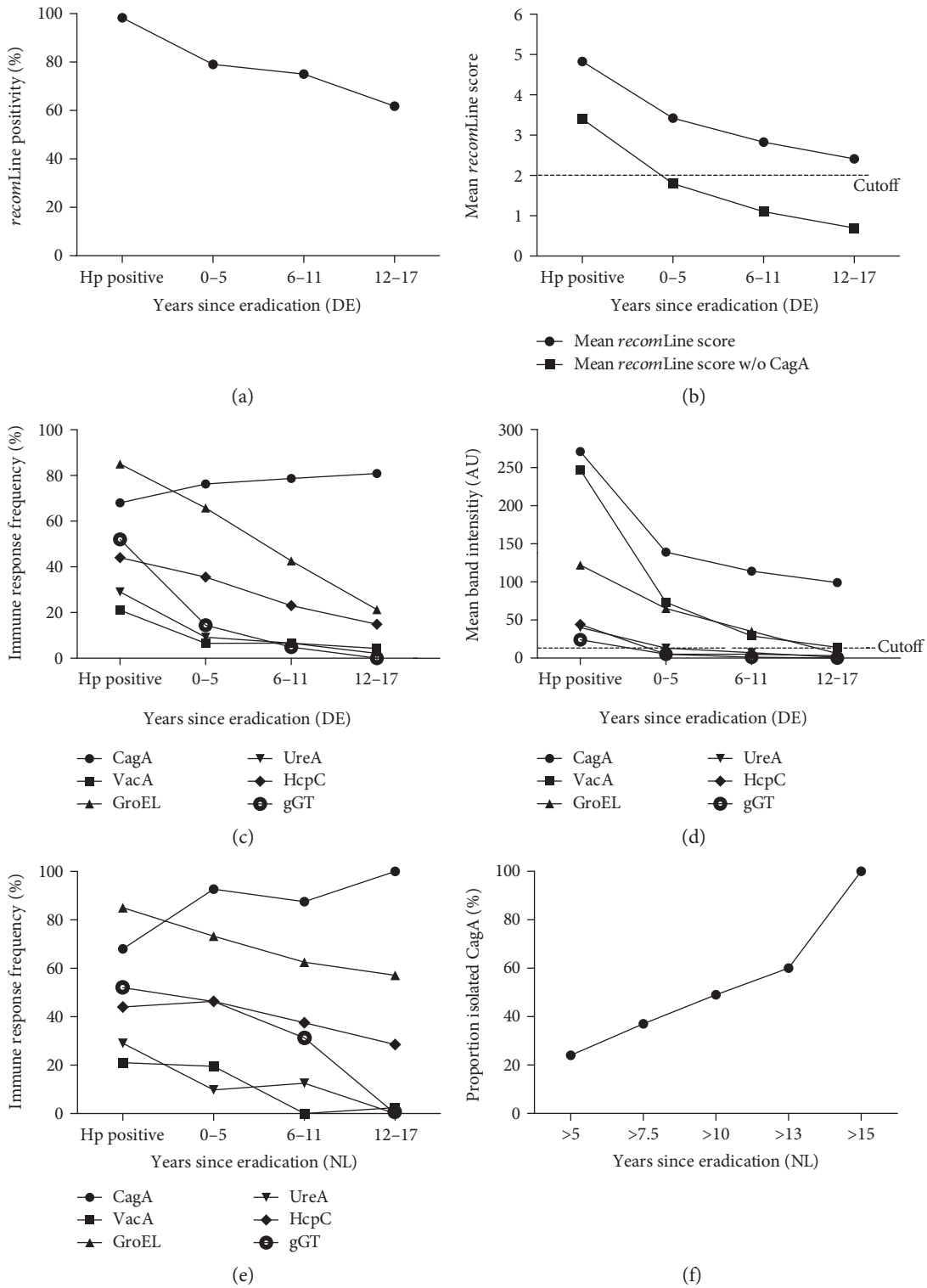

Figure 1: *recom*Line test results from patients without and with eradication therapy. (a) *recom*Line positivity, (b) mean *recom*Line score, (c) immune response frequency, and (d) mean immune response intensity of individuals with an active ongoing *H. pylori* infection ($n_{\text{Hp positive}} = 402$) compared to individuals having received antibiotic treatment in the past ($n_{\text{years since eradication}} = 210$), indicated as years since eradication and grouped (0–5, 6–11, and 12–17 years) analysing a German cohort (DE). Moreover, the relationship between (e) immune response frequency to individual antigens (●CagA, ■VacA, ▲GroEL, ▼UreA, ♦HcpC, and ○gGT) and (f) percentage of isolated CagA immune response and the time since eradication therapy analysing a Dutch cohort (NL) ($n = 65$). While the immune response for all antigens tested declines after eradication, the response towards CagA antibodies persists over a long period, resulting in an isolated CagA positivity, which remains above cutoff and is significantly increased ($p < 0.01$) in the 12–17 years group compared to all other antigens.

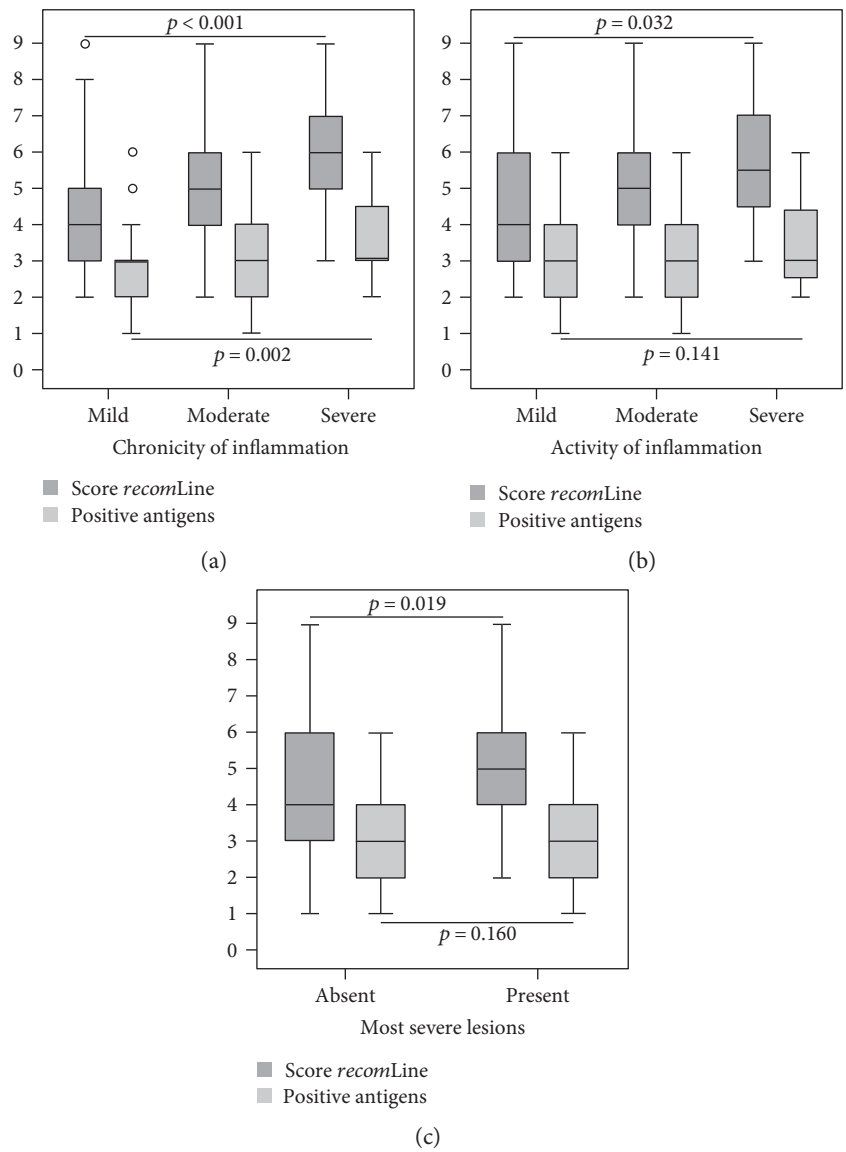

Figure 2: Box plot diagrams showing the association between *recom*Line test results (dark grey: score *recom*Line/light grey: number of positive antigens) and histological findings in *H. pylori* positive cases. (a) Correlation between *recom*Line test results and the chronicity of inflammation ($n = 391$). (b) Correlation according to the activity of inflammation ($n = 389$) as well as (c) pathological findings ($n = 95$) according to histology. Data are presented as box plot showing the median, 75th and 25th percentile, outlier, and extreme values. Significances were calculated using the Kruskal-Wallis test. The results show a significant correlation between the achieved *recom*Line score and histological findings. The sum of positive antigens only correlates significantly with the chronicity of inflammation.

markedly lower antibody responses after eradication except for the antibody response towards CagA (Figure 1(e)). As shown in Figure 1(f), depending on the eradication time point, the sensitivity to detect an individual with an isolated CagA immune response in this population increases up to 100% after 15 years. Based on these results, the serological response pattern showing an isolated CagA band might be used to discriminate *H. pylori* positive from eradicated patients when using this serology assay.

3.3. recomLine Results Correlate with Histological Findings in H. pylori Positive Patients. To examine the relationship between the *recom*Line test results and histological findings, we focused on the cases which are positive by histology. Of all the histologically positive cases, 98.3% (400/407) were *recom*Line positive, with the majority of cases being either CagA and/or GroEL positive. Hence, the serological response to the antigens VacA, UreA, and gGT or their combination was important to detect those cases that were CagA and GroEL negative. Moreover, the additional information gained from recognition of these antigens was valuable for the identification of patients with severe chronic and active gastritis as these antigens also have an impact on the overall score. As shown in Figure 2 compared to the number of recognized antigens, the mean *recom*Line score significantly correlates with the chronicity ($p < 0.01$) and activity ($p = 0.032$) of inflammation. Furthermore, the *recom*Line score significantly correlates ($p = 0.019$) with pathologic

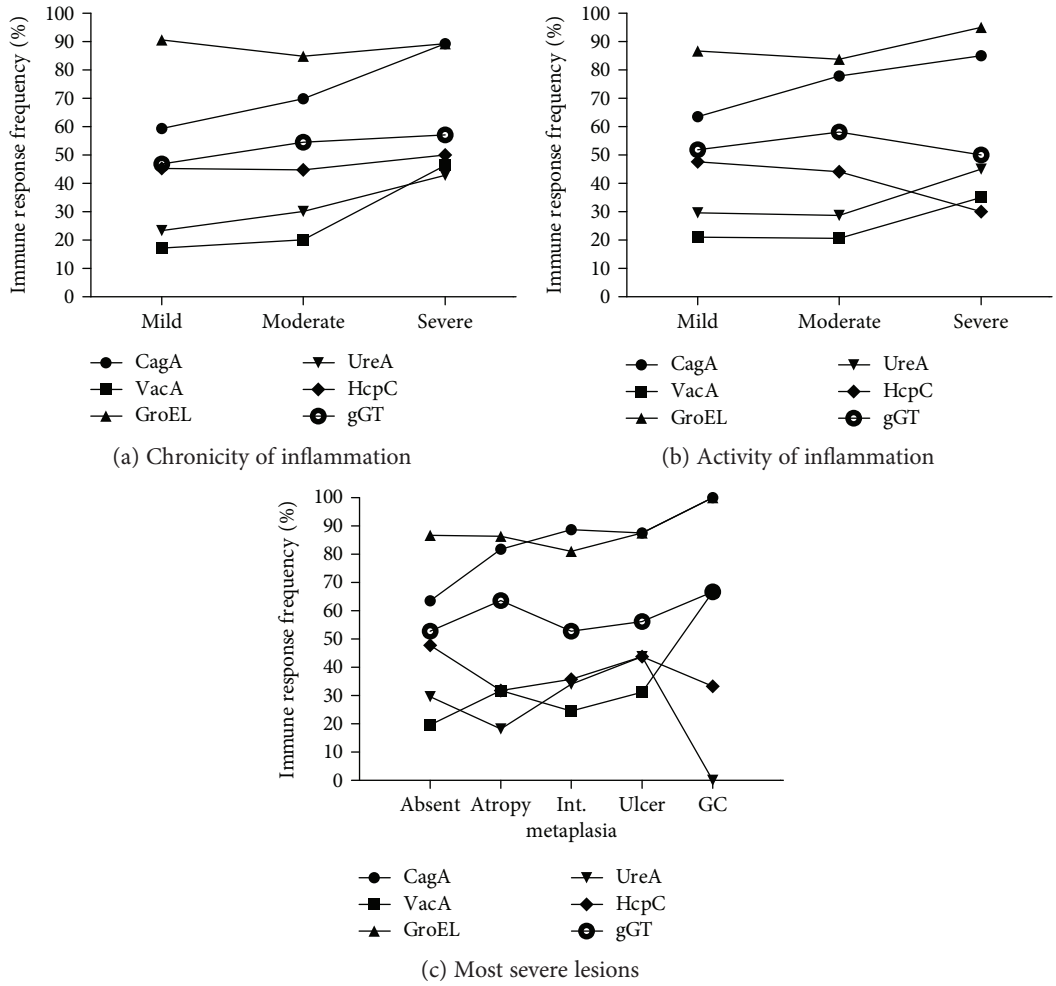

Figure 3: Association between positivity of individual antigens (●CagA, ■VacA, ▲GroEL, ▼UreA, ♦HcpC, and ○gGT) and histologic findings such as (a) chronicity and (b) activity of inflammation as well as (c) most severe lesions. The chronicity and activity are categorized as mild, moderate, and severe. Most severe lesions are categorized as either absent, atrophy, intestinal metaplasia, ulcer, or gastric cancer (GC) according to histology. A significant correlation could be found between CagA or VacA and the chronicity of inflammation ($p = 0.015$; $p < 0.01$, resp.), as well as CagA and the activity of inflammation ($p < 0.01$).

findings, which were grouped as most severe lesions due to small number of such findings in our population. The mean *recom*Line score was significantly higher ($p = 0.02$) in the group presenting with most severe lesions (5.7 ± 1.8) compared to the mean score of *H. pylori* infected asymptomatic individuals (4.6 ± 1.9).

On the level of individual antigens, a significant correlation could be found between CagA or VacA and the chronicity of inflammation ($p = 0.015$ or $p < 0.01$, respectively), as well as CagA and the activity of inflammation ($p < 0.01$) (Figure 3). The serological detection of the antigen UreA tends to increase with disease severity. This, however, was not significant, possibly because of its overall low prevalence in this setup. The antigens GroEL, HcpC, and gGT are stable between the groups analysed (Supplementary Table 3). Next, we looked for correlations between the individual antigens and pathological findings, such as ulcer or atrophy, metaplasia, and carcinoma. A significant correlation could again be seen for CagA ($p < 0.01$). Ulcer and carcinoma cases also showed high seropositivity rates for GroEL with 87.5% and 100%, respectively, but not at a significant correlation because of the high frequency in the control group. A change in seropositivity could also be shown for the antigen VacA, however not at a significant level. Detailed information is given in Supplementary Table 4.

The immune response towards a more virulent *H. pylori* type I was defined by CagA and/or VacA seropositivity and towards the less virulent type II strains by a lack of immune response towards CagA and VacA. Our data show a significant correlation between CagA seropositivity and classification of increasing pathology according to histology. Therefore, a significant correlation was also found for the type of immune response to *H. pylori*. Here, we looked at the 400 cases that were positive for *H. pylori* in histology as well as by *recom*Line. Of these, 71.5% were *H. pylori* type I and 28.5% type II, compared to 87.1% that were classified as type I in the eradicated collective. As shown in Figure 4, the proportion of type I immune response significantly increases with higher scores of chronicity or activity of inflammation ($p = 0.02$ or $p < 0.01$, respectively).

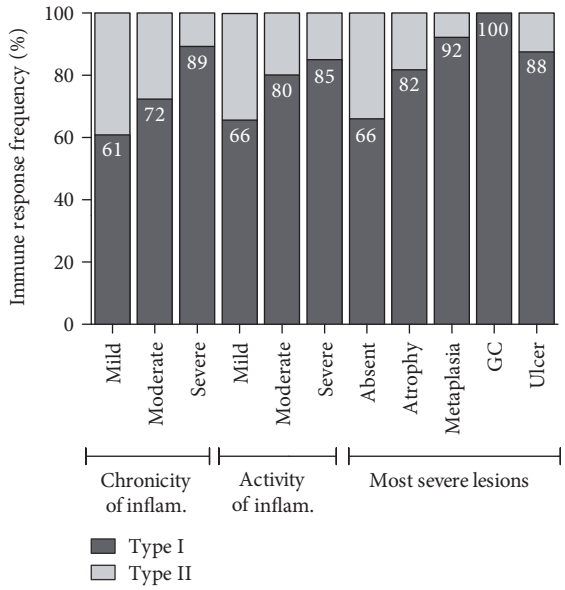

Figure 4: Association between the type of serological response (I/II) and the findings in histology analysing *H. pylori* positive cases. The presence of an immune response against *H. pylori* virulence factors CagA/VacA indicates a type I infection, and these are defined as the high-risk group. The chronicity and activity are categorized as mild, moderate, and severe. Most severe lesions are categorized as absent, atrophy, intestinal metaplasia, ulcer, and gastric cancer according to histology. The proportion of type I immune response increases with higher degrees of chronicity and activity of gastritis ($p = 0.02$ and $p < 0.01$, resp.). There is a significant increase in the portion of type I immune response if signs of advanced pathology are present in histology ($p < 0.01$).

Furthermore, there is a significant increase in the share of type I immune response if signs of advanced pathology are present in histology ($p < 0.01$), increasing up to a 100% type I immune response in the gastric cancer group.

4. Discussion

H. pylori plays an important role in the development of gastroduodenal diseases. Common serological methods for the detection of *H. pylori* do not allow distinguishing between patients with an ongoing *H. pylori* infection and patients that have received eradication therapy in the past. Furthermore, these serological tests are not capable of stratifying patients according to their individual disease state, even though several virulence factors have been known for years. The aim of the present study was to evaluate a new line assay (*recom*Line) with respect to identifying individuals that have received antibiotic treatment. Moreover, we evaluated the capacity of the *recom*Line to stratify patients according to their disease status. The present study confirmed a high sensitivity and specificity of 98.3% and 95.5%, respectively, for the detection of an *H. pylori* infection by the *recom*Line analysing this extended cohort. Nevertheless, serology still plays a minor role in the standard diagnosis of *H. pylori*, even though studies have shown that serological screening for *H. pylori* can reduce the endoscopy workload, which is especially important in countries where access to endoscopy is limited [28].

As our cohort includes a large proportion of individuals that had received antibiotic treatment, we investigated the behaviour of IgG immune responses after successful *H. pylori* eradication in this subpopulation. Current serological tests are incapable of distinguishing an ongoing from a past *H. pylori* infection. Histology as the gold standard in *H. pylori* diagnosis may show an Ex-*H. pylori* gastritis after successful eradication therapy, sometimes even after several decades. However, after due course, the gastritis can also disappear and previous *H. pylori* infection is indeterminable [29]. Furthermore, the urea breath test only allows the identification of an ongoing infection. Thus, it might be helpful to employ the *recom*Line to detect a past *H. pylori* infection. Our results show that a low test score especially in combination with an isolated CagA band may allow the differentiation of *H. pylori* positive and eradicated individuals. The mean band intensity and in parallel the immune response frequency decline after successful *H. pylori* eradication for all antigens tested except for CagA, which was particularly long lasting. Thus, this decline in immune responses has an effect on the test score and the overall *recom*Line positivity. This was corroborated in a prospective study, analysing a blinded Dutch cohort, in which most of the cases turned out to have undergone eradication therapy. Again, the results show a decrease in immune response frequency for all antigens except for CagA. In this cohort, the sensitivity of detecting a status of prior eradication through an isolated CagA band would increase up to 100% after 15 years. This study shows the feasibility of detecting individuals that have received antibiotic treatment by analysing the immune responses to individual antigens. One limitation of our study is that the time span since eradication in most individuals was quite long. For a more precise analysis, a prospective follow-up study with known baseline antigen patterns should be conducted, focusing on shorter time intervals. Furthermore, IgA immune responses should be analysed to draw further conclusions for this antibody class, as this class might be more accurate to detect *H. pylori* pre- and postinfection as suggested by Kato et al. [23].

In addition, we investigated whether or not the detection of immune responses to individual antigens or the overall test score might have the capacity to identify H. pylori positive patients at increased disease risk, if the *recom*Line allows the differentiation between the more virulent type I and the more attenuated type II strains, and if this differentiation might be associated with the disease outcome. The *recom*Line test score, compared to the number of positive antigens, shows a significant correlation with the severity of gastritis (activity and chronicity of inflammation) and most severe lesions. The *recom*Line score was determined by assessing the serological response to six *H. pylori* virulence factors CagA, VacA, GroEL, UreA, HcpC, and gGT. 393 of the 400 *recom*Line positive cases were either CagA and/or GroEL positive. The other four antigens were, thus, needed for detecting those rare cases not being CagA/GroEL positive. Moreover, they were valuable in the calculation of the *recom*Line score, which was employed for the differentiation

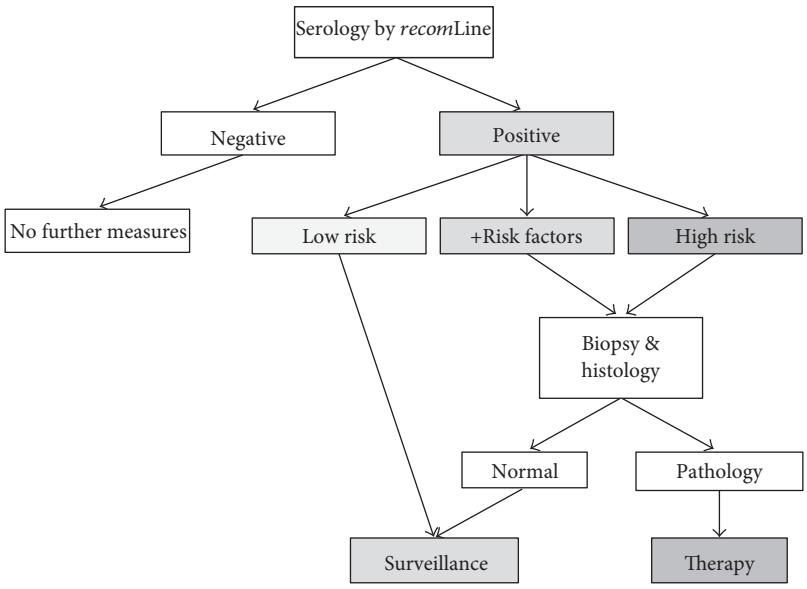

Figure 5: *H. pylori* diagnostic decision tree. Low risk: no seropositivity against CagA and VacA and no additional risk factors; +Risk factors: no seropositivity against CagA and VacA but additional risk factors such as smoking, diet, and genetic predisposition; High risk: seropositivity against CagA and VacA.

between *H. pylori* positive and eradicated patients. In addition, we show a significant correlation between the *recom*Line score and the chronicity and activity of inflammation. The more antigens were detected and, thus, the higher the *recom*Line scored, the more likely it was that the respective patient presented with a severe gastritis. The highest *recom*Line scores were found in patients with severe chronic and active gastritis, as well as gastric or duodenal ulcer, and gastric cancer patients. These patients were also more likely to have a more virulent type I *H. pylori* infection. Thus, the *recom*Line test can identify patients at increased risk to present with gastroduodenal disease and allows a further risk stratification concerning the pathogenicity of individual *H. pylori* strains. Thus, the recomLine is the first serologic test with can support risk stratification of *H. pylori* positive patients.

The more virulent *H. pylori* type I strains are in general defined through CagA positivity and secretion of "toxic" VacA s1m1 [30]. Due to the relatively high homology between the different VacA forms and numerous shared epitopes, serology cannot reliably differentiate the respective proteins. A distinction between VacA s1m1 (the more virulent form) and s2m2 (the more attenuated form) is not possible with the current *recom*Line test. However, CagA presence is mostly associated with the more virulent VacA s1m1 form [15]. In total, 285 cases were either CagA or VacA positive, the majority being CagA positive (97.5%). Only 7 cases were VacA positive but CagA negative, which were also calculated as type I immune response. Future *recom*Line optimization should enable the distinction in VacA positivity according to its s and m variants in order to allow a more accurate identification of *H. pylori* type I strains.

Previously, it was reported that antibody responses against GroEL correlate significantly with disease outcome [31]. In our study population, however, seroresponse against GroEL showed no significant correlation with increased pathology, due to its high frequency in the control group. It is known that GroEL has a high homology between *H. pylori* isolates. Therefore, the difference between our findings and published data might be due to antigen production, or the test format applied, which might lead to alterations in epitope recognition.

By identifying patients at increased disease risk, the *recom*Line can help to reduce the number of patients needed to treat and, thus, unnecessary side effects of eradication therapy are avoided. If in our study cohort only patients who carry a more virulent type I *H. pylori* strain (according to serology; $n = 278$) were to be treated in contrast to all *H. pylori* positive patients ($n = 400$), 122 patients (30.5%) could have been spared from probably unnecessary eradication therapy. The 278 cases of type I immune response include the majority of patients showing pathological findings in histology: all (100%) gastric cancer cases, 87.5% (14/16) of the gastric or duodenal ulcer cases (which would also be identified through clinical symptoms), 88.7% (47/53) of the metaplasia cases, 81.8% (18/22) of the atrophy cases, 85.0% (17/20) of the high activity of inflammation, and 89.3% (25/28) of the high chronicity of inflammation cases. Thus, only 18 patients (12.7%) with such findings in histology escaped recognition (10× score ≥ 4; 8× score < 4). If only patients with a type I immune response ($n = 278$) or a score of 4 or higher ($n = 58$, type II immune response) were to be treated, eight cases (5.6%) with pathological findings in histology would not receive treatment (1× ulcer, 4× metaplasia, 1× atrophy, and 2× severe activity of inflammation). However, these patients might be subjected to further endoscopic surveillance due to histological findings. Thus, the *recom*Line supports the identification of patients at higher risk. Nevertheless, histology still remains an important part of standard diagnosis in order to identify all patients with severe gastritis and changes in the gastric mucosa.

Our patients showed a high frequency of type I immune responses in the *H. pylori* positive collective (71.5%). As already mentioned, patients with severe gastritis, metaplasia, ulcer, and carcinoma showed high type I rates. However, patients with already a mild gastritis showed high rates of CagA positivity in both cohorts (61% and 65%, respectively). These patients might be at an early stage of their disease and thus at a milder level of inflammation, as the mean age of these patients (mild chronic and active inflammation and a type I immune response) was 53.9 (±14.6), compared to a mean age of 63.0 (±13.1) in patients with high chronicity and highly active gastritis. Further longitudinal studies are needed in order to assess disease development in such a group compared to a reference group of type II immune response patients in order to validate such prognostic patient stratification.

To further evaluate the predictive properties of the *recom*Line, a larger patient collective has to be screened, as the present cohort included only a minority of advanced stages of gastric disease. Moreover, the inclusion of additional antigens may provide further information concerning the pathogenicity of *H. pylori*. Especially, the discrimination between a type I and type II immune response needs to be further substantiated, for example, by distinguishing seropositivity of the more virulent VacA s1m1 from the s2m2 variant or by identifying new biomarkers.

We conclude that the *recom*Line is a sensitive, specific, and noninvasive test for the detection of the serum response to an *H. pylori* infection. Moreover, this assay has indicative capacity to identify patients at higher risk for *H. pylori* associated diseases. We, therefore, propose to include *recom*Line-based serology in the clinical handling of *H. pylori* infection according to the decision tree as suggested in Figure 5. We are aware that the practical implementation in the clinical practice presents a challenge for such test. As with many novel diagnostic tools, it may take years to incorporate them into clinical guidelines. Yet, since the first presentation of our data on scientific meetings, several gastroenterologists in Germany have started to use the line blot as an additional means to assess the putative disease risk of *H. pylori*-infected patients presenting with chronic gastritis. Clinical trials investigating the usefulness of such test in a prospective manner (i.e., before endoscopy is performed) are underway and may help to introduce the test into clinical practice. Further, we are currently performing cost-efficiency calculations of the algorithm proposed in Figure 5 to substantiate the advantages of such approach. Thus, the *recom*Line assay could present a valuable tool for an easy-to-perform, cost-effective primary patient screen, and could help to identify patients who require endoscopic evaluation and subsequent antibiotic therapy.

Conflicts of Interest

Markus Gerhard is the guarantor of the article. Gereon Göttner, Christina Nölting, and Erwin Soutschek are employees of Mikrogen GmbH. Markus Gerhard has licensed a patent to Mikrogen GmbH. All other authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Role of Proteinase-Activated Receptors 1 and 2 in the Regulation of Periodontal Tissue Metabolism and Disease

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Proteinase-activated receptors 1 (PAR_1) and 2 (PAR_2) are the most highly expressed members of the PAR family in the periodontium. These receptors regulate periodontal inflammatory and repair processes through their activation by endogenous and bacterial enzymes. PAR_1 is expressed by the periodontal cells such as human gingival fibroblasts, gingival epithelial cells, periodontal ligament cells, osteoblasts, and monocytic cells and can be activated by thrombin, matrix metalloproteinase 1 (MMP-1), MMP-13, fibrin, and gingipains from *Porphyromonas gingivalis*. PAR_2 is expressed by neutrophils, osteoblasts, oral epithelial cells, and human gingival fibroblasts, and its possible activators in the periodontium are gingipains, neutrophil proteinase 3, and mast cell tryptase. The mechanisms through which PARs can respond to periodontal enzymes and result in appropriate immune responses have until recently been poorly understood. This review discusses recent findings that are beginning to identify a cardinal role for PAR_1 and PAR_2 on periodontal tissue metabolism.

1. Introduction

Periodontium is characterized by the tissues that involve and support the teeth such as the gingiva, alveolar mucosa, cementum, periodontal ligament, and alveolar bone. Periodontitis, an oral disease which leads to alveolar bone loss, can be mediated by some of the endogenous host enzymes, as well as bacterial proteinases present in the periodontal pocket (e.g., neutrophil serine proteinase 3), mast cell tryptase, and gingipain from *Porphyromonas gingivalis*. Interestingly, it was recently shown that the biological activities of these proteinases can be mediated through specific proteinase-activated receptor (PAR) activation. PARs are members of the G-protein-coupled family, seven-transmembrane domain receptors, and their activation occurs through proteolytic cleavage of the N-terminal domain by proteinases, leading to the generation of a new N-terminal "tethered ligand," which binds to the receptor itself resulting in its autoactivation [1, 2]. Until now, four members of the PAR family were discovered: PAR_1, PAR_3, and PAR_4 which are activated by thrombin and PAR_2 that can be activated by trypsin, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, mast cell tryptase, membrane-tethered serine proteinase-1, or gingipains [3, 4]. PARs represent a component of the innate inflammatory response, being involved in neutrophil recruitment, increased perfusion, pain, and swelling. Thus, since PARs are present in periodontal epithelial cells and are capable of recognizing and responding to bacterial infections, it is believed that they can act as a first "alarm system" for bacterial invasions [5]. In addition, studies have suggested an important role for PARs in regulating the inflammatory response intensity to bacterial infection, as well as in periodontitis [5, 6].

Although the structures and mechanisms related to the activation of these receptors are similar, they can be expressed by different cells; hence, in each cell, their activation may lead to distinct roles in pathophysiological processes, such as growth, development, inflammation, tissue repair, and pain [2, 7–10]. PAR_1 is expressed by platelets, osteoblast endothelial cells, epithelial cells, fibroblasts, myocytes, neurons, and astrocytes, and it seems to play an important role in injured tissues. In the periodontium, the literature have also implicated PAR_1 in bone repair and homeostasis [11–13], as well as proliferation of gingival fibroblasts mediated by the

protein synthesis of endothelin-1 (ET-1) and subsequent activation of ET receptor type A [14] and transactivation of latent transforming growth factor beta 1 (TGF-β1) [15]. In addition, Rohani et al. [16] showed that in gingival epithelial cells, the PAR_1 activation by thrombin can result in the induction of chemokines leading to granulocyte attraction. Interestingly, gingipain-R (RgpB and HRgpA), a proteinase from *Porphyromonas gingivalis*, can activate PAR_1 in monocytic cells triggering an overproduction of proinflammatory cytokines [17] and in the surface of platelets leading to platelet aggregation [18]. This mechanism may constitute the biological plausibility of the association between periodontitis and cardiovascular disease and deserves further clarification by future studies.

PAR_3 and PAR_4 are expressed by platelets, endothelial cells, myocytes, and astrocytes, and their activation has been associated with the formation of pathologic thrombus [1]. The functional role of PAR_3 is controversial, whereas some investigators have described it as a nonsignaling receptor that acts along with PAR_1 and PAR_4, others have claimed that PAR_3 can signal independently of PAR_1 activation. Thus, since PAR_3 and PAR_4 are less abundant than PAR_1 in periodontal cells, and since their possible function on the periodontium still needs to be investigated [16], they are not going to be part of the present review.

PAR_2 has been demonstrated to be expressed by epithelial cells, endothelial cells, fibroblasts, osteoblasts, myocytes, neurons, astrocytes, lymphocytes, neutrophils, and mast cells [1, 17, 19–21], where it has been associated to several roles in the inflammation process such as increased vascular permeability, blood vessel relaxation, hypotension, granulocyte infiltration, release of cytokines, and pain [3, 4, 17, 22–26]. Moreover, inflammatory events in the joints, skin, colon, kidney, and airways have also been associated to PAR_2 activation [1, 2, 24, 27–29]. More recently, studies have shown that PAR_2 activation may play an important role in the inflammatory process and tissue breakdown in periodontitis [6, 30–33].

In this review, we will discuss the possible roles of the most highly expressed members of the PAR family in the periodontium, PAR_1 and PAR_2, as important molecules that mediate mammalian and bacterial enzyme's effects on cells in the regulation of periodontal inflammation and repair.

2. Potential Activators of PARs and Their Inhibitors in the Periodontium

A nucleophilic Ser residue at the active site of serine proteinases gives this class of enzyme its name. These proteinases play important roles in several biological functions, like clot formation and wound healing through the ability to activate PARs. The main PAR-activating proteinases found in the periodontal environment are neutrophil proteinase 3, thrombin, plasmin, tryptase, MMP-1, MMP-13, and gingipains (Table 1).

Neutrophil proteinase 3 is a multifunctional serine proteinase mainly located on the cell surface and in the azurophilic granules of neutrophils which are the predominant cell type in the periodontal pocket of chronic periodontitis and represent the first line of defense against infection. Interestingly, in inflammatory states, the intracellular proteinase 3 can be translocated to the cell surface, thus increasing the accessibility of proteinase 3 to bind to molecules such as PARs [34]. As neutrophil proteinase 3, elastase is a proteinase stored in the secretory granules which are released during the inflammatory process. These neutrophil proteinases differentially activate PARs 1 and 2 by a biased signaling mechanism that can both disarm the receptors from thrombin and trypsin activation and can cleave the receptors at distinct N-terminal residues to unmask different "noncanonical" receptor-activating tethered ligand sequences, triggering different signaling pathways from thrombin and trypsin [2, 35, 36]. In PAR_2, neutrophil proteinase 3, elastase, and cathepsin G (another neutrophil proteinase) can cleave the receptor downstream from the canonical trypsin site, serving as a deactivating proteinase and also as a biased PAR_2 agonist. Although the impact of these proteinases in PAR_2 activation is uncertain, it is believed that they play a role in inflammatory diseases [35].

Thrombin is another endogenous serine proteinase that can be released from fibrin clot following gingival tissue injury or inflammation. In gingival tissues, thrombin has been suggested to play a role in the healing processes, since it is known that thrombin-rich and platelet-rich plasma has been successfully used for periodontal regenerative surgery. Recently, it has been suggested that several cell functions that regulate inflammation, healing, and fibrosis in the periodontium can be mediated through the activation of PARs by thrombin [15].

Plasmin is a serine proteinase which not only acts on fibrin degradation leading to clot dissolution but also activates MMPs, growth factors, and proteinase-activated receptors (PAR_1). Studies have shown that plasmin may activate different cell types, playing a role in the process of tissue remodeling, repair, and host defense [37–39]. In periodontium, Sulniute et al. [40] showed that plasmin plays an important role in preventing the development of chronic periodontitis in mice. In addition, a recent study suggested that plasmin may reduce lipopolysaccharide- (LPS-) induced inflammatory osteoclastogenesis through PAR_1 activation [13]. Increased mast cell tryptase is found at the mucosal and subcutaneous connective tissue [41] and at the gingival crevicular fluid of patients with chronic periodontal diseases [42–45]. Holzhausen et al. [46] showed that the selective inhibition of tryptase with a compound named nafamostat mesilate leads to decreased gingival tissue granulocyte infiltration, decreased alveolar bone loss, and decreased PAR_2 expression in the gingival tissue of rats subjected to experimental periodontitis, therefore suggesting that tryptase may play a role in the pathogenesis of chronic periodontal disease through PAR_2 activation.

Interestingly, the zinc-dependent endopeptidases, MMP-1 and MMP-13, also have demonstrated the ability to activate PAR_1. MMPs cleave PAR_1 at noncanonical sites distinct from thrombin, generating unique tethered ligands which activate biased signaling pathways associated with thrombus initiation and thrombosis, atherosclerosis and restenosis, sepsis, angiogenesis, heart failure, and cancer. da Silva et al. [47]

Table 1: Potential activators of PARs in the periodontium.

Protease	Protease-3	Thrombin	Plasmin	Tryptase	MMP-1 & MMP-13	Gingipains	Dentilisin	Aa protease
Origin	Neutrophils	Fibrin clot	Fibrin clot	Mast cells	Monocytes/ macrophages	*Porphyromonas gingivalis*	*Treponema denticola*	*Aggregatibacter actinomycetemcomitans*
Type	Serine protease	Serine protease	Serine protease	Chymotrypsin-like protease	Collagenases	Cysteine proteases (i) Arginine (HRgpA and RgpB) (ii) Lysine (Kgp)	Chymotrypsin-like enzyme	Arginine and lysine proteases
PAR interaction	PAR_2 activation	PAR_1 activation PAR_3 activation PAR_4 activation	PAR_1 activation	PAR_2 activation	PAR_1 activation	PAR_1 activation PAR_2 activation	PAR_2 disarming	PAR_2 activation

PAR: protease activated receptor; MMP: matrix metalloproteinase; Aa: *Aggregatibacter actinomycetemcomitans*.

demonstrated that increased MMP-13 levels were associated with an increased PAR_1 expression at the gingival crevicular fluid of patients with chronic periodontitis after nonsurgical periodontal treatment.

In addition to host origin proteinases, exogenous serine proteinases originated by periodontopathic bacteria can also play a role in the innate response mediated by PARs. *Porphyromonas gingivalis*, for instance, produces and releases the cysteine proteinases, arginine-gingipain (HRgpA and RgpB) and lysine-gingipain (Kgp) which are strongly associated with periodontal breakdown and disruption of host defense. Some of the mechanisms played by gingipains are mediated by PARs 1 and 2, due to their potential to interact with host cell surface receptors modulating the innate response.

Other nonmammalian proteinases produced by periodontal pathogens have already been suggested to play a role on PAR_2 function. Dentilisin, a chymotrypsin-like enzyme produced by *Treponema denticola*, is suggested to cause PAR_2 disarming or inhibition to further activation [6]. Interestingly, a study by Euzebio Alves et al. [32] has demonstrated an inverse relationship between PAR_2 expression and the expression of dentilisin in the periodontal sites of moderate chronic periodontitis patients. Another bacterial proteinase, an arginine- and lysine-specific proteinase produced by *Aggregatibacter actinomycetemcomitans*, was shown to induce interleukin (IL)-8 and intercellular adhesion molecule-1 (ICAM-1) expression in gingival epithelial cells through PAR_2 activation [48]. It can be suggested that bacterial proteinases produced by other periodontal pathogens could also play a role on the activation or suppression of PAR_2 function or expression.

Interestingly, the plasma contains serine proteinase inhibitors (serpins) that can regulate proteolytic events in tissues [2, 49]. The easy accessibility of the reactive site loops of serpins guarantees the rapid inhibition of specific host proteinases, but it also makes them easy targets for bacterial proteinases, which can specifically inactivate them. In fact, the ability to resist inhibition by serpins is also important in host defense evasion by bacterial pathogens. Accordingly, Euzebio Alves et al. [32] have demonstrated that elevated levels of gingipain and proteinase 3 and decreased levels of secretory leucocyte proteinase inhibitor (SLPI) were associated to PAR_2 overexpression. SLPI is expressed by epithelial and immune cells where they play a role as an alarm proteinase inhibitor mediating anti-inflammatory and antimicrobial effects. In this study, decreased levels of SLPI were found in chronic periodontitis patients, whereas periodontal treatment led to its upregulation. The authors suggested that these results might be explained by the ability of the arginine-specific gingipains (Rgps) to degrade SLPI. Similarly, reduced SLPI levels and higher serine proteinase activities correlating with PAR_2 overexpression were found in the gastric mucosa of *Helicobacter pylori*-infected individuals. This fact may be associated to the loss of host protective capacity and increase susceptibility to breakdown from chronic infection. These data reinforce the role played by *Porphyromonas gingivalis* on PAR_2-mediated periodontal inflammation.

3. Biological Effects of PAR_1 Activation in Periodontal Cells and Tissues

PAR_1 involvement in periodontal tissue metabolism has been suggested by several in vitro studies which have shown expression of its receptor by the periodontal cells such as human gingival fibroblasts, gingival epithelial cells, periodontal ligament cells, osteoblasts, and monocytic cells and presence of its possible activators, thrombin, plasmin, MMPs, and gingipains, in the periodontal environment (Table 2).

The biological effects of PAR_1 activation on the periodontium are still not well clarified. Some studies have shown that PAR_1 activation has a tissue destructive profile, leading to the induction of proinflammatory mediators that regulate periodontal breakdown, while others highlighted its possible involvement with the repair of periodontal tissues [11, 12].

Uehara et al. [50] have showed that production of hepatocyte growth factor (HGF) by human gingival fibroblasts upon stimulation with gingipains occurred through PARs, specifically PAR_1 and PAR_2. HGF plays a role in wound healing, through its mitogenic activity to gingival epithelial cells, and enhances matrix metalloproteinases production, therefore playing a fundamental role in tissue remodeling. Moreover, HGF also stimulates blood vessel formation and promotes vascularization, a later process in wound healing.

In gingival fibroblasts, thrombin and a specific PAR_1-activating peptide-induced proliferation through endothelin-1 (ET-1) are involved in drug-induced proliferation of gingival fibroblasts [14]. In this context, an in vitro study [15] demonstrated that thrombin and PAR_1 agonist induced connective tissue growth factor (CTGF) synthesis and TGF-β1 activation in gingival fibroblasts. TGF-β1and CTGF are proteins that regulate many biological effects, such as cell adhesion, migration, differentiation, proliferation, extracellular matrix production, angiogenesis, and wound healing. As well as ET-1, it is suggested that its overexpression may be involved in gingival overgrowth.

Moreover, Rohani et al. [16] showed that activation of PAR_1 by thrombin in gingival epithelial cells leads to induction of chemokines which are important chemo-attractants for neutrophils and have a role in wound healing.

Thrombin exerts multiple effects upon osteoblasts including stimulating proliferation and inhibiting osteoblast differentiation and apoptosis. Some of these effects such as synthesis and secretion of growth factors and cytokines are believed to be mediated through PAR activation. In fact, Pagel et al. [51] demonstrated that thrombin induced TGF-1β, cyclooxygenase-2, tenascin C, fibroblast growth factors 1 and 2, connective tissue growth factor, and IL-6 expression in wild-type osteoblasts, but not in PAR_1 knockout mouse osteoblasts. In addition, PAR_1-specific activating peptide and thrombin induced release of both prostaglandin E_2 and IL-6 by osteoblasts, therefore suggesting the receptor participation in the earliest stages of bone healing.

The other evidence that links PAR_1 action to bone metabolism comes from the fact that in periodontal ligament cells, PAR_1 activation by thrombin induces the synthesis of

Table 2: Biological effects of PAR_1 activation in periodontal cells.

PAR_1	Periodontal destruction	Periodontal repair/protection
Oral epithelial cells	↑ IL-1α, IL-1β, IL-6, TNFα [52] ↑ CXCL 5 [16]	
Gingival fibroblasts		↑ HGF [50] ↑ endothelin-1 [14]
Osteoblasts	↑ COX-2 [51] ↑ IL-6 [51] ↑ PGE_2 [51]	↑ TGF-β [51] ↑ FGF-1/FGF-2 [51] ↑ CTFG [51]
Periodontal ligament cells		↑ osteoprotegerin [12]
Monocytic cells	↑ IL-8 [17]	

PAR: protease-activated receptor; IL: interleukin; TNF: tumor necrosis factor; CXCL: C-X-C motif chemokine; COX: cyclooxygenase; PGE: prostaglandin E; HGF: hepatocyte growth factor; TGF: transforming growth factor; FGF: fibroblast growth factor; CTFG: connective tissue growth factor.

osteoprotegerin, which is one of the key molecules that regulate bone homeostasis and prevent osteoclastogenesis [12]. Corroborating with these findings, a recent study found that the activation of PAR_1 in monocytic cells by plasmin diminished LPS-induced inflammatory osteoclastogenesis and bone resorption by inactivation of nuclear factor kappa beta (NF-κB) [13]. Conversely, Uehara et al. [17] showed that the gingipains Rgp and Kgp synergistically increase the secretion of proinflammatory cytokines such as IL-8 from human monocytic cells via PAR_1, PAR_2, and PAR_3 in combination with Toll-like receptors or NOD agonists (pathogen-associated molecular pattern receptors). This study was the first one to report that gingipains stimulate the secretion of cytokines from monocytic cells through the activation of PARs with synergistic effects by pathogen-associated molecular patterns (PAMPs). In addition, Giacaman et al. [52] have showed that selective cleavage of PAR_1 on oral epithelial cells by the gingipain Rgp upregulates expression of the proinflammatory cytokines IL-1α, IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α).

Interestingly, gingipains-R (RgpB and HRgpA) were also shown to activate PAR_1 in platelets leading to platelet aggregation [18]. This mechanism may explain the biological plausibility of the association between periodontitis and cardiovascular disease and deserves further clarification by future studies.

Taken together, the results from these in vitro studies show that PAR_1 is associated with both proinflammatory and reparative processes in the periodontium. However, Wong et al. [53] found no difference between $PAR_1^{+/+}$ and PAR_1 knockout mice with regard to alveolar bone loss in a *Porphyromonas gingivalis*-induced periodontal disease model, indicating that this receptor does not play a pivotal role in the progression of experimental periodontitis. Most recently, it was shown by Spolidorio et al. [54] that parstatin, a 41-amino acid peptide released upon PAR_1 activation, has potential anti-inflammatory effects since it decreases inflammatory cell infiltration, myeloperoxidase (MPO) activity, and proinflammatory mediators' levels, including IL-1β, IL-6, and TNF-α in gingival tissues of rats subjected to experimental periodontal disease.

Furthermore, a recent study by da Silva et al. [47] has suggested the first clinical evidence of the association of PAR_1 with periodontal repair. The authors demonstrated that PAR_1 expression was downregulated in chronic periodontitis patients and inversely correlated to gingival crevicular fluid levels of IL-6, IL-8, TNF-α, IFN-γ, and MMP-2. In addition, periodontal therapy resulted in PAR_1 overexpression by epithelial and immune cells from the gingival crevicular fluid, therefore suggesting the importance of PAR_1 mediating the known anabolic actions of thrombin in the periodontium.

4. Biological Effects of PAR_2 Activation in Periodontal Cells and Tissues

PAR_2 acts as a "sensor" of bacterial and host proteinases and modulates host immune defense playing a role in the host alarm system [55, 56]. PAR_2 has been localized in many cell types (Table 3) that can be found in periodontal tissues, including neutrophils, osteoblasts, oral epithelial cells, and human gingival fibroblasts [18, 21, 50, 57]. Gingipains from *Porphyromonas gingivalis*, neutrophil proteinase 3, and mast cell tryptase are the agonists that can possibly be found at the periodontal environment and that have already been studied for their ability to activate PAR_2.

Gingipains have been shown to activate PAR_2 in immunoinflammatory cells that play important roles in periodontal disease development. For instance, PAR_2 activation by RgpB leads to neutrophil activation as indicated by increased intracellular calcium concentrations [57]. In addition, gingipains (Rgps and Kgp) may activate PARs (PAR_1, PAR_2, and PAR_3) in monocytic cells increasing production of IL-6, IL-8, and monocyte chemoattractant protein- (MCP-) 1 [17]. Furthermore, Yun et al. [58] showed that RgpA activated the proteinase-activated receptors and induced T-cell activation. Taken together, these findings suggest that *Porphyromonas gingivalis*, through its gingipains, makes use of the host cell PAR_2 to exacerbate inflammation during chronic periodontal disease.

One of the most important mechanical barriers that the bacteria encounter to invade the periodontium is the epithelial tissue. Besides the physical barrier, the epithelial tissues have also the ability to produce antimicrobial peptides such as the β-defensins (hBD). Interestingly, a study by Chung et al. [59] showed that gingipains may also play a protective

Table 3: Biological effects of PAR_2 activation in periodontal cells.

PAR_2	Periodontal destruction	Periodontal repair/protection
Epithelial cells	↑ IL-1α, IL-1β, IL-6, IL-8, TNFα [4, 47] ↑ MCP-1 [17] ↑ IL-33 [61]	↑ β-defensin 2 [59]
Fibroblasts	↑ IL-8 [62]	
Osteoblasts	↑ RANKL [64]	↓ RANKL [63] ↓ RANKL : osteoprotegerin ratio [63]
Monocytic cells	↑ IL-6, IL-8, MCP-1 [17]	

IL: interleukin; MCP: monocyte chemoattractant protein; RANKL: receptor activator of nuclear factor kappa-B ligand.

role by increasing hBD-2 expression in gingival epithelial cells partially through PAR_2 receptor signaling pathway. In addition, Pereira et al. [60] have showed that in subjects with chronic periodontitis there are significantly higher levels of *Porphyromonas gingivalis* associated with increased salivary hBD-2 levels and gingival crevicular fluid PAR_2 mRNA expression than in healthy subjects and that periodontal treatment decreases both hBD-2 levels and PAR_2 expression. On the other hand, gingipains have also been shown to activate PAR_2 on oral epithelial cells leading to the production of proinflammatory mediators, such as IL-6 [18] and IL-8 [17] that could result in periodontal tissue breakdown. Moreover, Giacaman et al. [52] suggested that gingipains Rgp and Kgp may cleave and activate PAR_2 in oral keratinocytes upregulating the expression of IL-1α, IL-1β, IL-6, and TNF-α. Furthermore, a recent study by Tada et al. [61] found that the expression of IL-33, a cytokine that augments Th2 cytokine-mediated inflammatory responses, is increased during *Porphyromonas gingivalis* infection in human gingival epithelial cells via PAR_2 through gingipain-dependent activation.

As the bacteria challenge increases, an enhanced permeability of the small blood vessels of the subgingival plexus occurs resulting in an increased neutrophil migration through the junctional epithelium and into the gingival sulcus. Interestingly, activated neutrophils may secrete a proteinase (neutrophil proteinase 3) which was shown to activate human oral epithelial cells through PAR_2, inducing IL-8 and monocyte chemoattractant protein-1 production [17].

Increased levels of proinflammatory mediators and pathogenic bacteria in the soft tissues may lead to the disruption of the epithelial tissue, which in turn facilitates the access of bacteria and their products to the subepithelial connective tissue. The exposure of the residing periodontal connective tissue cells to the bacterial agents may transform them into major participants in the pathophysiological process of periodontal tissue destruction. The dominant cell type in periodontal connective tissue is the fibroblast. Interestingly, Uehara et al. [50] demonstrated that human gingival fibroblasts express PAR_2 and that its activation by a synthetic PAR_2 agonist peptide (SLIGRL) induces the production of IL-8 which has the ability to selectively stimulate MMP activity, responsible for collagen destruction within periodontitis lesions. *Porphyromonas gingivalis* may exacerbate this process since it was demonstrated that gingipains upregulate PAR_2 gene expression in human gingival fibroblasts [62].

Abraham et al. [21] demonstrated that PAR_2 is expressed by osteoblasts and that its activation by a specific synthetic peptide did not show any effect on osteoblast proliferation or differentiation. In addition, in this study, osteoblast-mediated osteoclast bone resorption was also not stimulated by PAR_2 activation. Furthermore, Smith et al. [63] showed that PAR_2 activation inhibits expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and suppressed the RANKL : osteoprotegerin ratio in osteoblasts. However, a study by Amiable et al. [64] showed that PAR_2 activation in osteoarthritis subchondral bone osteoblasts induced a significant upregulation of RANKL and significantly enhanced bone resorptive activity. Interestingly, these findings on the resorptive properties played by PAR_2 in osteoblasts are in agreement with data reporting the involvement of PAR_2 activity in periodontitis [6, 30–33, 65].

Accordingly, it has been shown that a selective PAR_2 agonist (SLIGRL) causes periodontitis in rats through a mechanism involving prostaglandin release and MMP activation [65] and that PAR_2-knockout mice infected with *Porphyromonas gingivalis* have decreased levels of proinflammatory mediators, such as prostaglandin E_2, interferon-gamma, IL-1beta, and IL-6, and less alveolar bone loss when compared to wild-type animals [6].

Wong et al. [53] also have shown that less alveolar bone resorption occurred in PAR_2-knockout mice. In addition, they showed that T-cells from *Porphyromonas gingivalis*-infected $PAR_2^{-/-}$ mice proliferated less in response to antigen than those from wild-type mice and that T-cells from infected or antigen-immunized PAR_2-null mice had a significantly different Th1/inflammatory cytokine profile from wild-type cells such as decreased gamma interferon, ILs (IL-2, IL-3, and IL-17), granulocyte-macrophage colony-stimulating factor, and TNF-alpha than wild-type controls. The absence of PAR_2 therefore appears to substantially decrease T-cell activation and the Th1/inflammatory response. In this study [53], increased numbers of mast cells in the maxillary tissue of infected $PAR_2^{+/+}$ mice were also shown, indicating that PAR_2 may also have a role in mast cell differentiation or infiltration into tissues. Thus, activation of PAR_2 expressed by mast cells by the arginine-specific gingipains from *Porphyromonas gingivalis* may lead to the release of inflammatory mediators that are pivotal to early inflammatory response in chronic periodontitis. It has been shown that activation of PAR_2 leads to degranulation of mast cells, causing the release of proinflammatory compounds that kill

pathogens and upregulate the immune responses. In addition, tryptase, released from the granules of mast cells upon degranulation, may also activate PAR_2, and therefore, these cells could play a role in periodontitis by causing the activation of the receptor on other cells in the periodontal tissues. Thus, the regulation of such proinflammatory mechanisms in T-cells and mast cells by PAR_2 suggests a pivotal role in the pathogenesis of the disease (Table 4).

In the gingival crevicular fluid from patients with periodontal disease, there are high levels of proteolytic activity characterized by a mixture of endogenous and exogenous proteinases which may mediate degradation of connective tissue [66]. Among these hydrolytic enzymes in the inflamed periodontal environment, neutrophil proteinase 3, mast cell tryptase, and gingipains have been isolated and are known to activate PAR_2. In fact, high levels of proteolytic activity derived from both *Porphyromonas gingivalis* and neutrophils are expected to be found in the periodontal pocket of chronic periodontitis, since they are, respectively, the major periodontal pathogen and the predominant cells (approximately 90%).

PAR_2 has been shown to be expressed by cellular elements found in gingival crevicular fluid, which may include epithelial cells, and leukocytes even in clinically healthy human gingival sulci [30]. In addition, PAR_2 expression is upregulated in chronic periodontitis patients compared to that in healthy controls (Table 5). Interestingly, PAR_2 upregulation in the inflamed periodontium is associated with an elevated gingival crevicular fluid trypsin-like activity, probably due to the increased prevalence of *Porphyromonas gingivalis*, and expression of neutrophil proteinase 3 [30].

Importantly, proteinase 3 has been shown to activate oral epithelial cells through PAR_2 leading to the production of IL-8 and monocyte chemoattractant protein-1 [17]. These findings clearly suggest that proteinase 3 actively participates in PAR_2-mediated inflammation at periodontal sites.

PAR_2 activation results in the synthesis of proinflammatory mediators including IL-6, IL-8, Il-1, IFN-γ, PGE_2, and MMP-9 [6, 67, 68] and activates signaling pathways such as those involving mitogen-activated protein kinase and nuclear factor-κB, which potentiates inflammatory responses [69]. Interestingly, proinflammatory mediators such as TNF-alpha and IL1-beta are reported to increase PAR_2 expression [30]. Accordingly, an increased PAR_2 expression has been demonstrated in deeper periodontal pockets compared to the expression of the receptor in shallower pockets, and it was associated with significant increased levels of proinflammatory mediators [30]. In addition, periodontal treatment statistically reduces PAR_2 expression [30, 32]. Furthermore, Fagundes et al. [31] demonstrated that the presence of *Porphyromonas gingivalis* is associated with an elevated expression of PAR_2 in human chronic periodontitis, thus suggesting that *Porphyromonas gingivalis* may disturb the host inflammatory responses not only by regulating PAR_2 function but also by enhancing its genetic expression. Taken together, these findings clearly suggest that PAR_2 overexpression is an essential element in inflammation severity.

Another study by Euzebio Alves et al. [32] showed that PAR_2-positive staining in gingival crevicular fluid cells was reflective of tissue destruction, and its overexpression was positively associated to inflammatory clinical parameters and to the levels of the proinflammatory mediators IL-6, IL-8, TNF-alpha, MMP-2, MMP-8, hepatocyte growth factor, and vascular endothelial growth factor (VEGF). Another interesting finding from this study was that periodontally healthy sites from chronic periodontitis individuals showed a diminished expression of PAR_2 mRNA and PAR_2 protein level, therefore suggesting that PAR_2 periodontal expression is influenced by the presence of infection and not merely a constitutive characteristic that may favor periodontal inflammation.

5. Specific Synthetic Agonists

Selective synthetic peptides, corresponding to the tethered ligand sequences, are able to activate selectively the receptors through direct binding to the body of the receptor without the need of proteolysis [22]. With the exception of PAR_3, all the other receptors have their selective agonist peptides. PAR_1, PAR_2, and PAR_4 can be nonenzimatically and selectively activated by TFLLR-NH2, SLIGRL-NH2, and GYPGQVNH2, respectively [1, 2]. Since their discovery, selective PAR agonists have been used in order to assess the specific impact of PAR_1, PAR_2, and PAR_4 signaling.

Noteworthy, earlier literature in many instances used the "TRAP" peptide (SFLLRN) to activate "PAR_1" not realizing that the peptide also coactivates PAR_2 [36]. Thus, the earlier use of the "TRAP" peptide may have generated results that reflect the coactivation of both PARs 1 and 2. Furthermore, the PAR_2-activating peptide (SLIGRL-NH2) can in some settings cross-react with the MAS receptor, whereas the use of a more potent 2-furoyl-LIGRLO-amide can prevent this issue [36].

6. Recent PAR Antagonists

The newest PAR_1 antagonist (Vorapaxar) has been used for the treatment of cardiovascular diseases. Vorapaxar acts by blocking the docking of the tethered ligand sequence preventing PAR_1 activation, hence platelet aggregation [10]. Regarding PAR_2 blockage, the antagonist GB88 can block PAR_2 activation by trypsin as well as PAR-activating peptide. GB88 has been shown to inhibit PAR2-induced proinflammatory cytokine release and attenuate inflammation in a rat model of colitis [10]. However, until now, none of these antagonists were studied in periodontics.

7. PARs: Drug Targets in Inflammation

Therapies focusing on the inhibition of proteinases or, more specifically, the use of PAR antagonists may constitute an important approach for the modulation of an infectious pathology such as periodontal inflammatory disease.

According to Yun et al. [58], PAR_2 blockage with the use of antagonists might promote adverse high proteolytic activities in the gingival crevicular fluid. Thus, it seems that the

Table 4: In vitro studies on PAR_1 and PAR_2 activation associated with periodontal tissue metabolism.

Author(s)/year	In vivo model	PAR	Experimental groups	Biological effect(s)	Mechanism(s) involved
Holzhausen et al. [65]	Male Wistar rats subjected to ligature-induced periodontitis (right mandibular first molar)	PAR_2	(i) Ligature + saline, $n = 32$ (ii) Ligature + SLIGRL-NH_2 (agonist peptide), $n = 32$ (iii) Ligature + LRGILS-NH_2 (control peptide), $n = 32$ (iv) Sham + saline, $n = 32$ (v) Sham + SLIGRL-NH_2, $n = 32$ (vi) Sham + LRGILS-NH_2, $n = 32$ Sacrifice at 3, 7, 15, and 30 days	PAR_2 agonist induced alveolar bone loss and granulocyte infiltration and exacerbated ligature-induced periodontitis.	Upregulation of COX-1, COX-2, MMP-2, and MMP-9.
Holzhausen et al. [6]	PAR2-deficient mice subjected to *P. gingivalis* oral infection	PAR_2	(i) $PAR_2^{-/-}$ mice + *P. gingivalis* oral infection, $n = 20$ (ii) PAR_2 WT mice + *P. gingivalis*, $n = 20$ Sacrifice at 42 and 60 days	$PAR_2^{-/-}$ mice showed less alveolar bone loss compared to WT mice.	PAR_2 activation in the presence of *P. gingivalis* infection increased inflammatory cell infiltration, prostaglandin-E_2, IFN-γ, IL-6, and IL-1β levels.
Wong et al. [53]	PAR2-deficient mice subjected to *P. gingivalis* oral infection	PAR_2	(i) $PAR_2^{-/-}$ mice + *P. gingivalis* oral infection, $n = 24$ (ii) PAR_2 WT mice + *P. gingivalis*, $n = 24$ Sacrifice at 30 days	$PAR_2^{-/-}$ mice showed less exposed root surface and less alveolar bone eroded surface compared to WT mice.	PAR_2-deficient mice showed decreased infiltration of mast cells in the periodontal tissues and impaired T-cell immune responses (decreased activation and Th1/inflammatory response).
	PAR1-deficient mice subjected to *P. gingivalis* oral infection	PAR_1	(i) $PAR_1^{-/-}$ mice + *P. gingivalis* oral infection, $n = 24$ (ii) PAR_1 WT mice + *P. gingivalis*, $n = 24$ Sacrifice at 30 days	No difference between $PAR_1^{-/-}$ and PAR_1 WT mice with regard to the exposed root surface.	
Holzhausen et al. [46]	Male Wistar rats subjected to ligature-induced periodontitis (right mandibular first molar)	PAR_2	(i) Control group: daily i.p. administration of saline, $n = 20$ (ii) Ligature group: ligature placement and daily i.p. administration of saline, $n = 20$ (iii) Nafamostat mesilate (NM) group: NM (0.1 mg/kg/day, i.p.) a potent tryptase inhibitor, $n = 20$ (iv) NM + ligature group: ligature and daily i.p. NM (0.1 mg/kg/day), $n = 20$ Sacrifice at 7 and 14 days	Tryptase inhibition decreased alveolar bone loss, MPO, and total proteolytic activity in animals subjected to ligature-induced periodontitis.	Tryptase inhibition led to a 1.6-fold decrease in gingival PAR_2 expression.

Table 4: Continued.

Author(s)/year	In vivo model	PAR	Experimental groups	Biological effect(s)	Mechanism(s) involved
Spolidorio et al. [54]	Male Wistar rats subjected to ligature-induced periodontitis (upper second molars)	PAR_1	(i) Ligature + intraoral 3 μg parstatin, $n = 16$ (ii) Ligature + intraoral PBS, $n = 16$ (iii) Sham, $n = 8$ Sacrifice at 8 and 15 days	Parstatin (peptide released upon PAR_1 activation) prevented periodontal tissue breakdown.	Parstatin suppressed inflammatory cell infiltration and decreased MPO, IL-1β, TNF-α, and IL-6.
Castro et al. [33]	Male Wistar rats subjected to ligature-induced periodontitis (mandibular first molars)	PAR_2	(i) No ligature and no treatment, $n = 20$ (ii) Ligature + placebo (0,9% NaCl solution), $n = 20$ (iii) Ligature + 5 mg subantimicrobial dose of doxycycline (SDD) by daily gavage, $n = 20$ Sacrifice at 3 and 15 days	SDD downregulated alveolar bone loss.	Downregulation of PAR_2, IL-17, IL-1β, and TNF-α.

PAR: protease-activated receptor; COX: cyclooxygenase; MMP: matrix metalloproteinase; *P. gingivalis*: *Porphyromonas gingivalis*; WT: wild-type mice; IL: interleukin; IFN-γ: interferon gamma; MPO: myeloperoxidase; PBS: phosphate-buffered saline; TNF: tumor necrosis factor.

Table 5: Human studies on PAR_1 and PAR_2 activation associated with periodontal tissue metabolism.

	Groups	Main findings
Holzhausen et al. [30]	(i) Controls: 40 subjects (ii) Chronic periodontitis with moderate destruction ($3 < PPD \leq 6$ mm; $CAL \leq 4$ mm), $n = 40$ (iii) Chronic periodontitis with advanced destruction ($PPD > 6$ mm; $CAL > 4$ mm), $n = 40$	(i) Chronic periodontitis patients showed higher PAR_2 mRNA expression and increased levels of IL-1α, IL-6, IL-8, TNF-α, total proteolytic activity, *P. gingivalis* prevalence, and proteinase 3 mRNA expression compared to controls. (ii) Periodontal treatment decreased PAR_2 mRNA expression.
Fagundes et al. [31]	(i) Moderate chronic periodontitis ($3 < PPD \leq 6$ mm; $3 < CAL \leq 6$ mm), $n = 35$	*P. gingivalis* presence was associated with the following: (i) higher levels of IL-1α, IL-6, and TNF-α, (ii) higher proteolytic activity, (iii) higher PAR2 mRNA expression.
Euzebio Alves et al. [32]	(i) Controls: 31 subjects (ii) Moderate chronic periodontitis ($3 < PPD \leq 6$ mm; $CAL \leq 4$ mm), $n = 31$	Chronic periodontitis patients showed overexpression of PAR_2, IL-6, IL-8, TNF-α, MMP-2, MMP-8, HGF, VEGF, gingipain, and proteinase 3 and decreased levels of dentilisin, SLPI, and elafin. Periodontal treatment decreased PAR_2 expression.
da Silva et al. [47]	(i) Controls: 37 subjects (ii) Moderate chronic periodontitis ($3 < PPD \leq 6$ mm; $CAL \leq 4$ mm), $n = 38$	Periodontal treatment resulted in PAR_1 overexpression, which was inversely correlated with PAR_2 expression and gingival crevicular fluid levels of IL-6, IL-8, TNF-α, IFN-γ, and MMP.

PAR: protease activated receptor; IL: interleukin; *P. gingivalis*: *Porphyromonas gingivalis*; PPD: probing pocket depth; CAL: clinical attachment level; TNF: tumor necrosis factor; P3: neutrophil serine protease 3; MMP: matrix metalloproteinase; HGF: hepatocyte growth factor; VEGF: vascular endothelial growth factor; SLPI: secretory leukocyte protease inhibitor; GCF: gingival crevicular fluid; IFN-γ: interferon gamma.

potential side effects that the concept of PAR_2 blockade encounters do not seem to overcome the beneficial aspects for the treatment of periodontal inflammation.

At a certain point in the inflammatory process, PAR 1 and 2 blockages may be necessary to prevent increased inflammation, whereas at later time points, PAR activation may be required to aid resolution. Further, the use of specific proteinase inhibitors, rather than PARs antagonists, may result in a "dual" blockage inhibiting both the degradation of host molecules and activation of PARs. Golub et al. [70] found that tetracyclines can inhibit tissue collagenolytic activity in in vivo, in vitro, and periodontal pockets presented by subjects with and without diabetes. Castro et al. [33] found that administration of a subantimicrobial dose of doxycycline (SDD) in a rat periodontitis model may result in PAR_2 modulation through a dual mode, downregulating its gene expression and decreasing its posterior activation by proteinases. Doxyxycline in a subantimicrobial dose is able to inhibit the activity of MMPs and thus reduce the degradation of collagen, fibronectin, and elastin in the periodontal tissues [71], and its clinical use in the modulation of the immunoinflammatory host response as coadjuvant to periodontal conventional therapy is approved by the Food and Drug Administration since 1998. Moreover, in periodontal inflammation, the inhibition of MMPs by drugs from the tetracycline family may not only attenuate the activation of PARs by MMPs but also can prevent extracellular matrix remodeling which can sequester cell-regulating polypeptide that in turn can act together with the PAR- promoting fibrosis [2]. However, additional studies are necessary to confirm the clinical benefits of tetracycline family and other highly specific inhibitors on PAR-mediated periodontal inflammation.

8. Concluding Remarks

PARs together with Toll-like receptors and NOD-like receptors are pattern recognition receptors that contribute to innate immunity. A new paradigm in microbial pathogenicity has been established in which bacterial proteinases manipulate host cell functions through PAR activation. An uncontrolled PAR activation will certainly result in a disruptive local inflammatory response, which can benefit the periodontal microbial community.

The in vitro studies described here highlight the differential actions of PARs on periodontal cells, suggesting that PARs, like other components of the innate immunity, act as a double-edged sword with both protective and destructive responses. However, in periodontal tissues, PAR_2 seems to be upregulated during inflammation, where it is believed to be activated by bacterial and host proteinases, whereas PAR_1 is upregulated during periodontal tissue repair. This counterregulation of PARs actions was also demonstrated by Xue et al. [72] in rheumatoid arthritis synovial fibroblasts, where PAR_2 activation was associated with an elevated TNF-alpha release and PAR_1 activation prevented the release of proinflammatory cytokines. Interestingly, da Silva et al. [47] showed that PAR_1 overexpression after periodontal treatment was inversely correlated to PAR_2 expression in gingival crevicular fluid cells. In addition, Zhang et al. [73] observed that PAR_2 is upregulated whereas PAR_1 is downregulated in human gingival epithelial cells during *Porphyromonas gingivalis* infection. Understanding the mechanisms that keep their functions in balance can bring new knowledge on the role of PARs in the development and treatment of periodontal inflammation.

Conflicts of Interest

The authors declare that they have no conflict of interests.

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Review Article

Bacteriophages and Their Immunological Applications against Infectious Threats

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Bacteriophage therapy dates back almost a century, but the discovery of antibiotics led to a rapid decline in the interests and investments within this field of research. Recently, the novel threat of multidrug-resistant bacteria highlighted the alarming drop in research and development of new antibiotics: 16 molecules were discovered during 1983–87, 10 new therapeutics during the nineties, and only 5 between 2003 and 2007. Phages are therefore being reconsidered as alternative therapeutics. Phage display technique has proved to be extremely promising for the identification of effective antibodies directed against pathogens, as well as for vaccine development. At the same time, conventional phage therapy uses lytic bacteriophages for treatment of infections and recent clinical trials have shown great potential. Moreover, several other approaches have been developed in vitro and in vivo using phage-derived proteins as antibacterial agents. Finally, their use has also been widely considered for public health surveillance, as biosensor phages can be used to detect food and water contaminations and prevent bacterial epidemics. These novel approaches strongly promote the idea that phages and their proteins can be exploited as an effective weapon in the near future, especially in a world which is on the brink of a "postantibiotic era."

1. Introduction

Bacteriophages (or phages), small viruses of about 20–200 nm in size, are probably the most ancient and ubiquitous existing organisms on Earth. They date back 3 billion years, and they specifically infect bacteria to replicate, therefore playing an important role in maintaining the equilibrium of every ecosystem where bacteria exist [1].

Despite controversy over claims for priority, bacteriophage discovery is independently attributed to both F.W. Twort (1915) and to F. H. d'Herelle (1917) [2–4]. The former observed a peculiar in vitro transformation of micrococcus colonies, "plaques or rings grew and the disease could be transferred through simple contact between colonies." In 1917, at Pasteur Institute in Paris, researcher d'Herelle discovered an infective agent able to selectively destroy cultures of *Shigella dysenteriae* bacteria. The microorganism responsible for that lysis was called "bacteriophage," coined by the combination of *bacteria* and the Greek *phagein*, which means *devour* [5].

Phage classification is based on morphology, nucleic acid characteristics, and properties, although other factors such as host spectrum, virus-host interaction, and immunological features should be considered [6]. Concerning morphology, there are phages with icosahedral proteic capsid and no tail, phages with contractile tails, phages without contractile tails, and filamentous phages. The specificity for the *E. coli* conjugative F pilus was found to be a distinguishing trait of a large group of phages (Ff), including the first isolated filamentous phages f1 [7–10]; many other species, including temperate and Gram-positive tropic ones, were later discovered [11].

Lytic phages, unlike temperate and filamentous phages, lyse the bacterial cell at the end of their life cycle, disrupting its metabolism and releasing newly formed phage particles. During the lytic cycle, an ecliptic phase occurs: the bacterial cell does not contain any complete virion yet, but the virus replicates and both host and early viral components are involved. Late viral proteins are instead structural elements necessary for new particle assembly and formation and for the lysis of the cell that occurs after maturation [12].

During the lysogenic cycle, the viral genome does not take control of host machinery but remains inside the cell and replicates together with the host genome in order to generate clones of infected cells, which are then able to grow and divide for long periods of time. Latent forms of the viral genome are named "prophage" [12].

Phage discovery occurred well before the development of antibiotics, thus raising interest within the worldwide scientific community.

By 1919, d'Herelle and his colleagues in Paris began using bacteriophages in a therapeutic way, launching the "phage therapy" [13, 14]. That first, small-scale clinical trial concerned four children successfully treated from bacterial dysentery; after receiving a single dose of phage preparation, all began to recover within 24 hours. At the same time, phage sample ingestion by healthy individuals proved the treatment safety. In 1921, Bruynoghe and Maisin published the first paper describing the efficacy of bacteriophages in the treatment of a staphylococcal skin infection: they injected the phage preparation around surgically opened lesions and the infection regressed within 24–48 hours [15].

Bacteriophage therapy rapidly developed globally and attracted the attention of pharmaceutical companies and, independently, the Russian and German army physicians, who started using phages to treat soldiers.

Nevertheless, those results were sometimes controversial, and antibiotics were discovered and industrially produced [13, 14]. Thus, enthusiasm for phage therapy began to decrease in the West during the 1940s and the 1950s, even if in the meanwhile Luria and Delbruck used bacteriophages as model organisms for their "Fluctuation test," leading to the understanding of the genetic basis of interactions between viruses and hosts and to the development of the first molecular techniques [16, 17]. However, scientific interest for bacteriophages arose again in the late 1980s, with the design of a novel technique: phage display. Since then, this application has proved to be extremely successful for the identification of monoclonal antibodies (mAbs) directed against a wide range of pathogens, and several companies currently use this technique for the discovery of their lead compounds.

A recent report from 2014, commissioned by the UK government, predicts the killing of 10 million people across the world by 2050 because of antimicrobial-resistant infections; the Centers for Disease Control and Prevention (CDC) categorized some bacteria as presenting urgent, serious, and concerning threats [18]. Therefore, the appearance of multidrug-resistant pathogens has renewed the importance of pioneering antibacterial strategies in order to prevent epidemic infections, with a close monitoring of hospitals as well as food industries. This is giving a boost in revisiting the practical applications of bacteriophages both for phage therapy and for rapid and precise detection of bacterial pathogens.

2. Phage Display Technique for mAbs Identification against Pathogens

Phage display technique was invented in 1985: since then, it has been successfully applied to many immunology domains, in particular against infectious diseases and cancer research [6]. This technique has led to important results in both translational and basic research, with the identification of mAbs and small peptides capable of inhibiting functions of their receptor target. Some of these molecules have been developed as therapeutics and are currently in clinical or preclinical trials; on the other hand, others have been crucial to describe protein-protein interactions and revealed important therapeutic targets [19, 20].

Briefly, this method focuses on the construction of a library of peptides or antibody variants, which are then selected for their affinity to the target of interest since they are fused to a phage-coat protein. All surface proteins of bacteriophages can be engineered for display, but the most used are pVIII and pIII from M13 filamentous phages. In fact, each virion contains about 2700 copies of the former protein, representing almost 87% of its mass, and they are half-exposed to the environment, thus allowing an efficient display of only short-sequence peptides due to virion architecture. On the other hand, pIII can be used for larger peptides, resulting only in a slight loss of infectivity in a few cases [21]. Each library is composed by phagemid vectors containing only the sequence of a phage-coat protein fused to the peptide of interest; therefore, a helper phage with a reduced packaging efficiency is needed in order to obtain a population of phages both infectious and composed by modified coating proteins. The biopanning procedure is then performed and phages are selected for their ability to bind the antigen of interest. Many factors must be taken into account: library variability, target conformation, affinity, and avidity of the molecules exposed on phages. As mentioned, phage display has been widely used to find novel therapeutics against pathogens, particularly mAbs. This has been possible through two different biopanning strategies: using specific molecular targets, such as enzymes or membrane receptors, or using whole viruses or bacterial cells. The main difference between these two approaches is that the second one allows the identification of pathogen structures not already identified as drug targets. Moreover, surface antigens often present motifs able to elicit non-neutralizing mAbs and elude host immune response, so the screening procedure of the biopanning outcome must be done properly in order to identify only the few effective molecules.

Several human infectious diseases have been successfully addressed for drug discovery using phage display. Using molecular targets, specific mAbs have been isolated against viruses such as HCV, HIV, HBV, JCV, HSV, influenza A, or bacteria like *H. pylori* [20, 22–29], while whole-cell approach resulted in the identifications of effective molecules against rabies virus, *L. monocytogenes*, *H. pylori*, *C. trachomatis*, and various *Bacillus* species [30–34].

As stated above, many of the molecules developed using phage display technique are currently in clinical trials or under evaluation at a preclinical stage. MedImmune developed motavizumab, a version of their lead compound against RSV infection (palivizumab) which was optimized by enhancing its affinity to the target [35]. Affitech A/S is a company whose phagemidic library has a diversity of 10^{10}, allowing the identification of several human mAbs, for example, bavituximab has been tested in several clinical trials against HCV and HIV chronic infections, and for the treatment of several types of cancer [36]. Its target is phosphatidylserine, a molecule that is located in the inner part of the cell membrane of healthy cells, but becomes exposed on the surface of infected cells or solid cancer cells, allowing their immune evasion. Moreover, Human Genome Sciences developed raxibacumab for anthrax using a part of the toxin itself, the *B. anthracis*-recombinant protective antigen protein [37]. Additionally, Isogenica developed an improved version of the phage display technique, called "CIS display": it provides DNA fragments encoding the peptides of the library fused to the gene of RepA, the DNA replication initiator protein, so that proteins or peptides are displayed in vitro directly bound to their encoding DNA [38].

3. Phages for Vaccine Development

Neutralizing mAbs are very useful as therapeutics but can also be of great importance for the identification of protective epitopes on pathogen structures. In fact, the characterization of these regions on viruses and bacteria could suggest which elements should be included into a vaccine formulation in order to be effective. To date, there are several human infectious diseases that cannot be treated with vaccination, since all the approaches tested so far proved to be unsuccessful. One of the causes of failure is the nature of the antigens included into the vaccine. In fact, the use of recombinant proteins may limit, though not eliminate, the mechanisms of immunodominance that pathogens use to evade host immune response. Immunodominant epitopes are those regions that can be mutated without affecting the pathogen protein functions, both rerouting the adaptive immune response against non-neutralizing epitopes and masking epitopes that can impair infection mechanisms. Thus, bacterial and viral proteins often elicit a varying humoral response, with a minimal neutralizing fraction, unable to defeat the infection [39–41]. Moreover, also antibody-mediated interference has been widely described [22, 42]. Dulbecco et al. first hypothesized the interfering effect of non-neutralizing Abs (non-nAbs) to explain the apparent inhibition of virus neutralization exerted by some serum samples [43]. Later observations in both chronic and acute infections confirmed this assumption. Two mechanisms have been proposed for this evasion strategy: non-nAbs interference by steric hindrance due to proximity of neutralizing and non-neutralizing epitopes; inhibition of binding of nAbs due to epitope conformational changes caused by non-nAbs binding to the pathogen protein. Furthermore, it has been speculated that non-nAbs may enhance infections through interaction with Fc receptors or complement receptors [44].

On the other hand, neutralizing mAbs as molecular probes could be extremely useful for a rational design of vaccine formulations: the administration of their mimotopes should elicit only an effective humoral response against pathogens [45, 46]. For this purpose, phage display technique has been widely employed: HCV, HIV, and *Plasmodium falciparum* proteins are just examples of molecular targets used in the biopanning procedure for epitope mapping [26, 47, 48].

In addition, phage particles themselves can induce both cellular and humoral immune response when recognised by the immune system [49]. Moreover, they proved to be unaffected by harsh physical and chemical conditions, resulting suitable for vaccine delivery [50]. In fact, these peculiar characteristics, as well as cost-effective production and ease of modification, made bacteriophages attractive for industrial development of phage-based vaccines. Two different approaches have been developed: the first one is based on phage particles with antigens transcriptionally fused to their coat proteins, resembling the phage display technique described above. This strategy proved to be effective against *Yersinia pestis* [51], where T4 phages displayed an engineered structural subunit of the pathogen on its capsid, and against HIV and influenza, using phage T4 and T7, respectively [52, 53]. The second approach consists of phage DNA vaccines: the antigen gene is delivered by phages into antigen-presenting cells to be expressed and processed by them, leading to enhanced immune response compared to naked DNA delivery. Vaccines against HBV and HSV type 1 infections have been developed using an engineered phage λ that carries a viral surface epitope gene [49, 54]. More recently, a phage λ-based vaccine against *Chlamydia abortis* elicited an immune response superior to the stimulation of the commercial vaccine Enzovax [55].

4. Phage Therapy: Last Updates

Due to their high level of organization, their unique morphology, and biological properties, bacteriophages appear as sophisticated nanomachines and, as described above, have been immediately employed for therapeutic purposes.

Typically, only lytic phages are exploited for phage therapy: firstly, because they kill the host bacteria in a more efficient manner; subsequently because, after lysogenic induction, temperate phages can transfer bacteria DNA fragments to other species, and if these fragments contain gene-encoding toxins or antibiotic resistance elements, they could generate new dangerous bacteria. On the contrary, lytic phages are unable to perform transduction, thus ensuring a safer profile.

Nowadays, many bacteria have collected multiple resistance mechanisms, thus rendering some antibiotic classes useless. Beyond the quite high level of natural resistance, patients are concomitantly treated with broad-spectrum antibiotic molecules, increasing the rate of blind selection of multidrug resistance isolates (MDR). In hospital settings, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* (ESKAPE) are examples

of multidrug-resistant strains that solicit novel therapeutic measures [56]. This challenge forced modern medicine to review methods against bacterial infections, reconsidering the beneficial potential of phages.

In 2006, the Food and Drug Administration (FDA) approved the first Phase I clinical trial evaluating the safety of an eight-phage cocktail able to kill *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *and Escherichia coli*; 42 patients with venous leg ulcers were treated and a high-safety profile was demonstrated (ClinicalTrials.gov Identifier: NCT00663091) (Table 1).

In 2009, another study assessed safety, tolerability, and efficacy of oral administration of T4 phages in young children with diarrhea due to enterotoxigenic *Escherichia coli* (ETEC) and/or enteropathogenic *Escherichia coli* (EPEC) infections. This study aimed at demonstrating the potential of a novel therapy for childhood diarrhea, a major cause of morbidity and deaths in Bangladesh and other developing countries, and thus a priority for improving child health: as shown in Table 1, oral phages showed a safe gut transit, even if it failed to achieve intestinal amplification and did not improve diarrhea outcome. This was possibly related to insufficient phage coverage and too low *E. coli* titres requiring higher oral phage dosage (ClinicalTrials.gov Identifier: NCT00937274).

Afterwards, a randomized, multicentric, open-label Phase I/II clinical trial started in 2015 is currently in a "recruiting phase." The aim of this trial is to assess tolerance and efficacy of a local bacteriophage treatment of wound infections due to *Escherichia coli* or *Pseudomonas aeruginosa* in burn patients. The treatment uses GMP-produced cocktails of anti-*E. coli* and anti-*P. aeruginosa* bacteriophages (Pherecydes Pharma), and the safety profile of this phage therapy will be compared to the safety profile of a standard of care. This is a European Research & Development (R&D) project funded by the European Commission under the 7th Framework Programme for Research and Development involving seven clinical sites in the EU (ClinicalTrials.gov Identifier: NCT02116010).

Finally, a French trial which is scheduled to start in 2017 aims to compare the efficacies of a standard treatment with the addition of phage therapy versus a standard treatment plus placebo for diabetic foot ulcers monoinfected by *Staphylococcus aureus*. It will be a randomized, multicentric, controlled, two-parallel-group, double-blind, superiority trial and will enrol 60 subjects (ClinicalTrials.gov Identifier: NCT02664740).

Phage therapy efficacy is still controversial and is practiced only in a few institutions worldwide, with the Eliava Institute of Bacteriophage (Tbilisi, Georgia) and the Institute of Immunology and Experimental Therapy of Polish Academy of Sciences (Wroclaw, Poland) being the leading centers; nevertheless, as mentioned above, many pharmaceutical companies involved in phage research are carrying out an increasing number of clinical trials.

As reviewed by Oliveira and colleagues, despite a growing appreciation for bacteriophages, different human bacterial pathogens remain to be targeted by phage preparations [59]. Among these, *Rickettsia*, *Ehrlichia*, and *Coxiella*, which can potentially cause severe diseases, should be investigated with the purpose of developing a competent phage therapy.

Neither immunological complications nor adverse effects have been shown after administration of extensive amounts of bacteriophages, but their usage in vivo can give rise to immune responses. For example, innate immunity and phagocytosis in the blood and liver can reduce phage titres after intravenous administration. An enhancement of major classes of immunoglobulin can also be induced, due by long-term treatments or phage exposure itself, and this can decrease the availability of active phages, reducing the effectiveness of the therapeutic approach. In order to prevent these undesirable immunological effects in the medical use, every individual phage immune response should be assessed, paying specific attention to route of administration, dose, accompanying compounds, and timing of exposure [60, 61].

At present, the choice of phage formulation is under debate because of the lack of information concerning the therapeutic outcome under different conditions. A simple aqueous formulation is the most applied, but long-term stability studies of different phage formulations would be pivotal to ensure the endurance of their therapeutic effect. Furthermore, an accurate study addressing the pharmacokinetic profile of phage preparations is essential for clinical applications. Indeed, phages are thought to control bacterial infections by means of "active" and "passive" treatments: the former one involves a secondary phage infection of bacteria before they are killed, while for the latter one, the initial dose of phages is sufficient.

Concerning phage therapy delivery, the parenteral (and more specifically intraperitoneal) route seems to be the most successful form of systemic administration; oral delivery was proved to be preferable for gastrointestinal *E. coli* infections, although the highly acidic and proteolytically active environment of the stomach represents a major issue since it undermines phage stability.

Moreover, timing of administration and concentration of phages are critical aspects affecting clinical results, and thus, they should be rigorously optimized by dedicated research for each phage or cocktail of phages. In this context, phage stability within different formulations should be taken into account to avoid any unacceptable loss of activity during the treatment [62].

5. Phage-Derived Proteins as Antibacterial Agents

The huge progress that has been made in molecular biology has opened up new possibilities in the design of recombinant phage-derived proteins. Encouraging results emerged for phage enzymes involved in the first step of viral infection responsible for bacterial envelope degradation, named depolymerases. Furthermore, proteins encoded by lysis-cassette genes (as holins and endolysins), which are responsible for progeny release during the phage lytic cycle, are arousing growing interest too. The main characteristics of these antibacterial agents and the results obtained regarding their biological activity in vitro and in vivo will be addressed in the next sections.

Table 1: Bacteriophage and phage-derived proteins tested in clinical trials.

Drug	Condition	Phase	Results	Identifier
WPP-201 bacteriophage	Venous leg ulcers	I	This study found no safety concerns with the bacteriophage treatment [57]	NCT00663091
T4 phage cocktail	Diarrhea	I/II	Oral phages showed a safe gut transit in children but failed to achieve intestinal amplification and to improve diarrhea outcome, possibly due to insufficient phage coverage and too low *E. coli* pathogen titres requiring higher oral phage doses [58]	NCT00937274
E. coli phage cocktail	Wound infection	I/II ongoing	No results published yet	NCT02116010
Topical anti-*Staphylococcus* bacteriophage therapy	Diabetic foot staphylococcal infections	I/II ongoing	No results published yet	NCT02664740
Lysin CF-301	*S. aureus* bloodstream infections	I ongoing	No results published yet	NCT02439359
VAPGH P128	Nasal *S. aureus* colonization *S. aureus*-infected venous ulcer	I/II completed	No results published yet	NCT01746654

5.1. Polysaccharide Depolymerases. As mentioned above, polysaccharide hydrolases (depolymerases) are enzymes used by phages for bacterial cell degradation, in particular targeting macromolecule carbohydrates within extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) [63]. Polysaccharides are of particular importance for survival of many encapsulated bacteria, as they promote host virulence by letting cell adherence to both biotic and abiotic surfaces, or by protecting cells from phagocytosis and antimicrobials [64]. They also represent a physical barrier to phages, protecting the receptors needed for adsorption and infection. Depolymerases are the enzymes they use for "stripping away" the extracellular bacterial polysaccharide, and they have therefore been tested as therapeutics [65]. To date, few therapeutic protocols have been tried in vivo. Dubos and Avery in 1931 were the first to demonstrate that these enzymes could protect mice from type III pneumococcal infection, acting on the capsule polysaccharides [66]. Later, other protection studies in mice, rabbits [67], and monkeys [68] described the control of lethal pneumococcal infections by a partially purified preparation of depolymerases. Finally, Mushtaq et al. showed that intraperitoneal administration of 0.25 mg ($\gg$0.83 mg/kg) of the endosialidase E-protected rats from neurotropic strains of *E. coli* infection [69], and Scorpio et al. similarly found that depolymerase capsule removal from *Bacillus anthracis* resulted in reduced resistance to phagocytosis and associated killing [70], protecting a mouse infection model [71].

Furthermore, these phage-derived enzymes may have a greater potential as therapeutic agents against biofilm formation, as the structural backbone of glycocalix is composed of bacterial EPS [72, 73]. Depolymerases can be effective in EPS removal, making the biofilm bacteria more susceptible to treatment by antimicrobials. Table 2 shows a list of studies where bacteriophages and their EPS depolymerases have been employed to fight experimental bacterial biofilms. All studies involved in vitro growth of biofilms.

5.2. Endolysins. Endolysins are enzymes produced at the end of the lytic phage lifecycle: they accumulate in the cytoplasm of the host bacterium until they translocate through holes formed by holins in the plasma membrane. Then, endolysins cleave peptidoglycan bonds in the cell wall causing cell bursting and release of progeny phages [81]. Their biological activity is of particular interest as they are able to lyse a target cell within seconds after contact [82, 83], causing holes in the bacterial wall with its consequent osmotic lysis and death [84]. These proteins are among the most active and safest substances able to kill bacteria, but their activity has a major constraint. In fact, endolysin antibacterial activity is particularly effective against Gram-positive bacteria, since they lack an outer membrane, unlike Gram negatives [85].

Lysin structure reflects this dissimilar biological activity. Endolysins derived from Gram-positive-infecting phages have a modular domain structure that shows a variety in its architecture [86]. It is composed of two highly conservative domains: a N-terminal catalytic domain and a C-terminal bacterial wall-binding domain, connected by a linker [87]. Endolysins can be divided into five different classes according to their enzymatic activity: (1) N-acetylmuramidases (lysozymes), (2) endo-β-N-acetylglucosaminidases, (3) lytic trans-glycosylases, (4) endopeptidases, and (5) N-acetylmuramyl-L-ala-amidases. Classes 1 to 3 can cleave sugars and class 4 peptides and class 5 serve to cleave peptide bonds between sugars and peptides. All endolysins are hydrolases, except for transglycolases; amidases and muramidases are the most represented classes [88]. The C-terminal domain specifically binds ligands on the bacterial wall, tethering the lysin to the proteoglycan: even if the number of binding domains varies between endolysins [86], the affinity is almost as strong as antigen-antibody binding [89].

Table 2: Biofilm control by EPS depolymerase studies in vitro.

Target pathogen	Observations	Concerns	References
Pseudomonas putida	Biofilm clearance: significant variations between bacterial strains upon biofilm aging	Reduction of aged biofilm susceptibility to phage infection because of their thickness and phenotypic changes	[74]
Klebsiella pneumoniae	Greater biofilm clearance if cotreated with ciprofloxacin	Possible inhibition of depolymerase activity by ciprofloxacin	[75]
Escherichia coli	Depolymerase-producing phage construct capable of biofilm clearance	Results obtained for an engineered T7 strain	[76]
Enterobacter cloace	Biofilm clearance with depolymerase-producing phage; enhancement of chemical antibacterial penetration after phage-free depolymerase treatment	Combinations of three phages required for eradication of single-species biofilms; ineffective treatment of dual-species biofilms	[77]
Pseudomonas aeruginosa	Old biofilm clearance (20 days)	Bacteriophage migration facilitated by reduction of alginate viscosity	[78]
Enterobacter agglomerans and *Serratia marcescens*	Phage-resistant bacteria biofilm clearance with treatment of phage or phage-free depolymerase	Little differences in the chemical composition of EPS prevent the degradation of the polymer	[79]
Enterobacter agglomerans	Dual-species biofilm clearance with phage-free depolymerase	Specific depolymerase: no degradation of single biofilms of *Klebsiella pneumoniae*'s EPS	[80]

On the contrary, lysins from phages that infect Gram-negative bacteria mainly present a globular structure and lack of cell wall-binding domain [90].

The modular structure of endolysins suggested the opportunity to engineer novel enzymes in order to improve their bacteriolytic potency, to broaden their lytic spectrum, or to avoid phage resistance. For example, chimeric enzymes (chimeolysins) can be obtained by substitution or addition of heterologous binding domains to expand the bacteriolytic spectrum outside the native endolysins species specificity [86, 91]. Further improvements to endolysin properties have been obtained so far after fusion to a peptide or a protein domain of nonendolysin origin (artilysins) [92]. Recent studies led to the design of some effective hybrid enzymes targeting Gram-negative pathogens: for example, a chimera of T4 lysozyme fused to the bacterial toxin pesticin, targeting FyuA, a major virulence factor for some *Yersinia* and pathogenic *E. coli* strains [93]; another artilysin was designed against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa* by fusing to the N-terminus of an endolysin, a sheep myeloid antimicrobial peptide, able to pass the outer membrane of Gram-negative bacteria via a self-promoted uptake pathway [94]. Finally, modular endolysins from Gram-negative-infecting phages are rather rare but endowed with stronger and faster activity than the globular ones [95]. They might represent potential candidates to control multidrug-resistant infections, and domain swapping may allow creating novel enzymes with higher specificity or efficiency, as for chimeolysins obtained from Gram-positive-infecting phages.

Purified endolysin activity against bacterial was first described in 1959 [96]. Since then, several endolysins have been characterized in in vivo studies. An overview of data obtained from in vivo preclinical trials, which addressed the use of these enzymes against animal models of human infections and diseases, is summarized in Table 3, organized by pathogen.

To date, there are currently no applications of phage-derived proteins approved or in Phase III in both European Union and USA [121]. However, several placebo-controlled clinical trials demonstrated safety of phage therapy, as the intravenous use of PlySs2 (CF-301) by ContraFect (ClinicalTrials.gov Identifier: NCT02439359), but no data have been published yet (Table 1).

The use of lysins as therapeutics offers distinct advantages compared to antibiotics for disease prevention and treatment. The biological action of these enzymes is rapid, as already described. They can be used on mucosae to kill colonizing pathogenic bacteria without altering the resident flora due to their specificity. For example, Nelson et al. described the activity of a lysin whose action is specific for streptococci of groups A, C, and E without any observed effect on several other oral streptococci or other commensal organisms of the upper respiratory tract [84]. Notably, they have less bacterial resistance issues than antibiotics [84] and seem to be effective as decontamination reagents [83]. Moreover, resistance development to endolysin catalytic activity is unlikely as there are no described cases of bacteria-losing sensitivity to these enzymes or resistant mutants obtained after in vitro exposure to sublethal concentrations of protein [122].

5.3. Virion-Associated Peptidoglycan Hydrolases (VAPGH). Another phage-derived protein is represented by the virion-associated peptidoglycan hydrolase (VAPGH, or tail-associated lysin). These enzymes are structural components of some phage particles and mediate the local hydrolysis of cell wall peptidoglycan, allowing the phage tail tube to access the cytoplasm for viral DNA transfer [123, 124]. VAPGH are present in phages infecting both Gram-negative and Gram-positive bacteria but show a high degree of similarity to endolysins: these proteins also present a modular structure, allowing engineering to broaden and increase their bacteriolytic activity.

Table 3: Summary of phage-encoded endolysins tested in vivo.

Target pathogen	Endolysin	Animal model	References
Acinetobacter baumannii	PlyF307	Bacteraemia	[97]
Bacillus anthracis	PlyG PlyPH	Sepsis Peritonitis	[98, 99]
Pseudomonas aeruginosa	LoGT-008 (Artilysin)	Gut decolonization	[100]
Staphylococcus aureus	ClyS λSa2-E-lyso-SH3b LysK/CHAPk LysGH15 MV-L PhiSH2 Phi11 PlySs2 Ply187AN-KSH3b SAL-1 Twort WMY 80α 2638A	Nasal decolonization Bacteraemia Sepsis Mastitis Endophthalmitis	[101–111]
Streptococcus agalactiae	PlyGBS/PlyGBS90–1 PlySK1249	Vaginal decolonization Oropharynx decolonization Bacteraemia	[112–114]
Streptococcus pneumonia	Cpl-1 Cpl-771 PAL	Bacteraemia Sepsis Endocarditis Meningitis	[115–120]
Streptococcus pyogenes	PlyC (formerly C1) PlyPy PlySs2	Oral decolonization Bacteraemia	[84, 97, 106]

The chimeric enzyme P128 is the only VAPGH therapeutically tested in vivo. A MRSA strain was instilled into nares of rats followed by 2 intranasal treatments of a hydrogel formulation of P128 (50 mg/dose), or as a 2% mupirocin ointment (30 mg/dose). P128 hydrogel treatment was able to have MRSA colonization, and no efficacy was observed with the second formulation [125]. Later, GangaGen performed a combined Phase I and Phase II clinical trial evaluating the intranasal use of P128 (ClinicalTrials.gov Identifier NCT01746654), but no results have been published yet (Table 1).

5.4. Holins. As described, endolysins are responsible for peptidoglycan degradation at the final stages of cell lysis. Their activity involves another class of enzymes, named holins, which act in two different pathways: holin-endolysin and pinholin-SAR (signal anchor-release) endolysin systems. In the former system, these enzymes are phage-encoded proteins involved in the massive permeabilization of the cytoplasmic membrane, allowing endolysins to translocate into the periplasm and attack the peptidoglycan [81]. In the latter system, endolysins are exported into the periplasm and accumulate as inactive proteins. De-energization of the cytoplasmic membrane by imbedded pinholes activates SAR endolysins [81, 126] that are localised in the periplasm before pinholin triggering; thus, the degradation of peptidoglycan occurs more evenly, in contrast to the cell rupture in the first system [81].

The bacteriostatic activity of the holin-like protein Tmp1 was firstly demonstrated by Rajesh et al. [123]. This gene could complement a holin-defective phage λ and produce visible plaques on *E. coli* lawns. Its overexpression strongly inhibited *E. coli* growth during induction, and lysates inhibited the growth of different Gram-positive bacteria [123]. Surprisingly, holin-promoted lysis was observed in strains that were insensitive to endolysin. Thus, the holin-LySMP mixture was able to extend the spectrum of the endolysin alone, showing an interesting activity against several strains of multidrug-resistant *S. suis* and *S. aureus* [127].

6. Pathogen Detection

Because of the lack of strict regulation of their use as therapeutics, phage-based products are more widely considered for detection of pathogens in areas, such as food industry, water quality surveillance, or bioterrorism, where contaminations are frequently responsible of gastroenteritis, respiratory and mucosa infections, and if not worse, *sequelae.*

6.1. Bacteria Biosensors. Gastrointestinal epidemics, as the outbreak occurred in Germany in 2011 caused by Shiga toxin-producing strain *E. coli* O104:H4, revealed a critical need of efficient tools for pathogen detection. Standard

microbiological methods for pathogen identification are time consuming; besides, molecular methods such as quantitative PCR (qPCR) or DNA hybridization require high-purity specimens [128]. Likewise, enzymatic assays such as ELISA are very sensitive but not suitable for high-throughput screenings. For these reasons, phage-based technology represents an alternative approach to prevent such outbreaks, and phages have been recently considered for biosensoring bacteria [129, 130]. To date, some systems have been developed with *E. coli* 0157:H7 as their target, and two main groups of applications are available: one uses inert phage particles or their proteins, the other requires infecting phages [131, 132]. In addition, phages are able to replicate at high titres and with low costs, they are more resistant to temperature or pH variations compared to antibodies and can be kept at room temperature without activity loss [133]. FASTPlaqueTB assay takes advantage of these characteristics for the detection of *Mycobacterium tuberculosis* in sputum: phages (Actiphages) that infect the slow-growing tubercle bacillus survive to the addiction of a virucide (Virusol) and are then detected through their plaques on nonpathogenic mycobacterial sensor cells [134].

6.2. Labelled Phages. Ulitzur and Kuhn (1989) were the first to incorporate a reporter for the bacterial luciferase gene (lux) into phage λ for detection of *E. coli*, allowing quick and sensitive detection by luminometer after its expression in bacterial culture. Other examples are the Listeria bacteriophage A511, in which Vibrio harveyi luxAB genes are inserted downstream of the major capsid protein, or bacteriophage for *Yersinia pestis* and *Bacillus anthracis*, whose rapid diagnosis is crucial [129, 130]. Luciferase assay is a fast and low-cost test, as it requires only 1 or 2 days to estimate luciferase expression into target cells detecting bioluminescence [135], so it can be used also in developing country laboratories [136]. Unfortunately, bacteriophage typing has important limitations such as phage resistance issues and difficulties related to phage stocks and propagating strains maintenance [137, 138]. In addition, other types of reporter genes can be inserted in phage genomes. For example, hyperthermophilic β-glycosidase gene has been tested for *Listeria* detection, with the important advantage that the amplification signal goes on as long as the substrate is provided, also when the host has already been lysed [139].

On the other hand, labelled phages can be chemically or genetically modified adding a fluorescent dye, nanoparticle, or protein, covalently bound onto the phage-coat surface in order to identify target bacteria by fluorescence microscopy. For example, Awais et al. constructed GFP-labelled phage PP01 specific to *E. coli* O157:H7: when propagated in bacteria, the intensity of green fluorescence increases. In addition, these phages have a defective lysozyme, so they are unable to mediate host lysis in order to avoid signal reduction due to bacteria lysis [131].

However, only a few phage kits for pathogen detection in human samples have reached the market, such as the previously described FASTPlaqueTB assay and the MRSA/MSSA Blood Culture Test (http://www.microphage.com/technology/) [140]. This is because these detection systems have to be disposable and single-use kits; thus, the nature of their components, especially if genetically modified, presents safety issues. Their usage must be constrained within an appropriate waste management protocol.

7. Conclusions

Bacteriophages, the most ubiquitous organisms on Earth, are viruses that infect bacteria and, for that reason, have been employed since their discovery in the development of therapeutics against infections. They are highly specific, very safe, and effective against their target pathogenic bacteria and rapidly modifiable in order to address new threats.

The advent of antibiotics, which saved patients' lives and had a pivotal role in the achievement of considerable advances in medicine and surgery, made this approach less exploited. Moreover, bacteriophage development has been obstructed by reduced financial resources, by scientific and regulatory hurdles (such as lack of quality control and of properly controlled studies), and by unfamiliarity with phages themselves. However, the emergence of an increasing number of antibiotic resistant species forced modern medicine to propose novel therapeutic strategies, and research on these viruses bloomed again.

Here, we presented an overview of different current applications of bacteriophages and their usages against infectious diseases. Phage display techniques allow the identification of neutralizing mAbs against several pathogens and have had a pivotal role in the rational design of effective vaccines. Phage therapy approaches, which take advantage of lytic phage characteristics, have inspired the start of many human clinical trials that have great potential as novel treatments of bacterial infections (Table 1). Phage-derived proteins gained appreciation as antibacterial agents and put their relevance into effect through polysaccharide depolymerases, endolysins, engineered endolysins, virion-associated peptidoglycan hydrolases, and holins. Finally, bacteriophages have proved useful as biosensors in pathogen detection.

On the other hand, because of their narrow specificity, the isolation of therapeutic phages could be technically demanding, and their successful use strongly depends on the ability to promptly identify the etiologic agent and to ensure in vitro the lytic efficacy of preparations against the appropriate bacterial strain. The selection of phage-resistant mutants could also be a possible issue of phage therapy, even if the mixture of different phage (i.e., "phage cocktails") should help preventing it by increasing the range of targets of phage preparations. In any case, phage production, purification, and characterization should be performed taking into account the latest findings and the state-of-the-art biotechnology.

In conclusion, although much work remains to be done, bacteriophages and their applications seem to be a valid alternative to traditional approaches for the prevention and treatment of bacterial epidemics.

Disclosure

Elena Criscuolo and Sara Spadini are co-first authors.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Elena Criscuolo and Sara Spadini contributed equally to this work.

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Research Article

Synthetic Peptides as Potential Antigens for Cutaneous Leishmaniosis Diagnosis

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This work's goal was to research new candidate antigens for cutaneous leishmaniosis (CL). In order to reach the goal, we used random peptide phage display libraries screened using antibodies from *Leishmania braziliensis* patients. After selection, three peptides (P1, P2, and P3) were synthesized using Fmoc chemistry. The peptides individually or a mixture of them (MIX) was subsequently emulsified in complete and incomplete Freund's adjuvant and injected subcutaneously in golden hamsters. Sera from the hamsters administered with P1 presented antibodies that recognized proteins between 76 and 150 kDa from *L. braziliensis*. Sera from hamsters which had peptides P2 and P3, as well as the MIX, administered presented antibodies that recognized proteins between 52 and 76 kDa of *L. braziliensis*. The research on the similarity of the peptides' sequences in protein databases showed that they match a 63 kDa glycoprotein. The three peptides and the MIX were recognized by the sera from CL patients by immunoassay approach (ELISA). The peptides' MIX showed the best performance (79% sensitivity) followed by the P1 (72% sensitivity), and the AS presented 91% sensitivity. These results show a new route for discovering molecules for diagnosis or for immunoprotection against leishmaniosis.

1. Introduction

Leishmaniosis is a disease with two principal manifestations: visceral and cutaneous form. These are among the major neglected parasitic diseases that are reemerging and affect about 350 million people worldwide [1]. The disease is caused by the protozoa of the genus *Leishmania* Ross, 1903. The parasites infect humans in the Americas, Africa, Asia, and Europe. In the New World, the cutaneous form of the disease is caused by different etiological agents: *Leishmania (V.) braziliensis, L. (V.) peruviana, L. (V.) guyanensis, L. (V.) panamensis, L. (V.) naiffi, L. (V.) lainsoni, L. (V.) shawi, L. (Leishmania) amazonensis*, and *L. (L.) mexicana*. In Latin America, the widest distribution is presented by *L. braziliensis* and *L. amazonensis.* Visceral leishmaniosis (VL) is caused by *L. infantum (*synonym: *L. chagasi)*, which is more severe and can cause death in the absence of correct diagnosis and early treatment [2].

Advances have been made regarding the diagnosis and prevention of cutaneous leishmaniosis (CL) over the past decade. Moreover, there is no single method that can be adopted as the gold standard [1]. Generally, the combination of two or more indirect techniques is needed to perform an accurate diagnosis [3–5]. The CL diagnosis is frequently based on clinical and epidemiological data associated with laboratory tests. Several laboratorial methods are applied for detecting antibodies and identifying the parasite *Leishmania*. Parasitological methods consist of isolating the parasite from skin ulcers (for cutaneous forms) or from bone marrow or lymph nodes (for visceral forms). Material may be examined while fresh or it can be inoculated in culture medium for multiplication of the promastigotes. Nevertheless, the parasitological method is an invasive practice and demands expertise. Another possibility is the use of techniques researching deoxyribonucleic acid (DNA) of the parasite. However, studies have indicated that parasitaemia may be episodic and may

lead to a low number of parasites at the time of collection and hence nondetection by polymerase chain reaction (PCR). In addition, molecular techniques require specific devices, and not all laboratories have such highly complex equipment [6–8]. When these approaches fail, immunological tests are used to provide indirect parameters for the diagnosis. The delayed hypersensitivity skin testing is used for the cutaneous form of leishmaniosis [9]. The tests can detect infection in a few weeks and evaluate cellular immunity. Serological tests that detect antibodies (humoral immune response) are also useful [6–8]. However, immunological tests using promastigotes or soluble protein as a sensitizing antigen source could limit test specificity [4]. The ELISA technique is an alternative method to diagnose cutaneous leishmaniasis (CL). It presents easy execution as an advantage and is able to analyze a great number of samples simultaneously. New antigens that are more specific and sensitive are necessary and could be obtained with the usage of new technology such as recombinant proteins or phage display.

Given this context, the search for purified antigens, with high sensitivity and safety, for the immunological diagnosis and prevention of CL is essential. In recent decades, many molecules have been described as candidate antigens for leishmaniosis serodiagnosis, including some that are purified molecules or soluble fractions of the parasite. In addition, antigens produced by genetic engineering or phage display have been investigated and validated for research on an antibody against *Leishmania* [10–14], especially for the visceral form [15, 16]. However, these antigens still require adequation regarding their sensitivity and specificity values for use in diagnosis and immunoprotection.

Phage display is being used to discover molecules for diagnosis and research in the selection of antigens to be used for the ELISA tests. The immunoscreening of phage-displayed peptide libraries represents an alternative when searching for new antigenic targets. The peptide or protein expressed on the surface of each phage particle can be selected for binding to the target molecules by an affinity selection process called biopanning [17]. Our group has successfully used this technique to identify the epitopes or mimotopes of different pathogens [18–21]. The technology also enables the selection of antigens useful in vaccine production given that there is no effective treatment for numerous diseases [22–24].

This study aimed to select and evaluate synthetic peptides obtained by phage display that can be used in cutaneous leishmaniosis serological diagnosis.

2. Materials and Methods

2.1. Preparation of Leishmania Antigens. The strains of *Leishmania* species were obtained from the cryobank of the Biotechnology Laboratory of the Federal University of Paraná (UFPR) and had previously been characterized by isoenzyme analysis. Antigen from the promastigote culture of *L. braziliensis* (MHOM/BR/94/M2903) was prepared as described by Szargiki et al. [3]. Briefly, cultured parasites were washed with saline 0.9, 0.3, and 0.9% and with phosphate-buffered saline (PBS), pH 7.2, respectively, resuspended in distilled water, and lysed by the freeze/thaw method, followed by sonication. The resulting product was centrifuged at 14,000g for 30 min at 4°C. The supernatant was filtered (in a 0.22 μm filter) and recovered, and it featured the soluble antigen (SA). The protein content of the SA was determined by the Lowry method [25]. Aliquots of the antigen were kept at −80°C until they were used.

2.2. Patients and Groups. Blood samples were collected from patients diagnosed with clinical CL caused by *L. braziliensis*. This is the main parasite that causes the cutaneous disease in this state of Brazil according to Szargiki et al. [3] and Ribas-Silva et al. [26]. Volunteers came from the Federal University of Paraná's Hospital or several regional departments of health in the same state. The sera obtained were tested by ELISA using the SAs of *L. braziliensis* as described by Szargiki et al. [3]. For parasitological diagnosis, smears from the lesion were taken, stained by May-Grunwald-Giemsa, and observed under an optical microscope (1000x) or skin biopsies of the lesion were ground and inoculated into Tobbie and Evans media, incubated at 24°C, and examined and subcultured every week [3]. As the negative control, serum samples were collected from thirty-seven patients who had no history of leishmaniosis or Chagas disease and no contact with patients infected with these diseases. To point out putative cross-reactivity, sera from patients suffering from other infectious diseases were included in the present study, namely, 10 patients with Chagas disease, 10 patients with leprosy, and 10 patients with tuberculosis. The study was approved by the local research ethics committee (Protocol 107/11-UP).

2.3. Anti-L. braziliensis Immunoglobulins. Immunoglobulins G (IgGs) of the sera from patients with positive ELISA tests were obtained by precipitation with ammonium sulfate followed by chromatography using protein G-agarose [27]. Anti-*L. braziliensis* IgGs were recovered from immunoblots. For that, *L. braziliensis* SAs were resolved by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [3] and transferred to a polyvinylidene difluoride (PVDF) membrane that was blocked with PBS (pH 7.4) with 0.3% Tween 20 (0.3% PBST), washed with 0.05% PBST (PBS + 0.05% Tween 20), and incubated with IgGs in 0.05% PBST. After washing, IgGs' binding antigens immobilized in the membrane were eluted with 0.1 M glycine and 0.15 M NaCl at pH 2.8 at room temperature for 30 min. Anti-*L. braziliensis* IgGs were dialyzed against PBS after neutralization with 1 M Tris-HCl (pH 9.0), and the protein concentration was determined by the Bradford method [28].

2.4. Phage Display. Four rounds of biopanning were performed by incubating four phage display random libraries, obtained from J. Scott (Simon Fraser University, Canada), which expressed 8-mer (LX_4), 12-mer (LX_8), 15-mer (X_{15}), and 17-mer (X_8CX_8) peptides [29], with anti-*L. braziliensis* IgGs. All steps were done according to Alban et al. [19, 20]. Initially, two immunotubes (Nunc, Roskilde, Denmark) were coated with 10 μg of protein G (Sigma-Aldrich, St. Louis, MI, USA) diluted in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) and incubated overnight at 4°C. The

immunotubes were washed with 0.05% Tris-buffered saline TBST (TBS-0.05% Tween 20), blocked with 3% bovine serum albumin (BSA) in 0.05% TBST for 1 h at 37°C, and washed again. In immunotube one, the incubation was done with 5×10^{12} phages from each library overnight at 4°C. In immunotube two, 5 μg/mL of anti-*L. braziliensis* IgG was used, and incubation overnight at 4°C was done. After the incubation period, the supernatant of immunotube two was discarded, and the supernatant of immunotube one was added to immunotube two, where it remained under incubation overnight at 4°C. After washing, the bound phages were eluted with 0.1 M glycine (pH 2.2) and 1 mg/mL BSA. After neutralization with 2 M Tris-HCl, pH 9.0, the eluted phages were amplified by infecting *Escherichia coli* K91 cells. In the second panning, 2.5 μg/mL of anti-*L. braziliensis* was used for coating and 1.5 μg/mL for the remaining ones. After four rounds, phage clones were isolated and screened by ELISA [19]. Briefly, microtiter plates were coated with 0.5 μg/mL anti-phage antibodies (Sigma-Aldrich, St. Louis, MI, USA), in 100 mM $NaHCO_3$, pH 8.6, overnight at 4°C. The bacterial supernatant containing phage particles was diluted at 1 : 2 in 2% casein and PBS (pH 7.4) and was added to each well. The plate was incubated for 1 h at 37°C, washed, and incubated for 1 h at 37°C with a patient serum pool that was positive based on ELISA, with *L. braziliensis* SAs diluted at 1 : 100 in the incubation buffer (0.25% casein in 0.05% PBST). After washing, the reaction was detected using an anti-human IgG (Fc-specific) peroxidase antibody. As a negative control, the supernatant of the *E. coli* K91 culture was used.

2.5. Peptide Sequencing and Synthesis. The most reactive clones in ELISA tests (those with absorbance at least twice as high as the negatives control) were selected for DNA sequencing and for the subsequent identification of the amino acid sequences inserted in the phages. Phage genomic DNA was extracted with QIAprep Spin Miniprep Kit (Qiagen). Peptide sequences of the phage were determined by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the reverse primer 5′-GCT GCA TCT TTT AGC AGC-3′. The peptide sequences were analyzed for similarity to protein sequences from *L. braziliensis* or *Leishmania (Viannia)* sp. using the BLAST program.

Syntheses were performed according to standard protocol by using solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with a ResPep SL automated peptide synthesizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany). The peptides were lyophilized, and their masses were confirmed by mass spectrometry using a MALDI-TOF/TOF Autoflex instrument (Bruker Daltonics, Bremen, Germany) and flexAnalysis software (Bruker Daltonics, Bremen, Germany).

2.6. Reactivity of Anti-Peptides' Antibodies against L. braziliensis Antigens. Peptides containing additional cysteine residue on the C-terminal end were conjugated to mariculture keyhole limpet hemocyanin (mcKLH) using the Imject Maleimide-Activated mcKLH Spin Kit (Pierce, Rockford, IL, USA). Four-week-old female golden Syrian hamsters (*Mesocricetus auratus*) were immunized with individual peptides (P1, P2, and P3) or with a MIX of them. Six groups, with ten hamsters for each antigen, were inoculated intradermally (ID), in the back foot with KLH peptide (20 μg for each one) dissolved in a saline solution (0.9% NaCl). For the first immunization, the antigens were emulsified in complete Freund's adjuvant. For the subsequent immunization, we used incomplete Freund's adjuvant. Three boosters were administered at 30-day intervals. As the control, a group received Freund's adjuvant alone under the same conditions. Nonimmune serum was used as the negative control, and immune serum was obtained after seven days of the final immunization. By the end of this period, the animals' blood was collected as well as serum obtained for detection of anti-peptide specific antibodies. The study was approved by research ethics committee (Protocol 26/2012-CEUA-UP).

To assess the production of anti-peptide antibodies, microtiter plates were coated with 2 μg/mL of peptide diluted in 0.05 M carbonate buffer solution, pH 9.6, and incubated overnight at 4°C. After blocking with 2% casein in PBS (pH 7.4), the hamster pool sera diluted at 1 : 50 in the incubation buffer were added to the wells, and the plates were incubated for 1 h at 37°C. After washing, the detection of the reaction was performed using the anti-hamster IgG (whole molecule) peroxidase antibody (Sigma-Aldrich, St. Louis, MI, USA). Western blot assay was performed to evaluate whether the anti-peptide antibodies produced in the hamsters recognized *L. braziliensis* antigens. To achieve this, SAs of *L. braziliensis* (100 μg) were electrophoresed on 15% polyacrylamide gel and transferred to a PVDF membrane, which, after blocking (0.3% PBST), was incubated with hamster anti-peptide pool sera diluted at 1 : 100 in an incubation buffer (3% BSA in 0.05% PBST). The reactivity was detected using anti-hamster IgG (whole molecule) peroxidase antibody at 100 ng/mL in an incubation buffer and revealed with 0.07% (p/v) of DAB (3,3′-diaminobenzidine tetrahydrochloride) in 50 mM Tris, 0.15 M NaCl (pH 7.6), and 0.08% (v/v) H_2O_2.

All procedures involving the animals were consistent with the recommendations laid out in the *Guide for the Care and Use of Laboratory Animals* of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br).

2.7. Evaluation of the Potential Diagnostics of Synthetic Peptide. To evaluate the antigenicity of peptides, ELISA procedures were optimized (data not shown). After determining the best condition, microtiter plates were coated with 100 μL of each peptide or with the mixture (MIX) at 5 μg/mL in 0.05 M carbonate buffer (pH 9.6). The plate was incubated overnight at 4°C. After washing with a solution containing 0.9% NaCl with 0.05% Tween 20, the plates were blocked with Protein-Free Blocking Buffer (Thermo Fisher Scientific) for 1 h at 37°C. Then, the plates were washed and incubated with the human sera diluted at 1 : 50 in phosphate-buffered saline (PBS) solution at pH 7.4 containing 0.1% BSA. The plates were kept at 37°C for 1 h, and later they were washed and incubated with anti-human IgG (Fc-specific) biotin antibodies at 0.25 μg/mL in 1% BSA, PBS pH 7.4 for 1 h at 37°C. The plates were washed and incubated with 1 μg/mL of

Table 1: Enrichment of anti-*L. braziliensis* IgG binding phage after four rounds of biopanning. The phages bound are expressed as the ratio of the phage output titer and the phage input titer.

Biopanning cycles	Phages added (CFU)	Phages eluted (CFU)	% of bound phages ($\times 10^{-2}$)	Enrichment
1	$2.9 \cdot 10^{11}$	$3.6 \cdot 10^{5}$	0.012	
2	$2.0 \cdot 10^{11}$	$3{,}8 \cdot 10^{7}$	1.900	158.3x
3	$2.0 \cdot 10^{11}$	$6.6 \cdot 10^{7}$	3.300	275x
4	$2.0 \cdot 10^{11}$	$9.8 \cdot 10^{7}$	4.900	408.3x

Table 2: Peptide sequences obtained after performing the phage display technique using immunoglobulins G specific to *Leishmania braziliensis*. The researches in GenBank were made using as microorganisms *L. braziliensis.*

Peptide 1	G	H	R	M	P	P	T	S	V	S	A	L	A	R	P	NCBI reference sequence
GP63	$\underset{59}{\mathrm{Q}}$	: Q	: H	R	**P**	**P**	G	**S**	**V**	**S**	**A**	**L**	: G	L	$\underset{73}{\mathbf{P}}$	XP_001562922.1
Peptide 2	T	M	V	P	K	E	P	N	P	L	S	G	L	R	K	
GP63	$\underset{204}{\mathrm{A}}$	: S	**V**	**P**	S	**E**	: **P**	G	V	: **L**	A	T	: A	V	$\underset{218}{\mathrm{I}}$	XP_001562922.1
Peptide 3	S	K	P	Q	P	N	N	F	K	L	N	S	L	G	S	
GP63	$\underset{276}{\mathbf{S}}$	: N	L	: R	G	: R	: D	: Y	: E	: V	P	V	**L**	: S	$\underset{290}{\mathbf{S}}$	XP_001562922.1

The numbering below the alignment refers to the amino acid position in the protein sequence.
Bold letters denote identical amino acid residues.
The colon symbol (:) indicates amino acid residues with similar properties. Among the properties that can be shared between amino acids are hydrophobicity, polarity, charge, and presence of aromatic rings.

NeutrAvidin-peroxidase at 12.5 ng/mL with 1% BSA diluted in PBS pH 7.4, for 1 h at 37°C. A cutoff point for optimal sensitivity and specificity for the ELISA tests was determined using the Receiver Operating Characteristic (ROC) curve analysis, as described by Metz [30] and Zweig and Campbell [31]. The ROC curve enables the evaluation of the overall accuracy through the area under the ROC curve (Area under Curve, AUC) and the study of sensitivity and specificity with various cutoff points. The information generated subsidizes the identification of the optimal cut point. The curve analysis was performed using MedCalc 13.2.0 (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1. ELISA Using Soluble Antigens (SAs) of L. braziliensis and Screening of Phage-Displayed Peptide Libraries against Anti-L. braziliensis IgG. Of the 82 serum samples obtained from patients with CL, 57 were positive against the SAs of *L. braziliensis*. Thus, the CL patients were divided into two groups: Group 1 (G1) consisted of 25 CL patients with positive culture and indirect ELISA test positive using SAs antigen and Group 2 (G2) consisted of 57 CL patients with clinical diagnosis and indirect ELISA test positive for SAs antigen. Anti-*L. braziliensis* immunoglobulins G obtained in Group 2 were precipitated, purified, and incubated with phages that displayed peptide libraries. For each selection round, enrichment was monitored for antibody-specific phage by calculating the percentage of the phage that was bounded. A progressive increase in the phage recovery after each selection round was observed, indicating that specific enrichment of anti-*Leishmania*-binding phages occurred (Table 1).

3.2. Peptide Sequences. The fourth selection cycle was chosen to infect a culture of *E. coli* and obtain isolated colonies on plates containing LB medium. Each clone was tested by ELISA using the culture supernatants and sera from patients with high absorbance (tested initially against soluble antigens (SAs) of *L. braziliensis* in ELISA tests presenting absorbance above 0.5). Thus, 428 clones were tested and only 36 of these were reactive against the sera of patients with cutaneous leishmaniosis and three different peptides sequences were identified.

The peptides sequences were compared with others deposited in the GenBank to verify homology to proteins of *L. braziliensis*. The analysis between the peptide sequences and *L. braziliensis* proteins showed that they are like a 63 kDa glycoprotein of *Leishmania* (Table 2). Bold letters denote the consensus sequences between the mimotopes and the *Leishmania* proteins sequence from GenBank.

3.3. Immunogenicity of Peptides In Vivo and Antibody Capacity to Recognize Leishmania. To evaluate the presence of anti-peptide antibodies in hamsters, sera from immunized animals with peptides were assessed by ELISA (Figure 1).

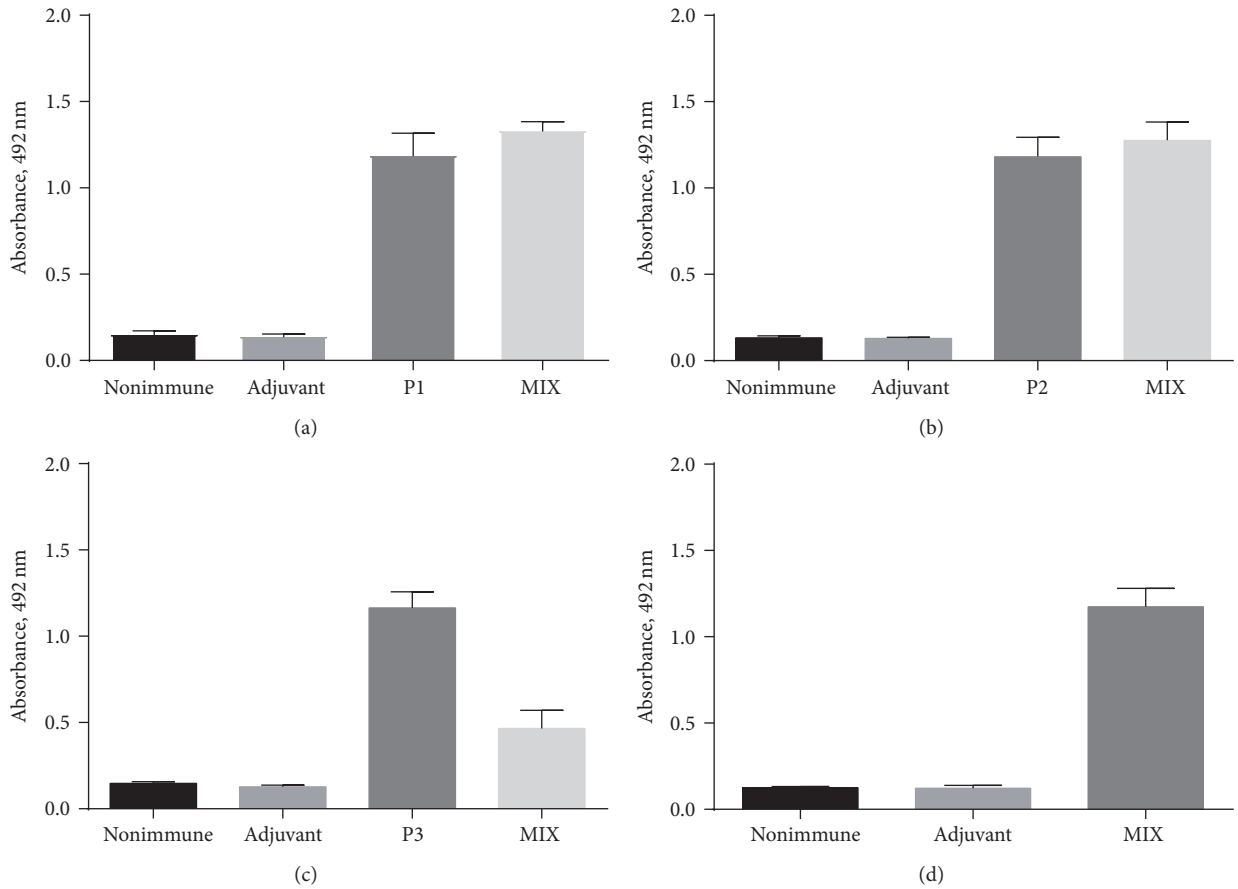

Figure 1: Reactivity of peptides with sera from immunized hamsters by ELISA. P1 peptide (a), P2 peptide (b), P3 peptide (c), and MIX of P1, P2, and P3 (d) were tested with anti-peptide serum P1 (P1), anti-peptide serum P2 (P2), anti-peptide serum P3 (P3), anti-peptide serum MIX (MIX), nonimmune serum, and serum against Freund's adjuvant. ELISA plates were coated with peptide (2 μg/mL) and incubated with hamster serum diluted at 1 : 50 (pool of sera with ten animals in each group). The detection reaction was conducted with anti-hamster IgG (whole molecule) peroxidase antibody (200 ng/mL).

All animals that were immunized with the peptide showed reactivity toward peptides 1, 2, and 3 alone and against the pool of peptides. Nonimmunized hamsters and those that were only given the adjuvant did not produce antibodies against the peptides. Anti-peptide antibodies were reactive to the SA of *L. braziliensis* in evaluation in vitro by western blot (Figure 2).

3.4. Performance of Peptides for Serodiagnosis of Cutaneous Leishmaniosis. The three individual peptides and the MIX of them were immunoreactive against the sera of patients in both patients groups G1 and G2. ELISA plates were coated with 5 μg/mL of individual peptide 1, 2, or 3 or MIX (equal concentrations of the three peptides totaling 5 μg/mL) and incubated with human serum diluted at 1 : 50. The detection was performed with anti-human IgG (Fc-specific) biotin antibody and NeutrAvidin-peroxidase. For the test with SA, the plates were coated with 0.5 μg/mL of SA and incubated with a 1 : 100 diluted human serum. The detection was performed with anti-human IgG (Fc-specific) peroxidase. The horizontal line represents the cutoff (provided by the ROC curve). The geometric shapes represent individual results (Figure 3).

The MIX of peptides showed 79% sensitivity and P1 showed 72%. The specificities of antigens ranged from 78 to 100%, demonstrating the ability to correctly diagnose the healthy individuals (Figure 4).

For the patients of the G1, when comparing antigens P1, P2, and P3 and the MIX of them with AS, a significant difference was found between MIX and AS ($p = 0.045$). For the patients of the G2, comparing antigens P1, P2, and P3 and MIX with AS, a significant difference was found between P3 and AS ($p < 0.001$) and between MIX and AS ($p < 0.001$). In both groups, no difference was found between P1 and AS and between P2 and AS. In summary, peptides P1 and P2 are not significantly different from AS and may present similar results in the diagnosis of CL.

The use of soluble antigen (SA) in serum of patients with Chagas' disease showed higher cross-reactivity (6/10). For P1, cross-reactivity was observed with Chagas disease (Table 3).

4. Discussion

Phage-displayed peptides are called mimotopes because they are not homologue sequences to the antigen. However, they

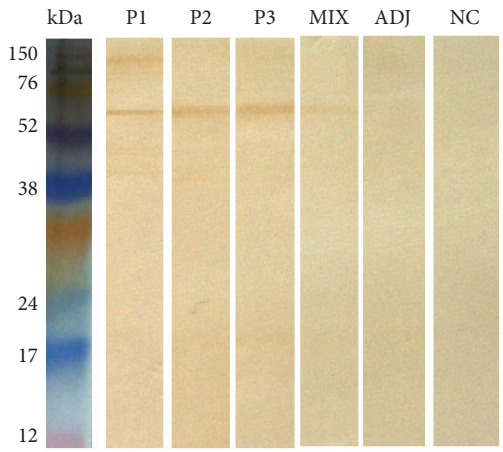

Figure 2: Anti-peptide antibody reactivity to *L. braziliensis* antigens by western blot. Forty micrograms of *L. braziliensis* antigen was separated by 15% SDS-PAGE and then, using western blot, was reacted with anti-peptide serum P1 (lane 1), anti-peptide serum P2 (lane 2), anti-peptide serum P3, anti-peptide serum MIX, sera against Freund's adjuvant (lane 5), and NC = negative control → nonimmune serum (lane 6). Sera from the immunized hamster were tested at a 1 : 100 dilution. The detection reaction was performed with anti-hamster IgG (whole molecule) antibody conjugated to peroxidase (100 μg/mL).

Table 3: Reactivity of peptides (P1, P2, and P3 and MIX) and soluble antigen (SA) against sera from patients with Chagas disease, leprosy, and tuberculosis. The cut-off was provided by the ROC curve.

Antigen	Cut-off	Chagas disease	Leprosy	Tuberculosis
P1	0.351	0/10	1/10	3/10
P2	0.327	1/10	2/10	1/10
P3	0.235	2/10	2/10	3/10
MIX	0.192	0/10	1/10	2/10
AS	0.101	6/10	2/10	0/10

MIX: P1 + P2 + P3; SA: soluble antigen of *Leishmania braziliensis*.

can induce antibodies that recognize the mimotope and the original antigen owing to conformational similarities between them [18]. Our group found evidence that supports phage-displayed peptides as promising biotechnological tools for the design of neglected disease diagnostic serological assays [18–23, 32, 33]. The phage display through specific antibodies and selected amino acid sequences can be identical or present physicochemical characteristics or spatial organization similar enough to the original epitope to induce an immunoprotective response [34]. In the present study, which aimed to select peptides for *L. braziliensis* diagnosis infection, we selected 36 of 428 phage clones that showed recognition against antibodies from the patients' pool sera that exhibited high titer with *L. braziliensis* SAs, in ELISA test. Three peptides were synthetized and hamsters were immunized with them. Antibodies from animals recognized proteins from *L. braziliensis* SAs. After that, we have shown that the mimotopes can identify human cutaneous leishmaniosis on ELISA approach. The peptides have induced antibodies that recognized 52–76 kDa protein in a SDS-PAGE assay. Analyzing the mimotopes' sequence, it was verified that possibly we are dealing with the GP-63 protein which is a *Leishmania* spp. surface protease that is involved in parasite virulence and host cell interaction [35, 36]. GP-63 is a glycoprotein that represents 1% of the total of the promastigotes and is crucial for evasion of host cells from immune response. It is responsible for inactivating the lysosomes enzymes and influences the CD4+ T-cells lymphocyte activity by reducing the cell-mediated immune response [37, 38]. In addition, it cleaves intracellular peptides, preventing the presentation of antigens and inhibiting the chemotaxis of macrophages [39]. Many researches have been conducted seeking to develop vaccines against leishmaniasis using the GP-63 [40–43].

In the second step of our work, all peptides, individually or in MIX, recognized antibodies against *Leishmania* in patient serum with cutaneous leishmaniosis. When tested in immunodiagnosis by ELISA assay, the P1 antigen showed the best sensitivity/specificity. In general, P1 and MIX showed the best results (64 to 72% and 72 to 79% sensitivity for groups G1 and G2, resp.) between the peptides here tested. The ELISA test using SA of *L. braziliensis* promastigote showed sensitivities of 80 to 91% in the analysis of patients of G1 (with parasites isolated) and G2 (only with clinical diagnosis). These different values may be related to the panel-evaluated sera with procedures performed during the production of antigens, such as the incubation time of the reagent, the blocking solutions that were used, and the types of plates that were used [44]. Variations in the studied population can also create differences in sensitivity profile and the specificity of the tests. In patients with recent injuries (1 to 6 months of evolution), frequent serologic negativity and greater sensitivity to parasitological testing have been detected [3]. Another concern regarding the use of SA in ELISA is the existence of different strains of *Leishmania* behavior in different culture media. Studies show that expression can occur from different antigenic epitopes when the parasites are grown in media with different formulations [45, 46].

The results obtained here with the peptides are of extreme importance considering the need to produce a new ELISA test for the diagnosis of CL and the scarcity of studies that have investigated this form of the disease. The ELISA test offers advantages in terms of its application for immunodiagnosis of leishmaniosis. This is because it has versatility: its results are not being dependent on the observer in the culture and can be adapted for automation, allowing its use on a large scale. Obtaining purified antigens for the diagnosis of infectious and parasitic diseases is promising because it provides reproducibility of the tests. These molecules are stable and do not depend on specific means for their production cultivation.

Most studies concerning the detection of leishmaniosis have investigated the diagnosis of the canine visceral leishmaniosis form of the disease. These studies reveal that the sensitivities and specificities found using synthetic peptides or recombinant proteins in ELISA were, respectively, 75 and 90% [12], 88 and 95% [47], 76 to 100% and 90 to 97% [15], and 100 and 98% [48]. As for human VL sensitivity and specificity,

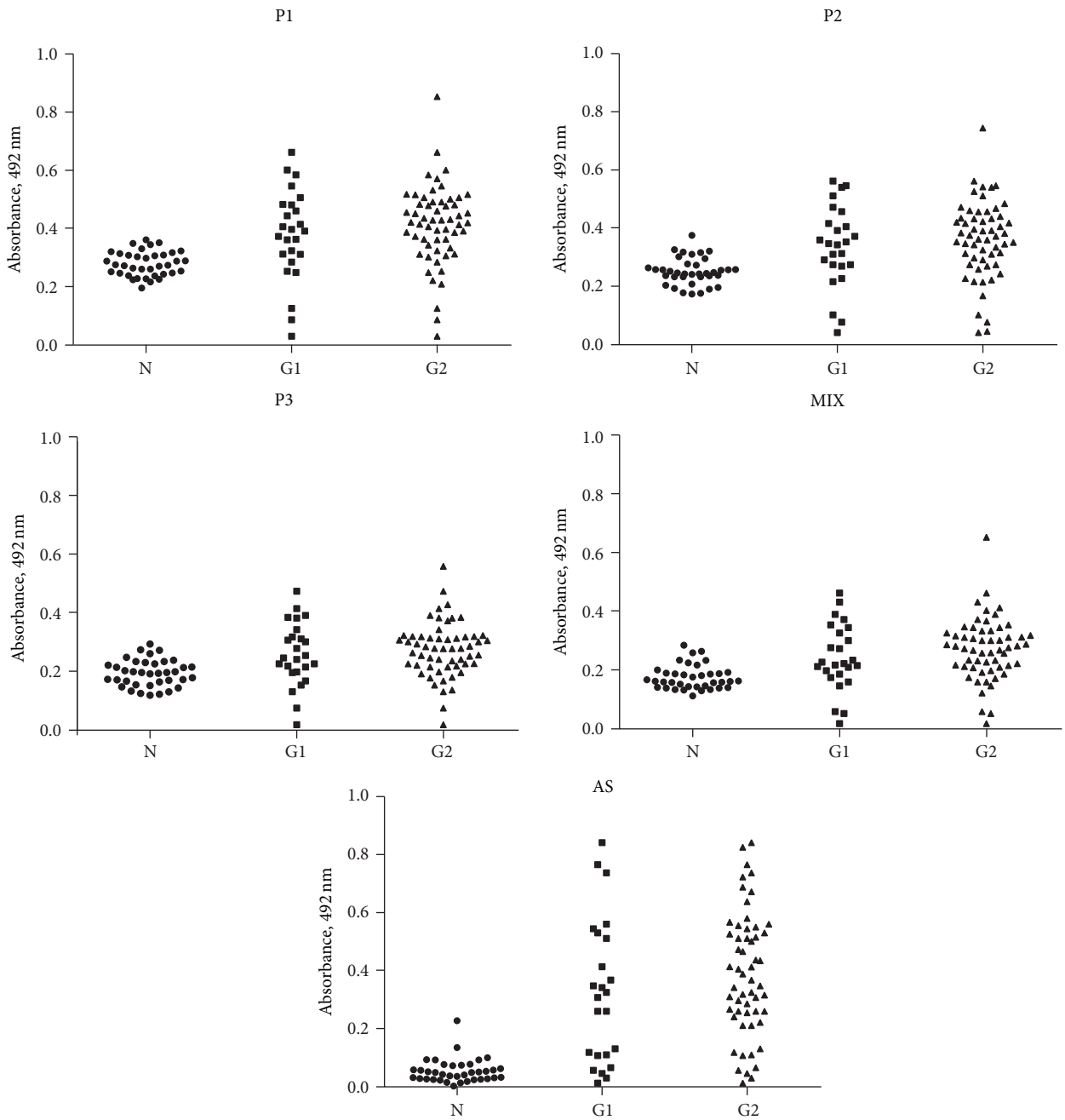

Figure 3: Reactivity of peptides P1, P2, P3, MIX, and SA against sera from CL patients (G1: Group 1, G2: Group 2) and negative control (N).

the figures stand at 100 and 100% [49], 81 and 10% [50], and 76 to 100% and 90 to 97% [15].

Analyzing the cross-reactivity, *Trypanosoma cruzi* is the agent with the highest reactivity in all antigens used. This can be explained by the phylogenetic relationship between *T. cruzi* and *Leishmania* [51]. This cross-reaction is more important in the visceral leishmaniasis form, given the clinical signs of the disease. The cross-reactivity was resolved when it was used with the MIX of the peptides. This information is of paramount importance, since only immunogenic molecules can generate specific antibodies. Since these three peptides have this property, they could prove useful as immunization tools and for use in other immunological strategies.

New researches highly recommend testing these antigens using other immunological methodologies, for example, multiplex ELISA that has the advantage of allowing simultaneous analysis of multiple peptides. Otherwise, in regions with few resources, immunochromatographic analyses or skin test could be performed like the Montenegro Test. In addition, the peptides obtained in this study show similarity with sequences of virulence factors associated with *Leishmania* spp. The next step will be to isolate the protein

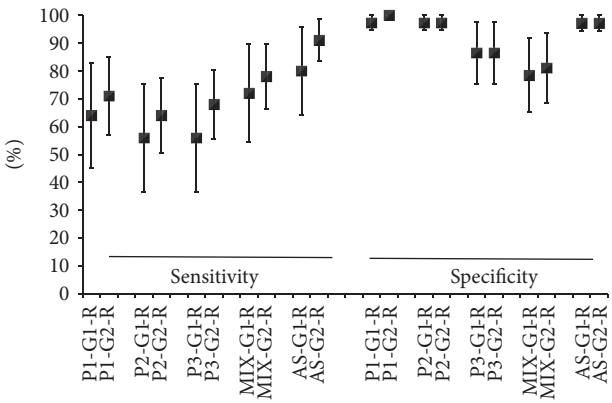

Figure 4: Performance of ELISA test with synthetic peptides and sera of patients with cutaneous leishmaniosis. P1: peptide 1; P2: peptide 2; P3: peptide 3, MIX: P1 + P2 + P3; G1: Group 1 (patients with clinical cutaneous leishmaniosis and parasites isolated); G2: Group 2 (patients with clinical cutaneous leishmaniosis and serological diagnosis positive); SA: soluble antigen.

and make the sequencing. It can be postulated that prior inoculation of these virulence factors could disrupt the course of a future infection, and thus these molecules could be used in immunoprophylactic strategies.

Competing Interests

The authors declare that they have no competing interests regarding the publication of this paper.

Authors' Contributions

Juliana Seger Link and Silvana Maria Alban contributed equally to this work.

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Research Article

Secreted Interferon-Inducible Factors Restrict Hepatitis B and C Virus Entry In Vitro

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Interferon-α (IFN-α) has been used for more than 20 years as the first-line therapy for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, because it has a number of antiviral effects. In this study, we describe a novel mode of its antiviral action. We demonstrate that the supernatant from IFN-α-treated cultured cells restricted HBV and HCV infection by inhibiting viral entry into hepatoma cells. The factors contained in the supernatant competed with the virus for binding to heparan glycosaminoglycans—the nonspecific attachment step shared by HBV and HCV. Secreted factors of high molecular mass that bind to heparin columns elicited the antiviral effect. In conclusion, IFN-α is able to induce soluble factors that can bind to heparan glycosaminoglycans thus leading to the inhibition of viral binding.

1. Introduction

Interferon (IFN) was first discovered in 1957, based on the observation that cells challenged with heat-inactivated influenza virus secreted a macromolecule that was able to "interfere" with several viruses, such as the infectious influenza virus or vaccinia virus [1]. This study and follow-up researches led to the development of IFN as a therapeutic tool to treat a number of infectious diseases, in particular chronic hepatitis B and hepatitis C. IFN elicits antiviral actions by inducing a wide array of IFN-stimulated genes (ISGs). By interacting with their specific receptors, IFN activates signal transducer and activator of transcription (STAT) complexes. Phosphorylated STAT then activates the classical Janus kinase-STAT signaling pathway and initiates the transcription of different ISGs [2].

The mechanisms by which these ISGs participate in the IFN-mediated response to hepatitis B virus (HBV) or hepatitis C virus (HCV) are not fully understood. Previous studies demonstrated that IFN inhibits HBV replication at multiple steps of its life cycle, by deaminating and degrading the viral transcription template covalently closed circular DNA (cccDNA) through apolipoprotein B mRNA editing enzyme catalytic subunit 3A [3–5], silencing cccDNA through epigenetic regulation by the STAT complex [6, 7], downregulating viral mRNA stability through antiviral zinc finger proteins [8, 9], inhibiting viral pregenomic RNA encapsidation via myxoma resistance protein 1 [10, 11], and reducing virion secretion by Tetherin [12]. IFN also promotes viral nucleocapsid degradation [13].

HCV and host ISGs show a much more complex interaction. For example, cellular pattern recognition receptors detect molecular patterns of HCV [14, 15], thereby forming a positive feedback loop to amplify IFN signaling. Many ISGs are reported to inhibit HCV RNA replication or viral protein translation, either directly or indirectly, including RNA-specific adenosine deaminase [16], viperin [17], and $2'$-$5'$-oligoadenylate synthetase [18]. IFN also induces transmembrane protein 1 (IFITM1) which was reported to inhibit HCV entry [19].

Virus entry can be a multistep process, in which the virus first attaches to cell type unspecific molecules, then binds to its specific receptor, and enters the cell. Heparan sulfate is present on the surface and in the extracellular matrix of

all mammalian cells and serves as an attachment factor or anchor for a number of enveloped viruses such as herpes simplex virus [20], respiratory syncytial virus [21], human immunodeficiency virus [22], cytomegalovirus [23], Dengue virus [24], HBV [25], and HCV [26], as well as nonenveloped viruses such as human papillomavirus [27] and foot-and-mouth disease virus [28].

Although the influence of ISG products on the replication of HBV or HCV has been studied extensively, little is known about the influence of IFN treatment on early steps of the virus life cycle. Here, we investigated whether IFN-α was able to induce soluble factors that would have extracellular antiviral activity. Our study reveals a novel antiviral mechanism of IFN-α. Upon IFN-α treatment factors are secreted that bind to heparan glycosaminoglycans—the attachment receptor of many viruses including HBV and HCV—thus leading to the inhibition of virus attachment and blocking infection.

2. Materials and Methods

2.1. Cell Cultures. HepaRG cells were cultured in Williams E medium (Gibco, Carlsbad, USA) supplemented with 10% fetal calf serum FetalClone II (HyClone, Little Chalfont, United Kingdom), 20 mM L-glutamine (Gibco, Carlsbad, USA), 50 U/mL penicillin/streptomycin (Gibco, Carlsbad, USA), 80 μg/mL gentamicin (Ratiopharm, Ulm, Germany), 0.023 IE/mL human insulin (Sanofi-Aventis, Paris, France), and 4.7 μg/mL hydrocortisone (Pfizer, Carlisle, USA) as described [29]. The cell cultures were maintained in a 5% CO2 atmosphere at 37°C. For infection, cells were maintained for 2 weeks in standard medium and then differentiated for 2 more weeks in medium supplemented with 1.8% DMSO (Sigma, Munich, Germany).

2.2. HBV Production. HBV was concentrated from the supernatant of HepG2.2.15 cells using centrifugal filter devices (Centricon Plus-70, Biomax 100.000, Millipore Corp., Bedford, MA) and quantified by HBV-DNA qPCR. Immediately after collection, the virus stock was divided into aliquots and stored at −80°C until use.

2.3. HBV Infection. The inoculation of differentiated HepaRG cells was performed with a multiplicity of infection of 200 (genome copies per cell) in differentiation medium containing 5% PEG 8000 (Sigma, Munich, Germany) for 16 h at 37°C. At the end of the incubation period, cells were washed three times with PBS and cultured in differentiation medium.

2.4. Analysis of HBV Replication. HBsAg was measured using the AXSYM system (Abbott, Chicago, USA), and HBeAg was measured by the BEP III system (Siemens, Munich, Germany). Total cellular DNA or DNA from cell culture supernatant were extracted from infected cells using NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Real-time quantitative PCRs (qPCRs) were performed using the LightCycler™ system (Roche, Manheim, Germany) and HBV-DNA and cccDNA were detected using specific PCR primers (Table 1). HBV-DNA from cell culture supernatant was quantified relative to an external plasmid standard. Intracellular HBV-DNA and cccDNA are expressed as normalized ratio to the genomic single copy gene of the prion protein (PRNP).

Table 1: Primers for qPCR.

Name	Sequence 5′-3′
cccDNA 92 fw	GCCTATTGATTGGAAAGTATGT
cccDNA 2251 rev	AGCTGAGGCGGTATCTA
PRNP fw	GACCAATTTATGCCTACAGC
PRNP rev	TTTATGCCTACAGCCTCCTA
rcDNA1745 fw	GGAGGGATACATAGAGGTTCCTTGA
rcDNA1844 rev	GTTGCCCGTTTGTCCTCTAATTC

2.5. Western Blot. Lysates from HepaRG cells were obtained by adding 200 μL "M-PER Mammalian Protein Extraction Reagent" (Thermo Scientific, Schwerte, Germany) onto cells per well and incubated at 37°C for five minutes. 50 μL "LDS sample buffer Nonreducing" was added and samples were shaken for five minutes at 800 rpm, 99°C. Proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, proteins were blotted onto a PVDF membrane. The membrane was blocked with 5% milk for one hour at room temperature followed by overnight incubation with polyclonal rabbit anti-HBV core antibody (gift from Heinz Schaller) at 4°C.

2.6. Virus Heparin Attachment Assay. 96-well plates were coated with 100 μL of a 25 μg/mL heparin solution per well and incubated at 4°C overnight. The wells were washed 3 times with PBS. Then 100 μL of samples was added and the plate was incubated overnight at 4°C. The samples were aspirated and the plate was washed 3 times with PBS. 300 μL of blocking buffer (1% BSA solution in PBS-T) was added to each well and incubated for 2 h at 37°C. After that, the plate was washed 3 times with PBS-T.

100 μL of blocking buffer, 1 μL of virus stock solution, and 50 μL Murex Conjugate solution (from Murex HBsAg Version 3 Kit, Saluggia, Italy) were added per well. The plate was incubated at 37°C for 1 h followed by 4 times with PBS-T washing. Then, 100 μL of substrate solution was added per well. After 1 h incubation at 37°C, 50 μL stop solution (1 M H_2SO_4 in ddH2O) was added per well. Light absorption was measured at 450 nm, with 670 nm reference wavelengths with an Infinite 200 (Tecan, Männedorf, Switzerland).

2.7. Enzymes Treatment Assay. Samples were treated with 50 U/mL N-glycosidase F beads (EDM Millipore, Bedford, MA) overnight at 37°C, or 0.0005% Trypsin (Gibco, Carlsbad, USA) for 1 hour at 37°C, or 0.1 μg/mL proteinase K for 1 hour at 37°C. After incubation, 5 mM protease inhibitor Pefabloc (Sigma, Munich, Germany) was added to the samples. Finally, the samples were purified with Vivaspin MWCO 3000 Spin-Column (Gelifesciences, Freiburg, Germany).

2.8. Preparation of Luciferase Reporter HCV Stocks. Huh7.5 cells were electroporated with reporter constructs pFK-Luc-Jc1 as described previously [30]. Culture supernatant of transfected cells was harvested and filtered through 0.45 μm Stericup® Filter Units (SCHVU11RE, Millipore, Germany) and precipitated with 8% PEG8000 at 4°C overnight. Virus was pelleted by centrifugation at 5000 ×g for 2.5 h at 4°C, resuspended in DMEM, and stored at −80°C before use. Each preparation was titrated for its infectivity by limiting dilution assay on Huh7.5 cells and 50% and the tissue culture infective dose ($TCID_{50}$) was calculated based on the method described [31].

2.9. HCV Infection and Luciferase Assay. Huh7.5 cells were seeded in a 96-well plate (1×10^4/well) for 24 h prior to HCV inoculation. 0.1 MOI/cell of HCV luciferase virus were used to infect the cells for 72 h. 10 μL of prepared "elution" and "concentrate" was present in the 200 μL culture medium during the whole process of HCV infection. Luciferase assays were performed using the assay kit (E1500, Promega, USA) following manufacturer's instructions. Luminescence was measured in programmed inject-then-read mode (Infinite F200, Männedorf, Switzerland). All luciferase assays were performed in triplicate.

2.10. Statistical Analysis. Student's unpaired two-tailed t-tests were performed with GraphPad Prism 5.0a (GraphPad Software, La Jolla, CA, USA). Data are means ± s.d. Two-sided P values < 0.05 were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Pretreatment with IFN-α Inhibits HBV Replication. We first sought to investigate whether pretreatment of HepaRG cells with IFN-a before infection with HBV would have an effect on establishment of HBV infection (Figure 1(a)). IFN-α pretreatment resulted in a decline of HBeAg and HBsAg at day 10 upon HBV infection (Figures 1(b) and 1(c)). Concomitantly, intracellular HBV replication markers at day 10 were analyzed, and qPCR analysis revealed more than 50% reduction of cccDNA and intracellular HBV-DNA after IFN-α pretreatment (Figure 1(d)). Western blot analysis showed the decline of intracellular HBV core protein production (Figure 1(e)). These results suggested that the pretreatment of cells with IFN-α for 24 hours is sufficient to induce an antiviral effect and the IFN-induced antiviral factors sustained this activity during infection. These results also implied that IFN-α induces an antiviral program preventing HBV early infection.

3.2. Interferon-Inducible Secreted Factors Restrict Early Steps of HBV Infection. In order to determine whether the IFN-α induced antiviral program would prevent initiation of HBV replication or inhibit attachment or entry into the host cell a conditioned medium was prepared from IFN-α treated cells (Figure 2(a)). The medium containing the ISG products (ISG+) was collected and added together with HBV to differentiated HepaRG cells. Compared with untreated cells, ISG+ medium treated cells showed decreased cccDNA and intracellular HBV-DNA demonstrating the inhibitory effect of ISG+ medium (Figure 2(b)). The ISG+ medium did not have an effect when it was applied to cells that had already been infected (Figure 2(c)).

To exclude that the antiviral action elicited by ISG+ medium was due to residual IFN-α, neutralizing antibodies (IFN-α-Ab) were applied. When either IFN-α or ISG+ medium was added concomitantly with the HBV inoculum, HBV infection was inhibited, but only the effect of IFN-α but not that of ISG+ medium was neutralized by IFN-αAb (Figure 2(d)). This proved that the antiviral activity of ISG+ medium was not elicited by residual IFN-α.

Taken together, the experiments show that ISG+ medium decreased HBV infection in cells treated before or during HBV infection, whereas no effect was observed if cells were treated following HBV infection. Thus we concluded that IFN-α treatment induces hepatoma cells to secrete soluble factors, and those soluble factors inhibit HBV infection by targeting an early step of HBV infection, most likely virus entry.

3.3. Interferon Induced Factors Interrupt HBV Binding. To further characterize the effect of interferon induced factors, we performed a virus attachment assay (Figure 3(a)). Differentiated HepaRG cells were incubated with mock (untreated differentiated HepaRG cell culture supernatant), ISG+ medium, heat-inactivated ISG+ medium (ISG+ inactivated), or the synthetic antilipopolysaccharide peptide Pep19-2.5, which served as a positive control as it has been shown to inhibit the binding step of many enveloped viruses including HBV [32]. Cells were then inoculated with virus particles for 4 hours at 4°C, because low temperature prohibits HBV entry into the host cell. Hereby, we found that interferon induced soluble factors could inhibit HBV binding as efficiently as Pep19-2.5, and heat inactivation led to attenuation of the antiviral effect (Figure 3(b)).

These results demonstrate that HBV binding to its host cell was impeded by treatment with ISG+ medium containing interferon induced soluble factors. We therefore speculated that the induced antiviral proteins associated with the virus itself or with one of the essential viral attachment factors or receptors.

3.4. Interferon Induced Factors Interrupt HBV Binding to Heparin Sulfate. Experimental evidence has been presented that HBV initiates infection of hepatocytes by binding to heparan sulfate proteoglycans [25]. We therefore analyzed whether IFN-α induced factors can interrupt the interaction between HBV and heparin, which is a close homologue of liver heparin sulfate [33]. We applied cell culture supernatant of untreated, differentiated HepaRG cells, ISG+ medium, or Pep19-2.5 on heparin columns and analyzed the residual binding capacity of HBV (Figure 4(a)). HBV virions efficiently bound to heparin-Sepharose under physiological salt conditions (mock) and could be eluted using high salt concentration (Figure 4(b)). HBV particle binding to heparin

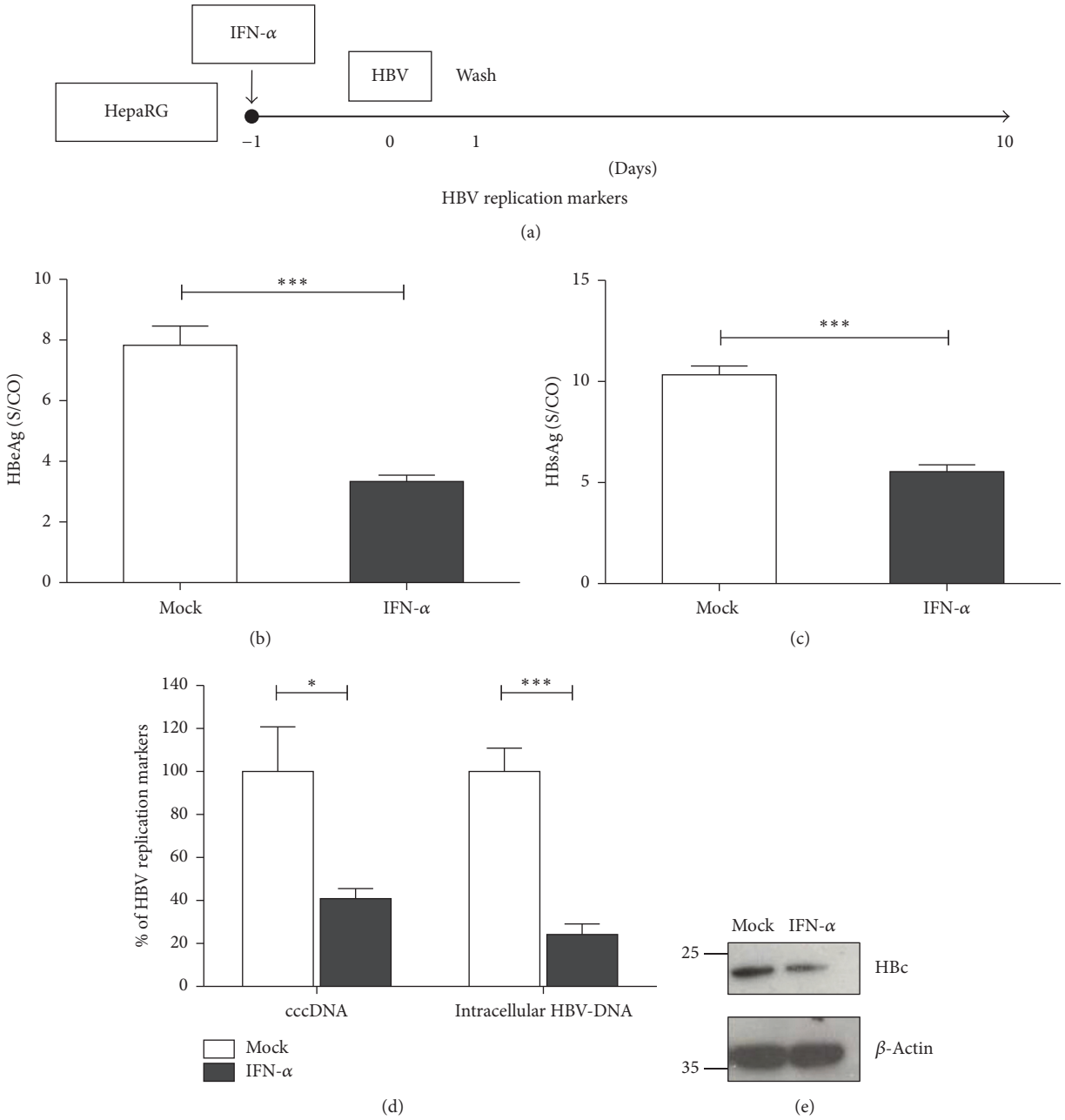


Figure 1: IFN-α pretreatment inhibits HBV infection. Differentiated HepaRG cells were treated with 1000 IU/mL of IFN-α for 1 day (IFN-a(−1)) and then infected with HBV (MOI = 200) (a). HBeAg from cell culture supernatant was measured by ELISA at day 10 (b). HBsAg was measured at day 7 and day 10 (c). HBV cccDNA and intracellular DNA were evaluated by qPCR (d). HBV core was detected by Western blot (e). Data are means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by Student's unpaired two-tailed t-test.

was reduced by 30% when particles were loaded after ISG+ medium. Hereby ISG+ medium inhibited HBV binding to heparin column almost as efficiently as Pep19-2.5 that has been described previously [32]. These results demonstrate that ISG+ medium competes with HBV for binding to heparin.

To further investigate our hypothesis that soluble factors in the ISG+ medium competitively bind to heparin in cultured cells, ISG+ medium was applied to the heparin column, and the flow-through (low heparin affinity part) and the elution (high heparin affinity part) were collected and applied to differentiated HepaRG cells (Figure 5(a)). The elution fraction from the heparin column containing high heparin affinity factors led to a reduction of HBV binding to HepaRG cells while the flow-through fraction showed no significant inhibition (Figure 5(b)). In conclusion, these results show that interferon induced factors inhibit initial binding of HBV to the cell surface by direct interaction with heparan sulfate.

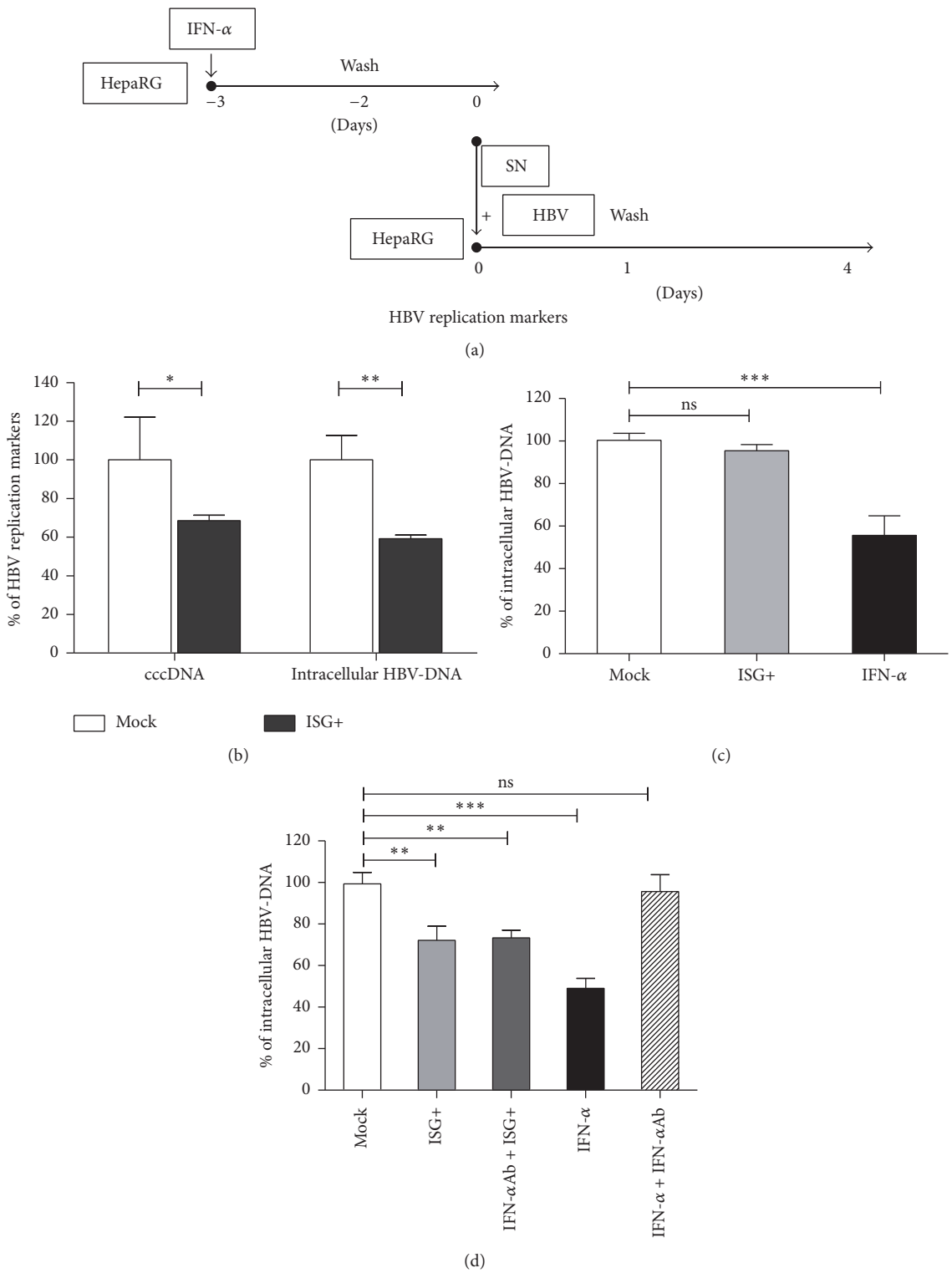


Figure 2: Secreted ISGs restrict early steps of HBV infection. (a) Differentiated HepaRG cells were treated with 1000 IU/mL of IFN-α. One day later, the medium was removed. Cells were washed three times with PBS and refilled with new medium. The resulting medium containing interferon induced factors (ISG+) was collected 48 hours later and then mixed with HBV and PEG. The mixture then transferred onto fresh differentiated HepaRG cell for HBV infection. (b) HBV cccDNA and intracellular DNA were evaluated by qPCR 4 days after infection. (c) Differentiated HepaRG cells were infected with HBV (MOI = 200) and 7 days later different treatments were applied as indicated. Intracellular HBV-DNA was measured 4 days after treatment. (d) Differentiated HepaRG cells were incubated with indicated treatment for 24 hours and then infected with HBV (MOI = 200). Intracellular HBV-DNA was measured 4 days after infection. Data are means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by Student's unpaired two-tailed t-test.

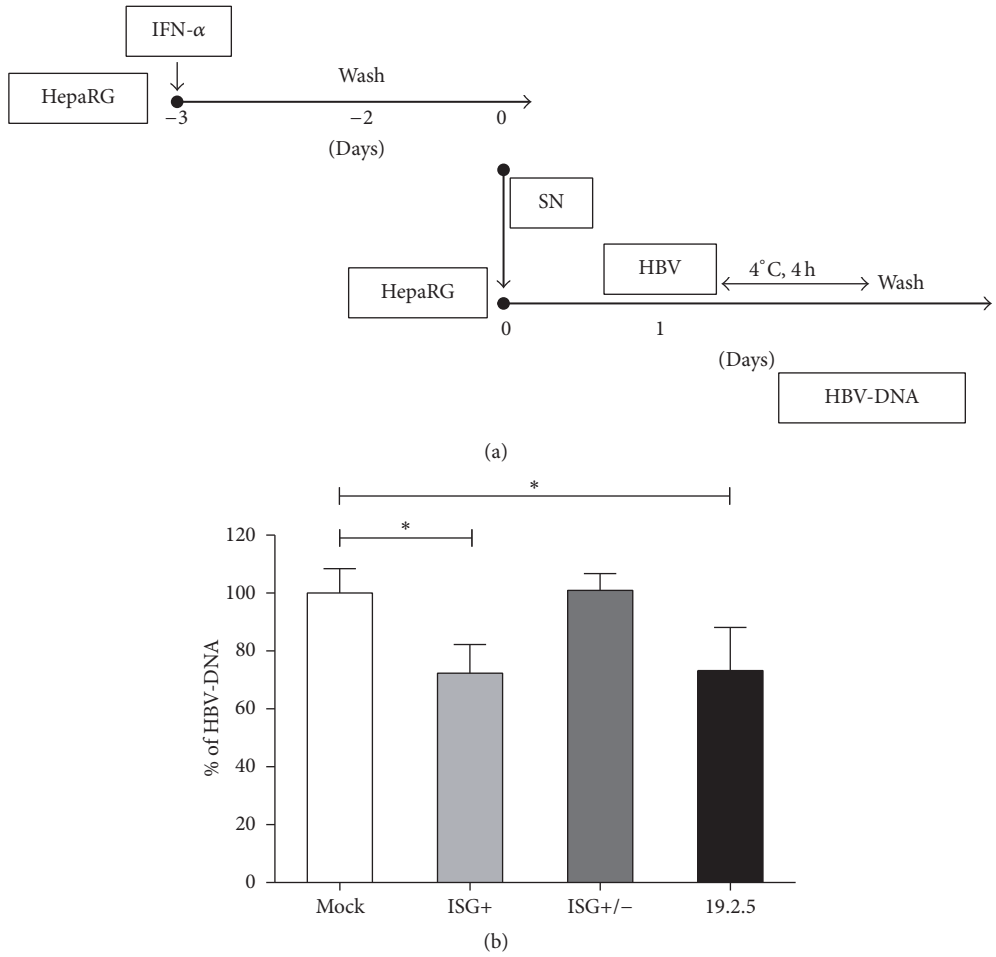


Figure 3: Secreted ISGs interrupt HBV binding. (a) Differentiated HepaRG cells were treated with 1000 IU/mL of IFN-α. One day later, the medium was removed. Cells were washed three times with PBS and refilled with new medium. The resulting medium containing interferon induced factors (ISG+) was collected 48 hours later. Part of the medium was heated at 99°C for 10 minutes to inactivate proteins (ISG+/−). The medium was incubated with fresh differentiated HepaRG cells for 24 hours followed by 4 hours of HBV incubation at 4°C. Peptide 19-2.5 was used as a positive control. (b) Cells were lysed and HBV-DNA were evaluated by qPCR. Data are means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by Student's unpaired two-tailed t-test.

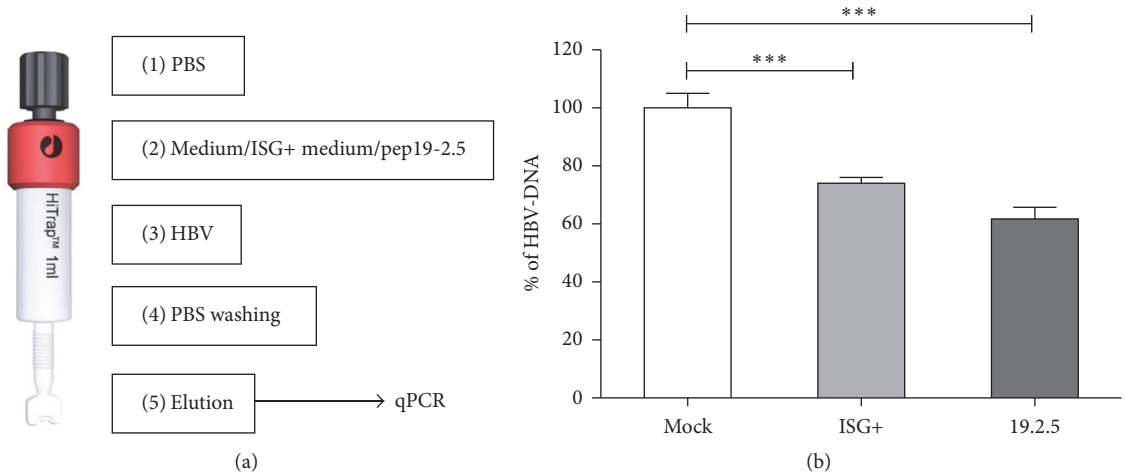


Figure 4: IFN-α induced products compete with HBV for binding to heparin column. (a) Heparin columns were washed with PBS and then applied with differentiated HepaRG cell culture medium (mock) or IFN-α treated differentiated HepaRG cell culture medium (ISG+) or the entry inhibitor Pep19-2.5. Then columns were applied with PBS containing HBV. After washing, HBV was eluted by elution buffer. (b) HBV amount from different elutions were analyzed by HBV-DNA qPCR.

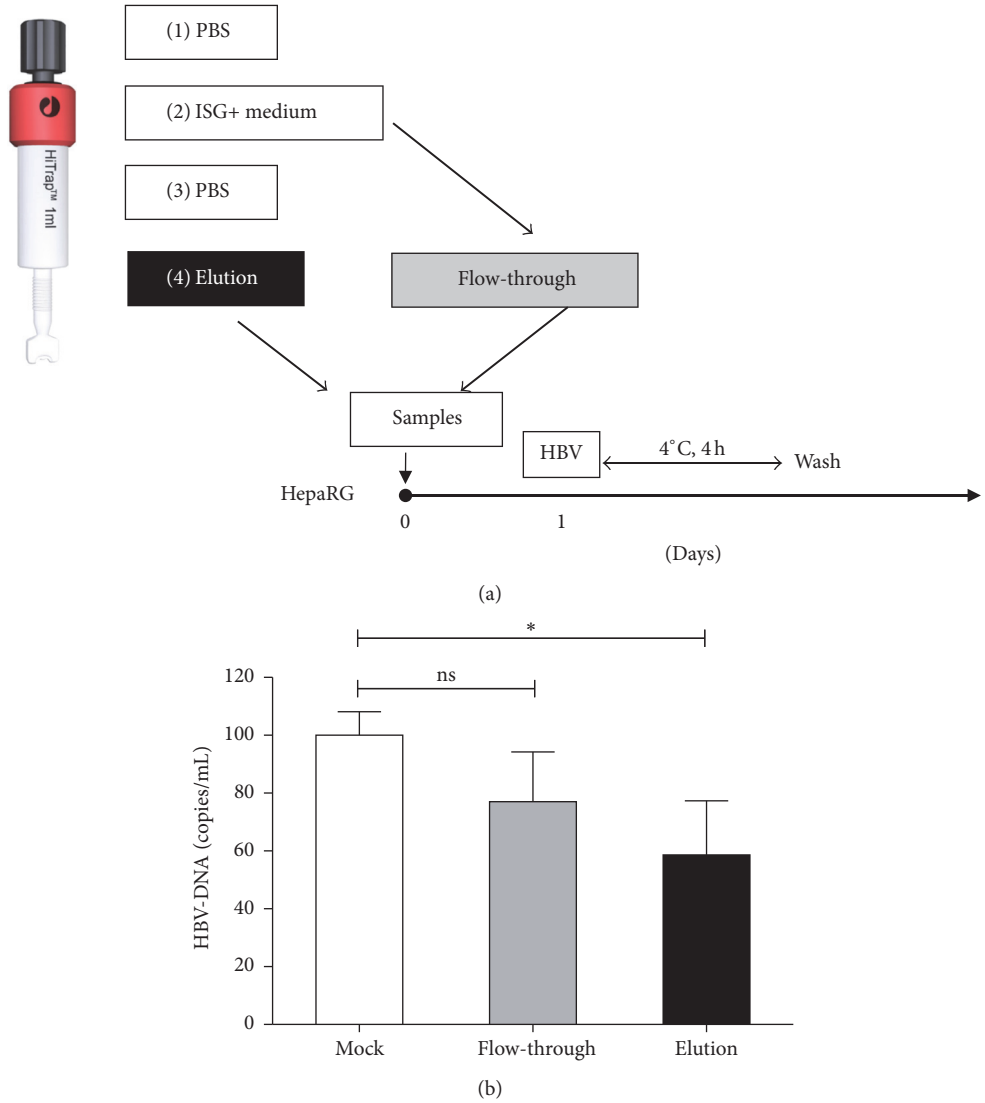
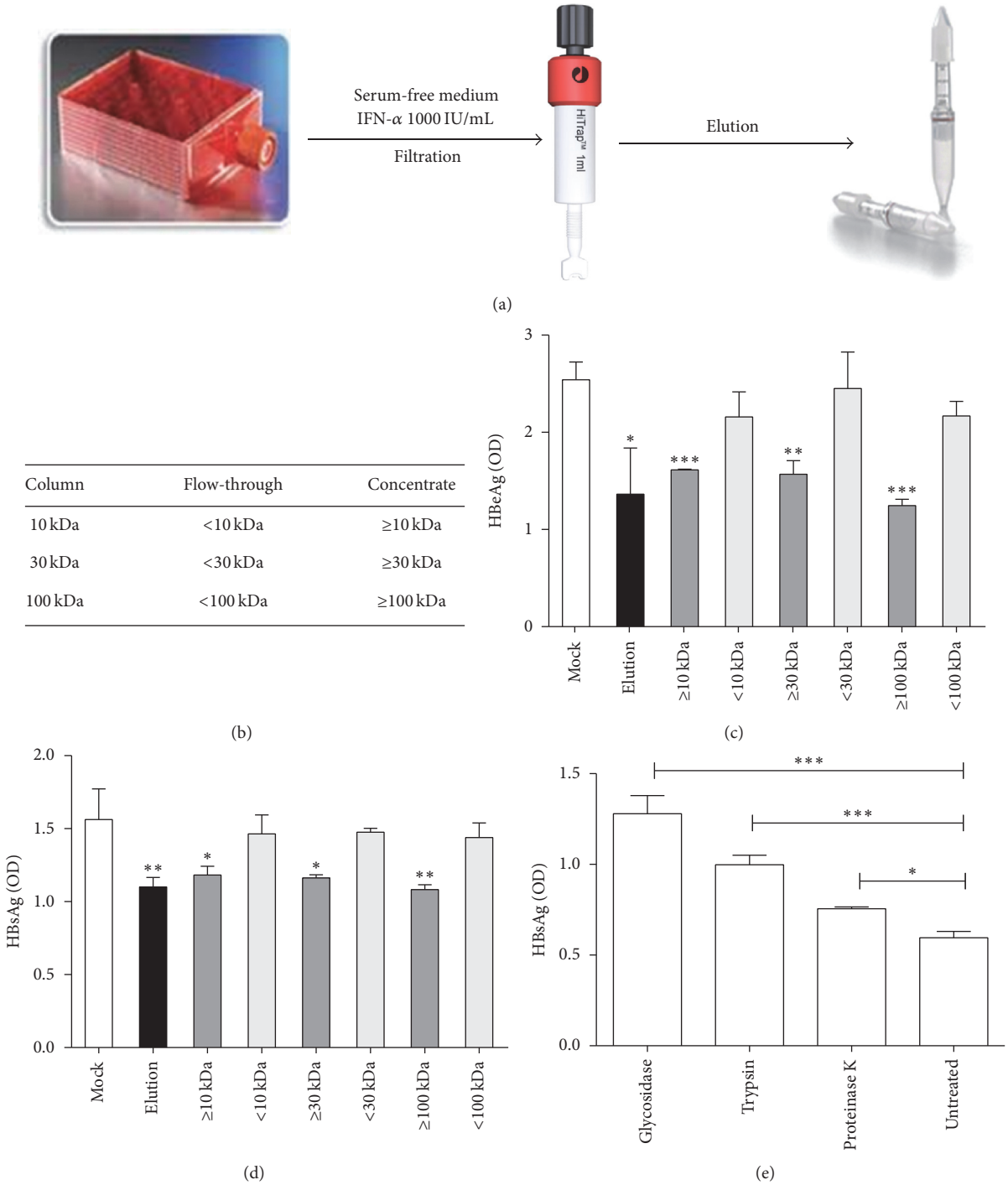


Figure 5: IFN-α induced products inhibit HBV binding to the cells. (a) Heparin columns were washed with PBS and then applied with IFN-α treated differentiated HepaRG cell culture medium. Then columns were washed with PBS. Differentiated HepaRG cells were incubated with elution or flow-through for 24 hours followed by 4 hours of HBV incubation at 4°C. (b) Cells were lysed and cellular HBV-DNA were evaluated by qPCR. Data are means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by Student's unpaired two-tailed t-test.

3.5. Characterization of IFN-α Induced Binding Inhibitors. In order to characterize the active factors in ISG+ medium in more detail, we determined their size using size exclusion chromatography. The heparin binding fraction was purified and applied to size exclusion columns with a cut-off of 10 kDa, 30 kDa, and 100 kDa, respectively, to further concentrate and separate the fractions by protein size (Figure 6(a)). Both flow-through and concentrate from each column were collected, and six different fractions containing a different size range of proteins (Figure 6(b)) were incubated on differentiated HepaRG cells before HBV inoculation. Elution from heparin columns inhibited HBV replication as expected. Among different fractions, all three concentrates containing proteins bigger than 100 kDa showed antiviral effects while all three flow-through fractions containing proteins smaller than 100 kDa did not reduce infection (Figure 6(c)).

To confirm binding specificity of the IFN-α induced factors to heparin, we coated a plate with heparin and added the elution from heparin columns or different fractions from protein concentration columns. We then determined the amount of HBV that could still bind to the heparin plate (Figure 6(d)). The elution fraction or the respective concentrates from all size exclusion chromatography columns competed with HBV for binding to heparin. The three flow-through samples did not show any inhibition of HBV binding. Taken together, this suggested that the interferon induced secreted factors that inhibit binding of HBV to heparin are larger than 100 kDa.



Column	Flow-through	Concentrate
10 kDa	<10 kDa	≥10 kDa
30 kDa	<30 kDa	≥30 kDa
100 kDa	<100 kDa	≥100 kDa

Figure 6: Size exclusion of IFN-α induced binding inhibitors. (a) HepaRG cells were cultivated in hyperflask. After differentiation, cells were stimulated with 1000 IU/mL IFN-α for one day and then fed fresh serum-free medium. The heparin binding fraction was purified from heparin columns and protein concentration columns with different cutoffs were used to further separate the fractions by protein size. (b) Protein size of each fraction. (c) Differentiated HepaRG cells were incubated with indicated samples for 24 hours and then infected with HBV. HBeAg was evaluated by ELISA 4 days after infection. (d) Highly purified HBV SVPs from chronic HBV carriers were added to heparin-coated (25 μg/mL) 96-well plate and treated with different fractions. Plates were incubated for 2 h at 37°C, and heparin-bound SVPs were detected using an HBsAg ELISA kit. (e) Differentiated HepaRG cells were incubated with elution treated by glycosidase, or trypsin, or proteinase K for 24 hours and then infected with HBV. HBsAg was evaluated by ELISA 4 days after infection.

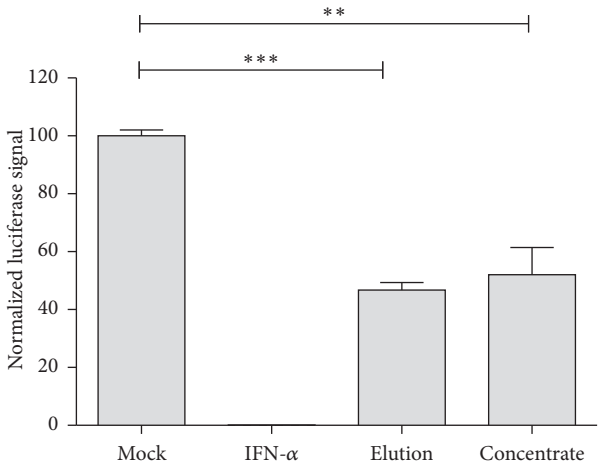


Figure 7: IFN-α induced factors inhibit HCV replication. Huh7.5 cells were seeded in 96-well plates in triplicate 24 h prior to virus infection. HCV luciferase reporter virus was used to infect cells at an MOI of 0.1 TCID50/cell. 1000 IU/mL of IFN-α, 10 μL of prepared "elution" or "concentrate" was added to the culture medium (total volume = 200 μL) from the beginning of inoculation until the cells were lysed 72 h later after HCV infection. Luciferase activity was determined and one of two independent experiments is displayed as mean ± SD.

To further characterize the IFN-α induced binding inhibitors, we treated heparin column purified ISG+ medium (elution) with glycosidase, trypsin, or proteinase K (Figure 6(e)). Interestingly, all three enzyme treatments enhanced HBV replication, which indicated that the antiviral factors present in the ISG+ medium are glycoproteins. The strong inhibitory effect of glycosidase on the factors suggested that carbohydrate groups of the glycoproteins might play an important role in the interaction with heparan sulfate.

3.6. Interferon Induced Secreted Factors Restrict HCV Infection. HCV is an alternative hepatotropic virus that uses heparan sulfate as a primary docking site for infection [26]. To investigate whether IFN-induced secreted factors were able to also inhibit HCV infection, IFN-α and heparin column elution or concentrate from 100 kDa protein concentration columns were added during infection with HCV luciferase virus (Figure 7). Importantly, both elution fractions from heparin columns and concentrates from size exclusion columns also restricted HCV infection by 50% (Figure 7). This result confirms that IFN inducible secreted factors block HCV binding to heparin sulfate proteoglycans and thus are able to block this common step of the two hepatotropic viruses, HBV and HCV.

4. Discussion

In the present study, we describe a novel antiviral mechanism of IFN-α that targets the HBV and HCV binding step. The supernatant of IFN-α treated cell cultures restricts HBV and HCV entry and infection. The inhibition is contributed by one or more secreted interferon induced proteins, which bind to heparin columns. This result indicates that the proteins in the IFN-α treated cell culture supernatant can bind to heparan glycosaminoglycans—the unspecific attachment receptor of many viruses.

Although ISG+ medium was prepared from IFN-α treated cells, the antiviral activity of ISG+ medium was unlikely due to residual amount of IFN-α present in the conditioned medium or de novo synthesized IFN-α/β in ISG+ medium treated cells. First, the residual IFN-α, if there was any, would be fast endocytosed and rapidly degraded by ISG producing cells [34]. Second, IFN-α treatment does not induce IFN-α/β production [35].

Initial interactions between an enveloped virus and its host cell are normally mediated through its membrane glycoproteins by binding to glycolipids and/or glycoprotein attachment factors, such as heparan sulfate proteoglycans, on the target cell surface [36]. For HBV, this first encounter is initiated via interaction of the "a" determinant(s) with heparan sulfate proteoglycans, resulting in the large envelope protein being able to bind to its specific receptor sodium-taurocholate cotransporting polypeptide to allow viral entry [25, 37]. Our enzyme digestion experiment suggested that IFN-α induced glycoproteins could compete with viral membrane proteins for binding to heparin sulfate proteoglycans thus blocking the whole entry process of virus infection.

IFN-α induced antiviral response is known to be multifunctional for a long time; however, its effects on the virus binding or entry steps are not well studied [38]. Until recently, the interferon-inducible transmembrane (IFITM) protein family has been shown to block early stages of viral infection [39, 40]. IFITM was originally identified through RNAi genetic screening and was shown to inhibit infections of vesicular stomatitis virus (VSV), influenza A virus, West Nile virus, and Dengue virus. Later IFITM proteins were found to potently restrict entry and infections by a number of highly pathogenic viruses, including human immunodeficiency virus (HIV), filoviruses, HCV, and SARS coronavirus [17, 19, 41–44]. Recently, researchers identified cholesterol-25-hydroxylase (CH25H) as a broad antiviral ISG [45]. CH25H converts cholesterol into a soluble antiviral factor, 25-hydroxycholesterol (25HC). 25HC treatment in cultured cells inhibited growth of a broad group of enveloped viruses including VSV, HIV, and herpes simplex virus. Interestingly, it also blocks HBV entry [46]. Since the molecular weight of IFITM or CH25H is less than 100 kDa, other factors that may contribute to the antiviral effect we observed.

The specific inhibition of virus entry is an attractive therapeutic target not only for acute but also for chronic viral infections. In the case of chronic infection entry inhibition prohibits infection from spreading to naive cells, which together with antiviral therapy eliminates infected cells providing higher chance of cure. For example, in HIV infection this has been accomplished with the interference of virus entry using a gp41 protein derived peptide, enfuvirtide, which prevents fusion of the virus with the host cellular membrane [47]. Virus entry inhibition also provides an opportunity to prevent recurrent hepatitis B after liver transplantation. Previous

studies demonstrate that acylated HBV preS-derived lipopeptides targeting viral envelope protein components could prevent the interaction of HBV with its cellular receptor, thus preventing de novo HBV infection in humanized mice [48]. Since hepatitis Delta virus uses the same receptor as HBV, HBV entry inhibitors are equally effective against both viruses [49]. Although there is no evidence indicating that HBV can propagate by cell-to-cell transmission, numerous enveloped viruses have been shown to employ modes of spreading involving both direct cell-cell transmission and the release of progeny viruses into the extracellular space [50]. Further study showed the myristoylated preS-derived peptide Myrcludex-B could block HBV cell-to-cell dissemination among human hepatocytes in the liver of humanized mice [51]. Similarly, in the context of HCV infection, an entry inhibitor targeting viral receptor claudin-1 used in monotherapy was shown to cure chronic infection in the infected humanized mice [52].

Altogether, recent studies results indicate that inhibition of HBV/HCV entry or binding, in combination with established therapies, might have potential applications in preventing vertical transmission during birth, reinfection after liver transplantation, or chronic HBV infection. In both HBV and HCV infection, hepatocyte turnover likely results in the reduced infection. Even more importantly, a recent clinical trial using entry inhibitor Myrcludex-B to treat patients with chronic hepatitis Delta has demonstrated that an entry inhibitor alone or in combination with IFN-α has a pronounced antiviral effect [53]. By blocking reinfection and protecting uninfected hepatocytes from de novo infection, IFN-induced viral entry inhibitors complement already known antiviral effects of IFN-α and their identification may open perspectives for novel therapeutic approaches for HBV and HCV infection.

5. Conclusion

In conclusion, this study reveals that IFN-α is able to induce soluble factors that bind to heparan glycosaminoglycans and lead to the inhibition of HBV and HCV binding and thus unravel a novel antiviral mechanism of action of interferons.

Disclosure

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Competing Interests

The authors have no conflict of interests to disclose.

Authors' Contributions

Yuchen Xia and Ulrike Protzer designed the study; Yuchen Xia, Xiaoming Cheng, Christoph K. Blossey, and Knud Esser conducted research; Yuchen Xia, Xiaoming Cheng, and Ulrike Protzer analyzed data and wrote the manuscript; Karin Wisskirchen revised the manuscript; all authors read and approved the manuscript.

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Research Article

Generation of Monoclonal Antibodies against Immunoglobulin Proteins of the Domestic Ferret (*Mustela putorius furo*)

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The domestic ferret (*Mustela putorius furo*) serves as an animal model for the study of several viruses that cause human disease, most notably influenza. Despite the importance of this animal model, characterization of the immune response by flow cytometry (FCM) is severely hampered due to the limited number of commercially available reagents. To begin to address this unmet need and to facilitate more in-depth study of ferret B cells including the identification of antibody-secreting cells, eight unique murine monoclonal antibodies (mAb) with specificity for ferret immunoglobulin (Ig) were generated using conventional B cell hybridoma technology. These mAb were screened for reactivity against ferret peripheral blood mononuclear cells by FCM and demonstrate specificity for CD79β^+ B cells. Several of these mAb are specific for the light chain of surface B cell receptor (BCR) and enable segregation of kappa and lambda B cells. Additionally, a mAb that yielded surface staining of nearly all surface BCR positive cells (*i.e.*, pan ferret Ig) was generated. Collectively, these MαF-Ig mAb offer advancement compared to the existing portfolio of polyclonal anti-ferret Ig detection reagents and should be applicable to a wide array of immunologic assays including the identification of antibody-secreting cells by FCM.

1. Introduction

The domestic ferret (*Mustela putorius furo*) serves as an animal model for the study of several viruses that cause human disease [1, 2]. Most notably, ferrets are naturally susceptible to human influenza virus and are capable of viral transmission [3–6]. Their application to influenza research began in 1933, when throat washings from human subjects were administered intranasally into ferrets [6]. These animals went on to exhibit outward symptoms of influenza including fever, sneezing, and lethargy. Transmission of influenza-like disease was also observed following transfer of nasal washings from an infected ferret or cohousing with a naïve contact. Due to the expression of both α2,6 and α2,3 sialic acid moieties along the respiratory tract, ferrets are permissive to infection with human seasonal and prepandemic avian influenza isolates [7]. Moreover, they recapitulate the extrapulmonary replication of highly virulent avian influenza subtypes such as H5N1 and H7N7 [8, 9]. Collectively, the ferret model has provided invaluable insights into the antigenicity, virulence and transmissibility of circulating and newly emerging influenza isolates [1, 10–12].

Despite the usefulness of the ferret animal model, the lack of ferret-specific reagents has severely hampered the ability to perform in-depth immunologic profiling. Recent studies have implemented more detailed methods for interrogating the immune response elicited in the ferret, including quantitative RT-PCR to measure cytokine and chemokine transcript levels and flow cytometric analysis of leukocyte populations utilizing cross-reactive monoclonal antibody (mAb) reagents [13–17]. However, mAb with defined specificity for B cell lineage surface markers, such as CD19 [18], that would facilitate identification of ferret B cells at various developmental stages by flow cytometry (FCM) are currently lacking. To circumvent this issue, others have identified ferret B cells on the basis of surface immunoglobulin (Ig) and/or CD79α

expression [14, 16]. While CD79α is an excellent marker for identification of B lineage cells because it is an obligate chaperone for surface expression of B cell receptor (BCR) [19], the epitope recognized by this mAb (clone HM47) requires intracellular staining and thus does not permit isolation of viable B cells [20]. Alternatively, polyclonal antibodies with reactivity against ferret Ig are conducive for surface staining but do not exclusively define B cells due to binding of ferret Ig by myeloid cells via Fc receptors [14]. Moreover, neither approach enables segregation of ferret B cells on the basis of heavy chain usage. Collectively, the suite of available reagents is still insufficient for an in-depth characterization of the humoral immune response, and specifically the identification of antibody-secreting cells by FCM. To begin to address this unmet need, several novel mAb with specificity for ferret immunoglobulin (Ig) were generated and characterized to define their specificity.

2. Materials and Methods

2.1. Animals. BALB/c mice (female, 8–10 weeks of age) from Jackson Laboratory (Bar Harbor, ME, USA) and Fitch ferrets (*Mustela putorius furo*, male or female, 6 to 12 months of age) from Triple F Farms (Sayre, PA, USA) were housed in cage units and fed ad libitum. All animals were handled in accordance with protocols approved by Institutional Animal Care and Use Committees and were cared for under USDA guidelines for laboratory animals.

2.2. Isolation of Ferret Peripheral Mononuclear Cells. Peripheral blood was collected via cardiac puncture into vacuum collection tubes containing sodium heparin (Becton Dickinson, Cat #367874) and gently inverted to prevent coagulation. Blood was then combined with phosphate buffered saline (PBS) (Corning, Cat #21-040-CV) to a final volume of 35 mL and overlaid on 10 mL Ficoll-Paque PLUS (GE Healthcare, Cat #17-1440) before centrifugation at 500 ×g for 25 min with the brake reduced to its lowest setting using a Sorvall Legend XTR (Thermo Scientific, Grand Island, NY, USA). Peripheral blood mononuclear cells (PBMC) at the interface were collected, washed with PBS, and then pelleted (400 ×g for 10 min). Following an additional wash with PBS, total cell number and viability was determined by Trypan blue exclusion using the Countess™ (ThermoFisher, Cat #C10227). Ferret PBMC were used immediately or resuspended in fetal bovine serum (FBS) (HyClone, Cat #SH30396.03) containing 10% DMSO (Thermo Scientific, Cat #20688) for long-term storage. Aliquots of 1-2 $\times 10^7$ viable ferret PBMC were stored in the vapor phase of liquid nitrogen until use and thawing of cells was according to similarly described methods [22].

2.3. Purification of Ferret Immunoglobulin. Serum from two ferrets (female, 7 months of age) was pooled and immunoglobulin (Ig) precipitated by drop-wise addition of an equal volume of saturated ammonium sulfate solution (4.1 M) (Sigma, Cat #A4418) while maintaining the solution under constant agitation at 4°C. Precipitated protein was pelleted by centrifugation at 11,5000 rpm for 20 min at 4°C and then dissolved in PBS. The protein solution was then transferred into a Slide-A-Lyzer dialysis cassette (ThermoFisher, Cat #66030) and dialyzed against PBS at 4°C for three days with daily buffer exchanges. Subsequently, the protein solution was clarified by centrifugation at 6000 rpm for 10 min at 4°C and then passed through a 0.2 μm syringe filter (ThermoFisher, Cat #09-719C). This material is referred to as crude ferret Ig. Ferret Ig was further purified by affinity chromatography using Protein A/G (ThermoFisher, Cat #20423). Briefly, crude ferret Ig protein solution was applied to Protein A/G and the column (Bio-Rad, Cat #7311550) was washed by gravity flow with PBS. Fractions (2 mL) were collected and absorbance (280 nm) was monitored using a PowerWave XS spectrometer (Biotek, Winooski, VT, USA). Once wash fractions returned to baseline, ferret Ig protein was eluted by addition of 0.1 M glycine, pH 2.5 (Amresco, Cat #M103). Eluted protein was immediately neutralized with 200 μl 1.5 M Tris, pH 8.8 (Amresco, Cat #M151) and protein containing elution fractions were pooled, buffer exchanged into PBS containing 0.05% sodium azide (Sigma, Cat #S2002), and concentrated using a Spin-X UF filter (Sigma, Cat #CLS431489). This material is referred to as purified ferret Ig. Protein concentrations of the crude and purified ferret Ig solutions were determined according to the manufacture's instructions using a micro BCA™ assay kit (ThermoFisher, Cat #23235).

2.4. Protein Gel Electrophoresis. To assess purity of the respective ferret Ig containing protein solutions, 5 μg of crude or purified ferret Ig was loaded into Bolt™ 10% Bis-Tris Plus precast protein gels (ThermoFisher, Cat #NW00102) and resolved at 150 V for 50 min. Protein samples were diluted in either 2x Laemmli sample buffer (Bio-Rad, Cat #161-0737) with or without β-mercaptoethanol (JT Baker, Cat #P62405) or 6x SDS-sample buffer (reducing) (Boston BioProducts, Cat #BP-111R). Reduced samples were heated at 100°C for 10 min and placed on ice prior to loading. Gels were stained with PageBlue™ protein staining solution (ThermoFisher, Cat #24620) and then destained in water before imaging using the myECL Imager (ThermoFisher, Waltham, MA, USA). Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher, Cat #26634) was included in all gels and used for molecular marker reference.

2.5. Generation of Murine Monoclonal Antibodies. Female BALB/c mice were immunized with 100 μg of purified ferret Ig containing the Imject alum adjuvant (Thermo Scientific, Cat #77161) via the intraperitoneal route. Serum was collected via the submandibular vein on day 21 and assessed by ELISA for antibody reactivity (refer to ELISA subsection). The two mice with the highest antibody titer received a booster immunization, via the intraperitoneal route, containing 100 μg of purified ferret Ig in PBS on Day 28. Three or four days after the booster immunization, splenocytes were harvested and used to perform a fusion with the SP2/O myeloma (kindly provided by Dr. Lawrence Wysocki, University of Colorado Denver) using polyethylene glycol 1450 (ATCC, Cat #50-X). Hybridomas were selected by addition of hypoxanthine (Acros Organics, Cat #1220100) and azaserine (Sigma, Cat #A4142) at a final concentration of 200 μM and 11.5 μM respectively in RPMI 1640 (Sigma,

Cat #R6504) containing 10% FBS, 23.8 mM sodium bicarbonate (Fisher Scientific, Cat #BP328), 7.5 mM HEPES (Amresco, Cat #0485), 170 μM Penicillin G (Tokyo Chemical Industry, Cat #P1770), 137 μM Streptomycin (Sigma, Cat #S9137), 50 μM β-mercaptoethanol, and 1 mM sodium pyruvate (ThermoFisher, Cat #11360070). Eleven days after the respective fusions, culture supernatants were screen for reactivity by ELISA (refer to ELISA subsection). Positive wells were further expanded and maintained under drug selection, and hybridoma cell lines of interest were subcloned by limiting dilution. Hybridomas were subsequently expanded in media containing 5% IgG-stripped FBS (Hyclone, Cat #SH30898.03) and monoclonal antibody (mAb) purified by affinity chromatography (Protein A/G) as described above.

2.6. ELISA. The enzyme-linked immunosorbent assay (ELISA) was used to assess antibody reactivity against purified ferret Ig and to determine the IgG subclass and concentration of the respective mAb. To measure antibody binding against the purified ferret Ig antigen, CoStar high binding ELISA plates (Corning, Cat #3590) were coated overnight at 4°C with 2 μg/mL purified ferret Ig in carbonate buffer pH 9.4 containing 5 μg/mL fraction V bovine serum albumin (BSA) (Equitech-Bio, Cat #BAC69). Alternatively, plates were coated with 5 μg BSA in carbonate buffer alone. Plates were blocked with ELISA block buffer, PBS containing 0.2% BSA, 0.1% bovine gelatin (Sigma, Cat #G9391), and 0.05% Tween 20 (Sigma, Cat #P7949), for 90 min at 37°C prior to addition of culture supernatant or antibody solutions. Culture supernatants were diluted 1 : 2 for hybridoma screening, and purified mAb were diluted to 3 μg/mL in ELISA blocking buffer prior to 3-fold serial dilution. Plates were incubated for 90 min at 37°C and washed with PBS to remove unbound antibody. For hybridoma screening, horseradish peroxidase conjugated goat anti-mouse IgG (γ-specific) (Southern Biotech, Cat #1030-05) secondary antibody diluted in blocking buffer was added to ELISA plates. Alternatively, binding of mAb to ferret Ig was revealed by addition of horseradish peroxidase conjugated goat anti-mouse IgG1 (γ1-specific) (Southern Biotech, Cat #1070-05) secondary antibody. After addition of secondary antibody, plates were incubated for 60 min at 37°C and then washed extensively with PBS prior to addition of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Amresco, Cat #0400) substrate. Plates were incubated at 37°C for development and colorimetric conversion was terminated by addition of 5% sodium dodecyl sulfate (SDS) (Teknova, Cat #S0294). Optical density was measured at 414 nm (OD_{414}) using a PowerWave XS spectrophotometer.

To determine the IgG subclass or determine the concentration of the respective mAb, CoStar high binding ELISA plates were coated overnight at 4°C with 1 μg/mL goat anti-mouse IgG (γ-specific) capture antibody (Sigma, Cat #M1397) in carbonate buffer pH 9.4 containing 5 μg/mL BSA. Plates were then blocked with ELISA blocking buffer for 90 min at 37°C. Culture supernatants from clonal hybridoma lines of interest, diluted mouse anti-ferret Ig (MαF-Ig) mAb or mouse IgG1,κ (Biolegend, Cat #401402), were serially diluted in ELISA blocking buffer and plates incubated for 90 min at 37°C. Plates were washed five times with PBS, horseradish peroxidase conjugated goat anti-mouse IgG1 (γ1-specific) secondary antibody diluted in ELISA blocking buffer added and the plates incubated for 60 min at 37°C. Following extensive washing with PBS, ABTS substrate was added and plates incubated at 37°C for development. Colorimetric conversion was terminated by addition of 5% SDS solution, and OD_{414} was measured using a PowerWave XS spectrophotometer. The concentration of individual mAb was then interpolated based on a nonlinear regression of the IgG1,κ standard using PRISM 6.0 (GraphPad Software, La Jolla CA, USA).

2.7. Competitive ELISA. A competitive ELISA was performed using unlabeled and biotinylated MαF-Ig mAb (refer to Protein Conjugation) to identify overlapping epitope recognition. CoStar high binding ELISA plates were coated overnight at 4°C with 2 μg/mL purified ferret Ig in carbonate buffer pH 9.4 containing 5 μg/mL BSA. Plates were then blocked with ELISA blocking buffer for 90 min at 37°C. Unlabeled MαF-Ig mAb (5–50 μg/mL starting concentration, determined using BCA assay kit) were serially diluted in ELISA blocking buffer, followed by addition of biotinylated MαF-Ig mAb, and plates incubated overnight at 4°C. Plates were washed five times with PBS, horseradish peroxidase conjugated streptavidin diluted in blocking buffer added, and the plates incubated for 60 min at 37°C. Following extensive washing with PBS, ABTS substrate was added and plates incubated at 37°C for development. Colorimetric conversion was terminated by addition of 5% SDS solution, and OD_{414} was measured using a PowerWave XS spectrophotometer. The percent of maximal signal was determined using the formula 100 × [OD_{414} experimental sample − OD_{414} blank/OD_{414} maximal signal − OD_{414} blank].

2.8. Western Blot. To characterize the specificity of individual MαF-Ig mAb, 1 μg of purified ferret Ig or 0.5 μl of ferret serum was reduced and resolved by protein gel electrophoresis as describe previously. Protein transfer to polyvinylidene difluoride (PVDF) membranes was performed using the Trans-Blot Turbo RTA Mini PVDF transfer kit (Bio-Rad, Cat #1704272) and a Trans-Blot Turbo Blotting system (Bio-Rad, Hercules, CA, USA) according to the manufacture's instructions. The membrane was blocked with PBS + Tween 20 (0.1% v/v) (PBST) containing 5% BSA (VWR, Cat #0332) at room temperature (RT) with constant agitation. The PVDF membranes were then cut into strips and probed with 15 mL of PBST containing 0.1 μg/mL of individual MαF-Ig mAb overnight at RT. The following day, PVDF membranes were washed three times with PBST before incubation for 60 min at RT with 10 mL PBST containing horseradish peroxidase conjugated goat anti-mouse IgG1 (γ1-specific). Following extensive washing with PBS, membranes were treated with 4 mL Clarity™ Western ECL Substrate (Bio-Rad, Cat #1705060) and imaged using myECL Imager. Postacquisition analysis was performed using myImageAnalysis™ Software (ThermoFisher).

2.9. Flow Cytometry. To evaluate the specificity of commercially available anti-ferret Ig reagents by flow cytometry

(FCM), ferret PBMC were labeled with FITC conjugated goat anti-ferret IgM (μ-specific) (Sigma, Cat # SAB3700807) or biotin conjugated goat anti-ferret IgG (GαF-Igγ BIO) (Sigma, Cat #SAB3700796). Binding of GαF-Igγ BIO was revealed using phycoerythrin conjugated streptavidin (SA-PE) (Biolegend, Cat #405204). The reactivity of individual mAb against ferret leukocytes was assessed by direct or indirect staining. Initially, culture supernatants from clonal hybridoma lines were diluted to 1 μg/mL in FCM staining buffer (PBS + 2% FBS + 0.1% NaN_3) and used to stain ferret PBMC. Because this screening method was dependent on revealing binding of the murine mAb using a polyclonal goat anti-mouse Ig Alexa Fluor 647 (GαM-Ig AF647) reagent (BD, Cat #51-9006588BK), we were unable to utilize a cross-reactive anti-CD79β murine mAb (clone CB3-1) to identify B cells [16, 23]. Ferret B cells were instead identified using GαF-Igγ BIO, which was revealed using SA-PE. Binding of murine mAb to ferret PBMC was revealed with GαM-Ig AF647. All staining was performed on ice in 100 μl of volume and cells were stained with LIVE/DEAD Aqua (ThermoFisher, Cat #L34957) prior to surface staining to enable exclusion of nonviable cells.

For direct staining, ferret PBMC were initially stained with LIVE/DEAD Aqua and then pretreated with irrelevant Mouse IgG2a,κ (Biolegend, Cat #401502) and Rat IgG2a,κ (Biolegend, Cat #400502) to exclude nonviable cells and minimize nonspecific binding. Ferret PBMC were then stained with anti-CD79β (Biolegend, Cat #341408) and DyLight 488, DyLight 650, and/or biotin conjugated MαF-Ig mAb (refer to Protein Conjugation). Biotinylated mAb were revealed by secondary staining with SA-PE. In order to putatively identify kappa light chain expressing B cells, ferret PBMC were stained with DyLight 650 conjugated recombinant Protein L (ThermoFisher, Cat # 21189) and anti-CD79β simultaneously with DyLight 488 and biotin conjugated MαF-Ig mAb. Acquisition was performed on the LSR II cytometer (BD Biosciences, San Jose, CA, USA) and analysis performed with FlowJo Version 10.0.8 (Tree Star, Ashland, OR, USA).

2.10. Protein Conjugation. Protein A/G purified mAb or recombinant Protein L were conjugated to DyLight 488 (ThermoFisher, Cat #46402), DyLight 650 (ThermoFisher, Cat #62265), or EZ-Link™ NHS-LC-Biotin (ThermoFisher, Cat #21336) according to the manufacture's instructions. Unreacted DyLight or NHS-LC-Biotin was removed by multiple buffer exchanges into PBS containing 0.05% sodium azide using Spin-X UF filters (30 kDa MWCO).

2.11. Sequencing Hybridoma V Region Genes. Variable genes of B cell hybridomas were cloned by RT-PCR and 5′ rapid amplification of cDNA ends (RACE) using pairs of constant region and anchor primers according to previously described methods, with minor modifications [24]. Briefly, total RNA was isolated from hybridoma lines using RNeasy (Qiagen, Cat #74104) and first-strand cDNA synthesis performed using SuperScript III RT (ThermoFisher, Cat #18080051) using oligo-dT primer. First-strand cDNA was isolated using QIAquik PCR spin columns (Qiagen, Cat #28104) and then dG-tailed with TdT (NEB, Cat #M0315S) and dGTP (ThermoFisher, Cat #10218014). Variable *IgH* or *Igκ* genes were then amplified from dG-tailed cDNA templates using Phusion (NEB, Cat# M0530S). A poly-A tail was added to products following completion of the second round of PCR by addition of 5 units recombinant Taq polymerase (ThermoFisher, Cat #EP0402) directly into the reaction and incubation at 72°C for 10 min. Products from *Igκ* PCR were further purified with QIAquick PCR spin columns before digestion with restriction enzymes PflFI (NEB, Cat #R0595S) or PflmI (NEB, Cat #R0509S) to disrupt the rearranged *Vκ21-12* gene from the SP2/0 fusion partner. After 2% agarose electrophoresis, the uncut Vκ products were isolated using the QIAquik gel extraction kit (Qiagen, Cat #28704) and eluted with autoclaved water. Variable region genes were cloned into pCR4-TOPO (ThermoFisher, Cat #K4575J10) or pSC-A (Agilent, Cat #240205) plasmids according to the manufacture's instructions. Plasmid DNA was purified using QIAprep spin columns (Qiagen, Cat #27104) and submitted to Macrogen (Rockville, MD, USA) for sequencing. Heavy and kappa variable region genes were identified using IMGT V-Quest [21].

2.12. Statistics. Statistical analyses were performed using PRISM 6.0.

3. Results

3.1. Commercial Reagents against Ferret Immunoglobulin Lack Heavy Chain Specificity. Expression of a class-switched B cell receptor (BCR), such as IgG or IgA, can be used as a marker of memory B cells, while naïve B cells express an IgM BCR [25]. As a first attempt to segregate ferret B cells into naïve and memory compartments on the basis of surface BCR expression, ferret PBMC were stained with polyclonal goat anti-ferret IgM (GαF-Igμ) or goat anti-ferret IgG (GαF-Igγ) antiserum. Additionally, the mouse anti-human CD79β mAb (clone CB3-1), which cross-reacts with ferret leukocytes (Supplementary Materials available online at https://doi.org/10.1155/2017/5874572), was included in the staining solution to identify surface BCR^+ cells [16]. The GαF-Igμ antisera labeled ~99% of the CD79β^+ population, while the GαF-Igγ antisera costained ~66% of the CD79β^+ population (Supplementary Materials). To extend these observations, ferret PBMC were next stained with both the GαF-Igμ and GαF-Igγ simultaneously. The majority of CD79β^+ ferret PBMC exhibited staining with both the GαF-Igμ and GαF-Igγ reagents. Collectively, these findings indicate that surface staining with anti-CD79β enables identification of ferret B cells and currently available anti-ferret Ig reagents are insufficient to discriminate B cells on the basis of heavy chain expression.

3.2. Purification of Ferret Immunoglobulin. Ferret Ig was first crudely enriched from serum through ammonium sulfate precipitation and the resulting protein solution was predominantly IgG (Figure 1, lanes 2 and 3). Next, ferret Ig was further purified by affinity chromatography using Protein A/G. This second purification step removed the majority of contaminating proteins and produced a highly pure ferret Ig preparation (Figure 1, lanes 4 and 5). Reduction of the purified ferret

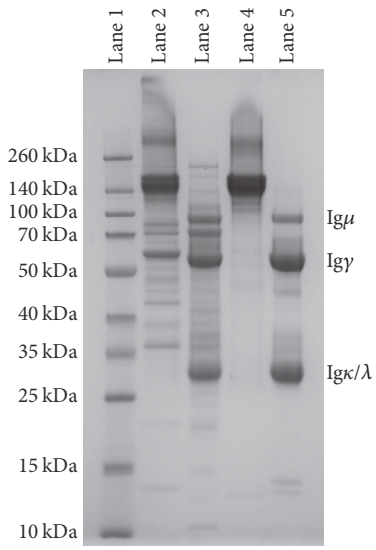

Figure 1: SDS-polyacrylamide gel electrophoresis of purified ferret immunoglobulin. 5 μg of crude (lanes 2 and 3) or Protein A/G purified (lanes 4 and 5) ferret Ig preparations were resolved by SDS-PAGE under nonreducing (lanes 2 and 4) or reducing (lanes 3 and 5) conditions. Molecular marker references are indicated (lane 1). Image was acquired using the myECL imager and contrast adjustment of the entire image was performed using myImageAnalysis software. Image is representative of two independent purifications.

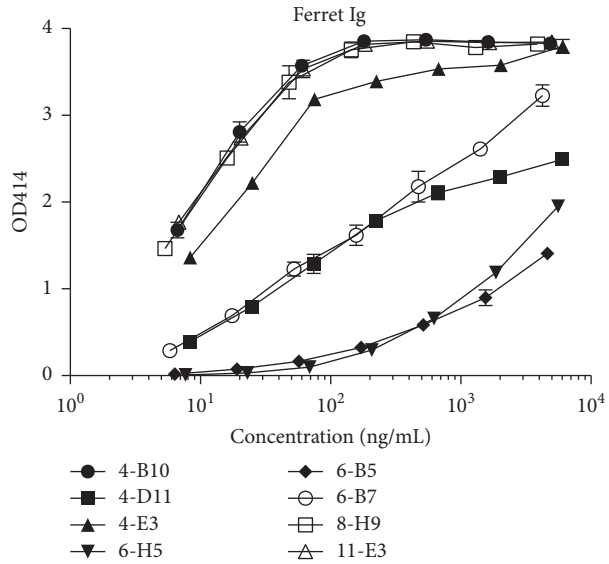

Figure 2: Monoclonal antibody binding to ferret immunoglobulin. Mouse mAb were evaluated for binding to purified ferret Ig by ELISA. Optical densities are plotted versus normalized IgG1 input (x-axis). Data are representative of two or more independent experiments that yielded similar results.

Ig into heavy and light chain components confirmed the presence of both Igμ and Igγ on the basis of their differential sizes (Figure 1, lane 5) [26].

3.3. Immunization with Purified Ferret Immunoglobulin. Mouse IgG2a mAb have increased nonspecific binding to ferret leukocytes relative to IgG1 (data not shown). In order to elicit an antibody response utilizing the IgG1 subclass, BALB/c mice were immunized with purified ferret Ig prepared with the Imject alum adjuvant [27]. Following a single immunization with ferret Ig and adjuvant, antigen-specific reactivity was detected by ELISA and western blot (Supplementary Materials). Moreover, reduction of ferret Ig in the SDS-PAGE enabled discrimination between antibody reactivity with the light (Igκ/Igλ) and heavy (Igμ or Igγ) chain components of ferret Ig.

3.4. Characterization of Monoclonal Antibodies by ELISA. Based on their reactivity with ferret Ig, two mice (#3 and #5) were chosen for mAb generation. Eight $IgG1^+$ mAb, collected from two independent fusions, reacted with purified ferret Ig. Each of these mouse anti-ferret Ig (MαF-Ig) mAb reacted with ferret Ig by ELISA (Figure 2). Additionally, normalization of the input IgG1 concentration highlighted the distinct binding curves of several MαF-Ig mAb.

3.5. Assessment of Monoclonal Antibody Reactivity by Western Blot. As an additional technique to further characterize the reactivity of the respective mAb with ferret Ig, individual MαF-Ig mAb were used to probe reduced ferret Ig antigen and serum from three naïve ferrets in a western blot screen. Three of the eight MαF-Ig mAb demonstrated specific reactivity with ferret Ig antigen using this assay (Figure 3). Both MαF-Ig mAb *4-B10* and *8-H9* reacted with ferret Ig light chain protein (Figures 3(b) and 3(c)). Additionally, mAb *11-E3* reacted with a protein species corresponding to the Igγ chain (Figure 3(d)). Of note, these MαF-Ig mAb reacted with both purified ferret Ig antigen and serum samples, suggesting the epitopes recognized by the respective mAb are not polymorphic.

3.6. Assessment of Monoclonal Antibody Reactivity by Flow Cytometry. In spite of earlier observations suggesting that the GαF-Igγ antisera was unlikely to define IgG^+ ferret B cells exclusively (Supplementary Materials), this reagent was incorporated into a flow cytometric screening assay to evaluate the ability of each MαF-Ig mAb to bind ferret PBMC because it enabled resolution of these cells into distinct populations on the basis of staining intensity (Figure 4(b)). The two populations of ferret PBMC that demonstrated staining with the GαF-Igγ antisera are referred to as IgG^{int} and IgG^{hi}, respectively, for the sole purpose of detailing the staining patterns observed for the respective MαF-Ig mAb (Figure 4 and data not shown). Using this flow cytometric screening approach, both *4-B10* and *8-H9* had similar staining patterns of ferret PBMC and labeled ~50% of the IgG^{hi} population (Figures 4(c) and 4(d)). Moreover, both of these MαF-Ig mAb also reacted with a small population of IgG^{int} cells. Three additional MαF-Ig mAb (*4-E3*, *4-D11*, and *6-H5*) had similar reactivity patterns (data not shown). A distinct staining pattern was observed using two MαF-Ig mAb (*6-B5* and *6-B7*) (Figure 4(e) and data not shown). Specifically, pretreatment of ferret PBMC with these mAb resulted

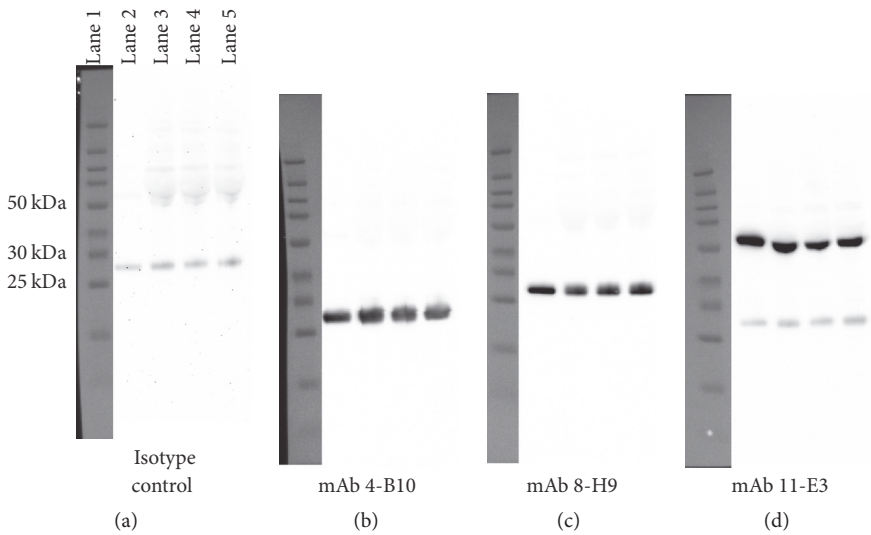

Figure 3: Reactivity of monoclonal antibody against reduced ferret immunoglobulin. 1 μg of ferret Ig (lane 2) or 0.5 μl of ferret serum ($n = 3$) (lanes 3–5) was resolved by SDS-PAGE under reducing conditions and transferred to PVDF membranes. Membranes were then cut into strips and probed with (a) irrelevant IgG1, (b) *4-B10*, (c) *8-H9*, or (d) *11-E3*. Images were acquired using the myECL imager and contrast adjustments of the entire image were performed using myImageAnalysis software. Visible light images of the molecular markers (lane 1) are presented next to the corresponding chemiluminescent image for reference. Images are representative of two independent experiments.

in the disappearance of the IgG^{hi} population, likely due to BCR internalization or blocking of epitopes targeted by the GαF-Igγ antisera. Additionally, a subset of the IgG^{int} population was labeled by *6-B7* on the basis of GαF-Igγ staining (Figure 4(e)). Finally, the *11-E3* mAb had a third staining pattern with low-level binding to IgG^{int} cells but did not bind to IgG^{hi} cell population.

As a next step to further characterize the reagents, directly labeled MαF-Ig mAb were used to stain ferret PBMC in combination with anti-CD79β (Figure 5). Based on previously observed western blot and flow cytometric staining patterns (Figures 3 and 4), the MαF-Ig mAb were segregated into two classifications. The first group of MαF-Ig mAb comprised *6-B5*, *6-B7*, and *11-E3* and these mAb had a reactivity profile consistent with Ig heavy chain specificity. Despite evidence of reactivity with IgG^{hi} cells in the indirect screen (data not shown), biotinylated *6-B5* did not stain CD79β^+ ferret B cells (Figure 5(a)). By contrast, biotinylated *6-B7* stained almost all of the CD79β^+ cell population (Figure 5(b)). In addition, *11-E3* stained the entire CD79β^+ cell population with a low-level of fluorescence, as well as a small population of CD79β^{neg} cells (Figure 5(c)). Similar results were also observed using these same mAb following DyLight conjugation (data not shown).

The second group of MαF-Ig mAb (*4-B10* and *8-H9*) exhibited a reactivity profile consistent with light chain specificity. However, double-labeling CD 79β^+ ferret PBMC with these mAb revealed staining of distinct populations of cells (Figure 6(a)). Moreover, nearly the entire CD79β^+ population stained positive with either *4-B10* or *8-H9*, with few cells reacting with both mAb. Collectively, these findings suggested that *4-B10* and *8-H9* define distinct populations of ferret B cells on the basis of light chain expression.

The remaining MαF-Ig mAb (*4-E3*, *4-D11*, and *6-H5*) also had light chain reactivity. These mAb were categorized on the basis of costaining with *4-B10* or *8-H9* single-positive ferret B cells (Figures 6(b)–6(d)). Using a multilabeling approach, *4-E3* was found to costain with nearly the entire 4-$B10^{pos}$ population, while simultaneously not labeling 8-$H9^{pos}$ cells (Figure 6(b) and data not shown). Similarly, 8-$H9^{pos}$ cells were labeled by *4-D11* or *6-H5*, and these mAb exhibited minimal reactivity with 4-$B10^{pos}$ cells (Figures 6(c) and 6(d)). Of note, we routinely observed a small population of 8-$H9^{pos}$ cells that were not stained by *4-D11* (Figure 6(c)). Collectively, these data indicate that *4-B10* and *4-E3* recognize a common light chain protein that is distinct from the light chain recognized by the other mouse anti-ferret light chain (MαF-IgL) mAb (*8-H9*, *4-D11*, and *6-H5*).

3.7. Identification of Overlapping Epitope Recognition through Competitive Binding ELISA. To further characterize whether the MαF-Ig mAb were recognizing overlapping or distinct epitopes on ferret Ig, competitive binding assays were performed. Specifically, unlabeled MαF-Ig mAb were used as competitors and evaluated for their ability to inhibit binding of biotinylated mAb (*4-B10*, *4-E3*, *6-B7*, *8-H9*, and *11-E3*) to the purified ferret Ig antigen. Consistent with the double-labeling FCM studies in which *4-B10* and *8-H9* recognized distinct populations of ferret PBMC (Figure 6(a)), these MαF-Ig mAb did not exhibit inhibition of each other in the competitive binding ELISA (Figures 7(a) and 7(c)). In addition, despite recognition of the same population as assessed by double-labeling FCM (Figure 6(b)), *4-E3* exhibited only subtle inhibition of *4-B10* at the highest concentration tested (5 μg/mL) (Figure 7(a)). In stark contrast, *4-B10* competed for binding with biotinylated *4-E3* (Figure 7(b)). Moreover,

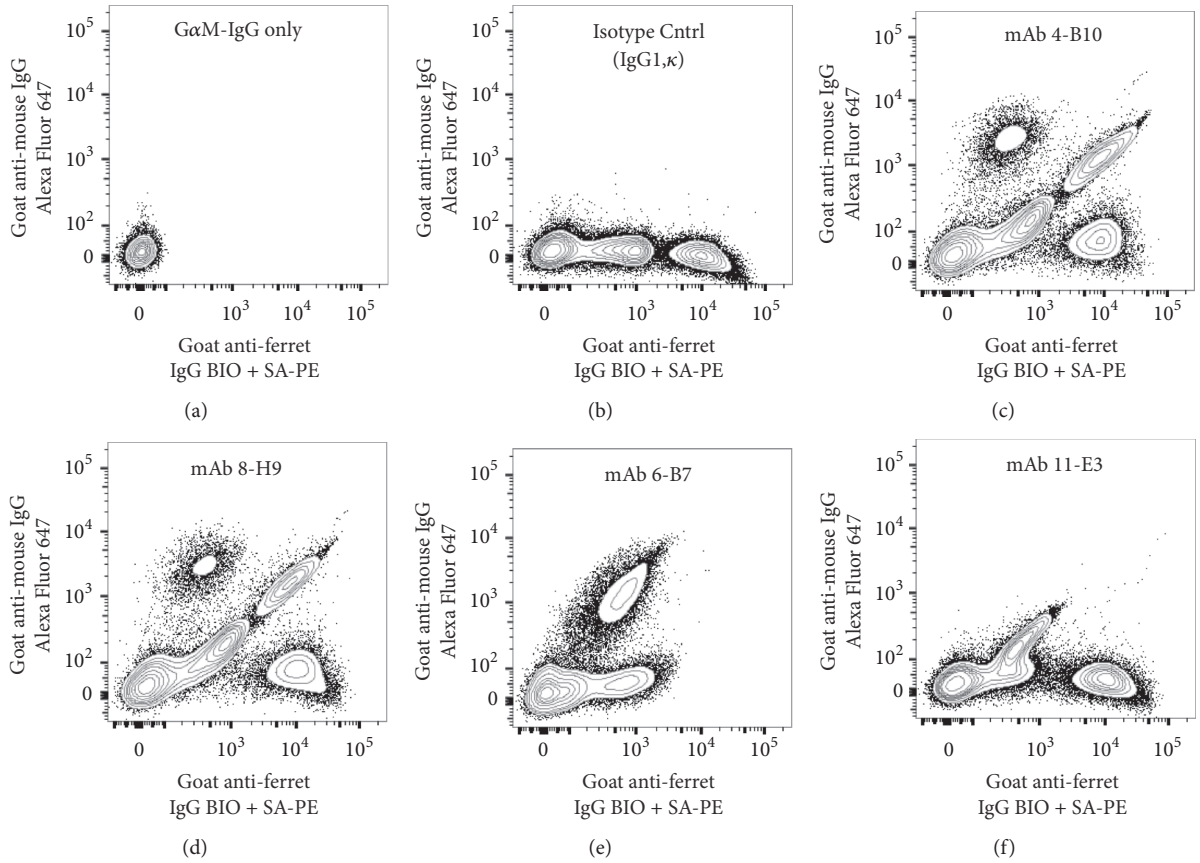

Figure 4: Flow cytometric assessment of monoclonal antibody reactivity. Reactivity of mouse anti-ferret Ig mAb with ferret PBMC was evaluated by flow cytometry. Binding of mouse Ig to ferret PBMC was revealed by secondary staining with Alexa Fluor 647 conjugated goat anti-mouse IgG (GαM-IgG). Additionally, ferret B cells were identified by costaining with biotinylated goat anti-ferret IgG, which was revealed by secondary staining with phycoerythrin conjugated streptavidin (SA-PE). (a) Reactivity of the GαM-IgG secondary antibody with ferret PBMC. (b–f) 1 μg of an IgG1 control (b) or mouse anti-ferret Ig mAb (c–f) was used for indirect surface staining of ferret PBMC. The presented data were generated using PBMC from a single ferret and are representative of two or more independent experiments that yielded similar results.

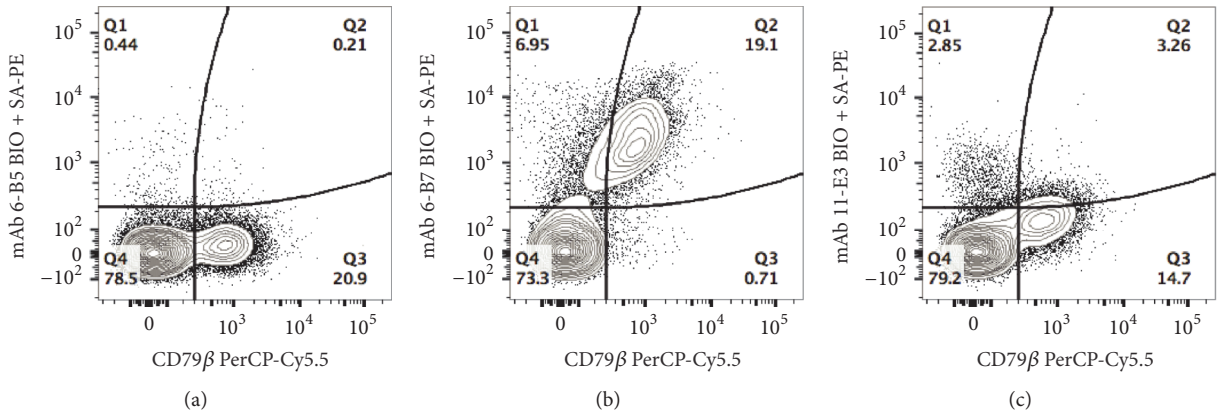

Figure 5: Surface staining with heavy chain reactive monoclonal antibodies. Biotinylated mouse anti-ferret Ig mAb were used in combination with anti-CD79β to evaluate surface staining of ferret B cells. Binding of biotinylated (a) *6-B5*, (b) *6-B7*, or (c) *11-E3* was revealed by secondary staining with phycoerythrin conjugated streptavidin (SA-PE). Frequency of CD79$\beta^{neg/pos}$ ferret PBMC that costained with the mouse anti-ferret Ig mAb are indicated. Data shown were generated using a single ferret and are representative of two or more independent experiments that yielded similar results.

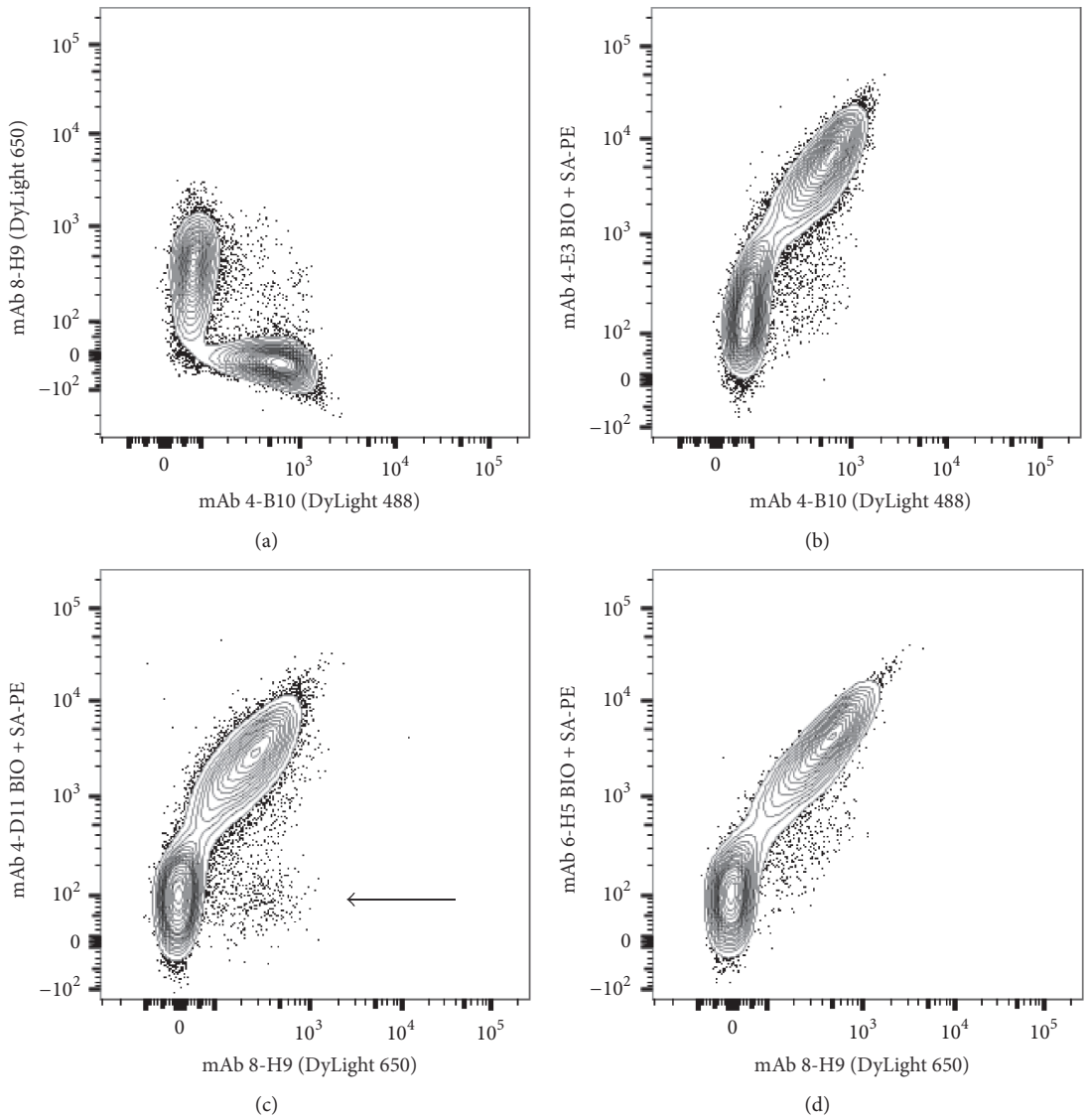

Figure 6: Characterization of light chain reactive monoclonal antibodies. Mouse anti-ferret Ig mAb were used in combination for surface staining of ferret PBMC. Plots are pregated on B cell receptor expressing cells (CD79β^+) as shown in Supplementary Materials. (a) Dual staining of ferret B cells with *4-B10* and *8-H9*. (b) Dual staining of ferret B cells with *4-B10* and *4-E3*. (c) Dual staining of ferret B cells with *8-H9* and *4-D11*. Arrow indicates population of 8-H9$^+$ B cells that lack costaining with *4-D11*. (d) Dual staining of ferret B cells with *8-H9* and *6-H5*. Data are representative of two or more independent experiments comprising $n \geq 6$ individual ferrets.

4-B10 exhibited superior competition with biotinylated *4-E3* relative to the homologous unlabeled competitor. No competition of biotinylated *8-H9* was observed by any MαF-Ig mAb tested. Strikingly, even unlabeled *4-D11* at 20 μg/mL failed to compete with *8-H9* despite these mAb labeling a common population of ferret PBMC by FCM (Figures 6(c) and 7(c)). Similarly, none of the MαF-Ig mAb exhibited competition with biotinylated *11-E3* (Figure 7(d)). However, a number of MαF-IgL mAb were found to compete with biotinylated *6-B7*, despite assignment of this mAb as heavy chain specific (Figure 7(e)). Specifically, both unlabeled *4-B10* and *8-H9* demonstrate definitive competition with biotinylated *6-B7* (Figure 7(e)). Moreover, *4-E3* and *4-D11* (only at high concentrations) were also capable of inhibiting binding of biotinylated *6-B7* to the ferret Ig antigen, albeit to a less extent relative to *4-B10* and *8-H9*.

3.8. Determination of Light Chain Specificity. Although the individual MαF-IgL mAb were already segregated into two distinct groups on the basis of light chain reactivity, their precise specificity remained undefined. To determine which group of MαF-IgL mAb was specific for the Igκ chain, a fluorescently labeled bacterial protein with specificity for Igκ chains from a variety of species [28, 29], Protein L, was used to label ferret B cells. Approximately 25% of CD79β^+ ferret B cells stained brightly with the fluorescently conjugated Protein L reagent (Figure 7(a)) and these cells were putatively assigned as Igκ-expressing B cells. Next, these CD79β^+

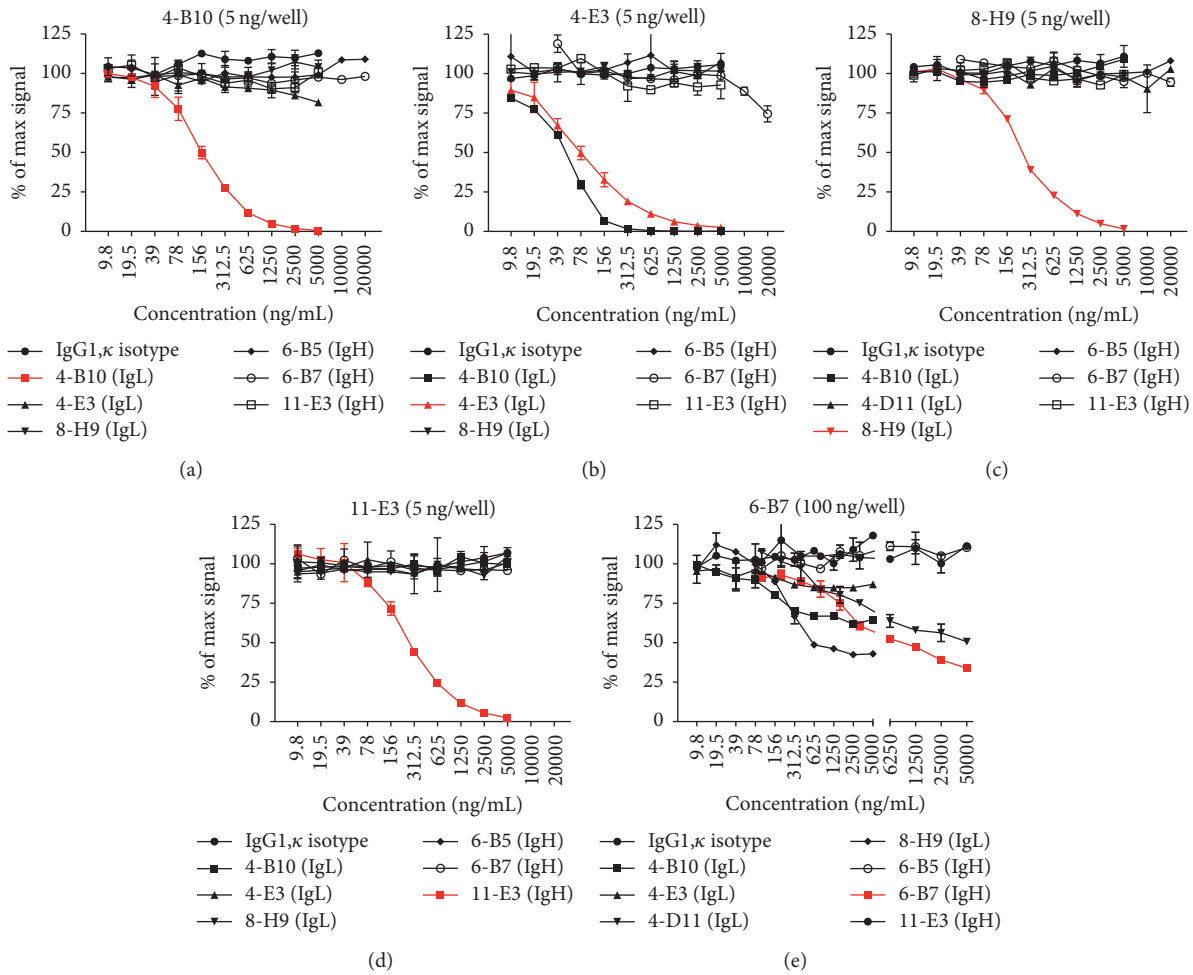

Figure 7: Mapping of monoclonal antibodies through competitive ELISA. Inhibition of biotinylated mouse anti-ferret Ig mAb binding to purified ferret Ig was performed to identify overlapping epitope recognition. Competitive inhibition of (a) *4-B10*, (b) *4-E3*, (c) *8-H9*, (d) *11-E3*, and (e) *6-B7* were performed using the indicated concentration of biotinylated mAb. The *x*-axis indicates concentration of unlabeled mouse anti-ferret Ig mAb competitor, and the homologous unlabeled mAb are depicted in red. Percent of maximal binding by the respective biotinylated mAb is plotted ± SD of duplicates. Data in (a)–(e) are representative of two independent experiments.

Protein L^{hi} cells were evaluated for costaining with *4-B10* or *8-H9*, which defined distinct subsets of ferret B cells. The majority (~80%) of CD79β^+ Protein L^{hi} cells were costained with *4-B10* (Figure 7(b)) and indicated that *4-B10* was specific for ferret Igκ. Conversely, this result indicated that *8-H9* was specific for Igλ. Using these assignments, the distribution of Igκ and Igλ expression by circulating CD79β^+ ferret B cells was determined (Figure 7(c)).

4. Discussion

In this study, eight individual mAb with specificity for ferret Ig were characterized (Table 1). Five of these mAb had specificity for ferret Ig light chain, while the remaining three mAb did not recognize a distinct surface expressed heavy chain. Additionally, we generated a mAb *(6-B7)* that yielded surface staining of nearly all surface BCR positive cells (*i.e.*, pan ferret Ig). Collectively, these MαF-Ig mAb offer advancement in ferret Ig detection compared to the existing portfolio of polyclonal antisera reagents. Moreover, several of these mAb (*4-B10*, *8-H9*, and *11-E3*) are likely to be applicable to a wide array of immunologic assays, including the identification of antibody-secreting cells by FCM.

Pooled serum was chosen as the source of material for subsequent purification of ferret Ig because both IgM and IgG antibody classes were abundant and this biological fluid was readily available [26]. Moreover, the purified ferret Ig preparation had the potential to elicit mAb with specificity for the heavy chain (Igμ or Igγ) and the light chain (Igκ or Igλ) using a single antigen. However, due to the complexity of the ferret Ig antigen, antigen-specific binding in the ELISA format was insufficient to determine the precise specificity of the respective MαF-Ig mAb (Figure 2). To this end, additional screening approaches, such as FCM, western blot and competitive binding assays, were necessary to further define the specificity and categorized the individual MαF-Ig mAb.

Implementation of an indirect FCM screening method during the initial characterization enabled identification of

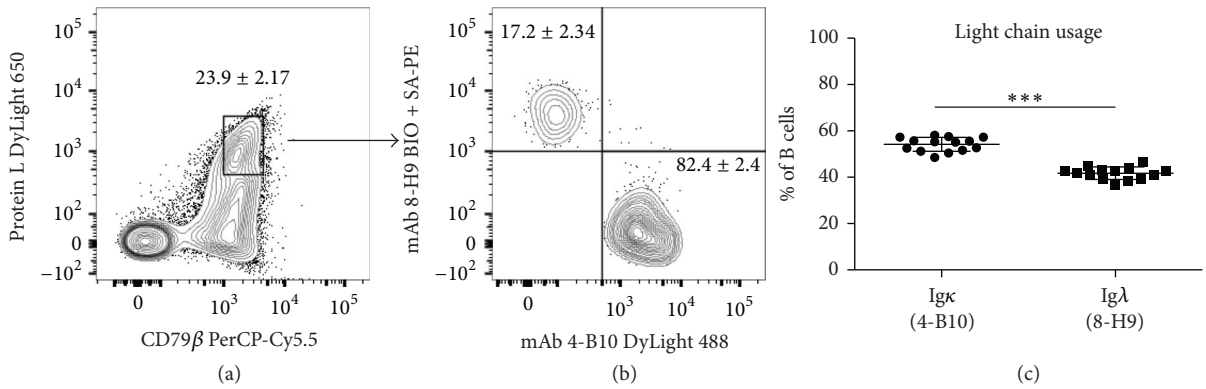

Figure 8: Designation of light chain reactive monoclonal antibody specificity. Mouse anti-ferret Ig mAb (*4-B10* and *8-H9*) were used in combination with anti-CD79β and DyLight 650 conjugated Protein L to assign mAb reactivity with kappa light chain expressing B cells. (a) Representative surface staining of ferret PBMC with anti-CD79β and Protein L. Kappa light chain expressing B cells were defined as CD79β^+ cells that stained intensely with DyLight 650 conjugated Protein L and were gated as shown in (a). The mean frequency of total B cells ± SD that stained intensely with Protein L is indicated. (b) Representative costaining of kappa light chain expressing B cells with *4-B10* or *8-H9*. The mean frequency ± SD of total kappa B cells exhibiting costaining with *4-B10* or *8-H9* is indicated. Data in (a) and (b) are combined from two independent experiments ($n = 6$). (c) Distribution of kappa or lambda light chain expression by total B cells (CD79β^+) was determined on the basis of costaining with *4-10* or *8-H9*. Data in (c) are combined from three independent experiments ($n = 14$). Statistical significance was determined using a paired Student's t-test. $^{***}p < 0.001$.

Table 1: Summary of MαF-Ig mAb.

mAb	Ig subclass	V_H [a]	V_L [a]	Specificity
4-B10	IgG1	IGHV4-1	IGKV6-13	Light chain (Igκ)[c,d]
4-D11	IgG1	IGHV2-3	IGKV16-104	Light chain (Igλ)[d]
4-E3[b]	IgG1	IGHV1-20 or IGHV1-37	IGKV3-12	Light chain (Igκ)[d]
6-B5	IgG1	IGHV5-6	IGKV19-93	Ferret Ig
6-B7	IgG1	IGHV5-6	ND[e,f]	Heavy chain (IgH)[g]
6-H5	IgG1	IGHV5-17	IGKV5-48	Light chain (Igλ)[d]
8-H9	IgG1	IGHV14-3	IGKV6-32	Light chain (Igλ)[c,d]
11-E3	IgG1	IGHV1S81	IGKV6-17	Heavy chain (Igγ)[c]

a: identified using IMGT V-Quest (see [21]). b: unable to unambiguously assign IGHV. c: determined by western blot. d: determined by flow cytometry. e: not determined. f: negative for intracellular Igκ by flow cytometry. g: determined by competitive ELISA.

MαF-Ig mAb that recognized epitopes present on ferret leukocytes. Moreover, incorporation of the GαF-Igγ antisera in this screen enabled resolution of ferret PBMC into distinct populations on the basis of staining intensity, and provided insight into the specificity of the respective MαF-Ig mAb (Figure 4 and data not shown). Specifically, each of MαF-IgL mAb exhibited a similar staining pattern in which approximately half of the sIgG^{hi} population was intensely labeled by the respective mAb. Moreover, these MαF-IgL mAb also labeled a small subset of the sIgG^{int} population at a similarly intense level, while the remainder of the sIgG^{int} population exhibited a low-level of staining (Figures 4(c) and 4(d) and data not shown). This staining pattern indicated that surface Ig$^+$ B cells constituted a fraction of the sIgG^{int} population and was consistent with the observation that the GαF-Igγ antisera as well as other commercially available polyclonal anti-ferret Ig antisera fail to label all B cells (data not shown and [14]). Additionally, this staining pattern implied that myeloid cells constituted a portion of the sIgG^{int} population and that IgG acquired via FcR was bound in such manner that the light chain was accessible to the respective MαF-IgL mAb. Furthermore, segregation of the IgG^{int} and sIgG^{hi} populations in the FCM screen also facilitated perception of the distinct binding specificities exhibited by the heavy chain reactive mAb *6-B7* and *11-E3* on the basis of their differential reactivity profile with the sIgG^{hi} population (Figures 4(e) and 4(f)). Lastly, in the absence of the apparent disappearance of the sIgG^{hi} population that resulted following pretreatment of ferret PBMC with *6-B5* (data not shown), this MαF-Ig mAb would not have been characterized further.

Despite the perceived reactivity of *6-B5* in the antigen-specific capture ELISA and indirect FCM screening during the initial characterization, definitive staining of ferret B cells using this mAb, when directly coupled, was not observed (Figure 5(a) and data not shown). It is likely that biotinylation of *6-B5* was inefficient, rather than that conjugation destroyed the binding specificity of this mAb. Specifically, both unlabeled and biotinylated *6-B5* exhibited comparable binding to ferret Ig in an ELISA when using a goat anti-mouse IgG1 secondary reagent (data not shown). By contrast, the

same biotinylated *6-B5* exhibited a near absence of reactivity against ferret Ig when streptavidin was used to reveal binding. Consequently, it will be necessary to explore alternative chemistries for conjugation of *6-B5* to fully realize the utility of this mAb for flow cytometric applications.

The competitive binding ELISA contributed to the characterization of the respective MαF-Ig mAb. First, the data generated using this approach served to reaffirm prior observations, such as the distinction between *4-B10* and *8-H9* determined by double-labeling FCM. Second, the competitive binding and antigen-specific capture ELISA data were in agreement and reflected differences in functional affinity by several of the MαF-Ig mAb. Most notably, *4-B10* exhibited stronger avidity for the ferret Ig antigen relative to *4-E3* (Figure 2) and also demonstrated superior competition of biotinylated *4-E3* compared to the homologous mAb (Figure 7(b)). Thirdly, the competitive binding ELISA also provided additional insight into the epitopes recognized by the respective MαF-Ig mAb. While it was already presumed that the MαF-IgL mAb were targeting the constant region of kappa or lambda light chain based on their FCM staining patterns (Figure 6), it remained unclear if these anti-kappa or anti-lambda mAb were targeting distinct or overlapping epitopes. On the basis of competitive inhibition, it is likely that *4-B10* and *4-E3* are recognizing similar, but not completely overlapping epitopes on the kappa constant region since the inhibition was unidirectional (Figures 7(a) and 7(b)). By contrast, *4-D11* failed to inhibit binding of biotinylated *8-H9* despite the use of an elevated concentration (20 μg/mL), suggesting that these mAb recognize distinct epitopes on the lambda constant region. However, it remains plausible that the large difference in functional affinity between these mAb contributed to the lack of competition (Figures 2 and 7(c)). Additionally, biotinylated *4-D11* failed to produce a sufficiently high binding signal against the ferret Ig antigen to enable evaluation of reciprocal inhibition by *8-H9* (data not shown). Similar to *8-H9*, no inhibition of biotinylated *11-E3* was observed using any of the MαF-Ig mAb tested, further supporting the notion that this mAb recognizes a distinct epitope (Figure 7(d)). Surprisingly, several of the MαF-IgL mAb were capable of inhibiting binding of biotinylated *6-B7* to the ferret Ig antigen. This was unexpected since *6-B7* was categorized as heavy chain reactive based on the observed FCM staining pattern (Figure 5(c)). However, the ability of *4-B10* and *8-H9*, as well as *4-E3* and *4-D11* to a less extent, to compete with *6-B7* supports the conclusions that *6-B7* is recognizing an epitope that is restricted to the heavy chain. Moreover, these data suggest that *6-B7* recognizes an epitope on the heavy chain of ferret Ig that is proximal to either the kappa or lambda constant region. To this end, we hypothesize that *6-B7* would maintain reactivity with a Fab fragment of ferret Ig.

In spite of strong reactivity with purified ferret Ig by ELISA (Figure 2) and the Igγ chain by western blot (Figure 3(d)), intense staining of ferret B cells using directly labeled *11-E3* was not observed (Figure 5(c)). However, low-level staining of the entire CD79β^+ B cell population, as well as a small population of non-B cells in bulk ferret PBMC, was observed using *11-E3*. Collectively, these observations indicate that the epitope recognized by *11-E3* may be restricted to secreted ferret IgG. Further experimentation will be necessary to resolve the utility of *11-E3* for studying ferret B cells and derived Ig.

The assignment of lambda light chain specificity to *8-H9* was based upon several independent observations. First, the near absolute segregation between populations of 4-B10$^+$ and 8-H9$^+$ ferret B cells indicated that these mAb recognized fundamentally distinct light chains (Figure 6(a)). Of note, this observation also indicates that allelic exclusion of dual light chain expression is largely intact in the ferret model [30]. Secondly, 8-H9$^+$ ferret B cells were severely underrepresented in the population of B cells that bound the kappa-specific Protein L reagent at high levels (Figures 8(a) and 8(b)). While there was a population of 8-H9$^+$ B cells in this gate (~20%), it is plausible that these Igλ^+ B cells acquired labeling with Protein L either due to inherent BCR specificity or through the association of secreted Igκ with surface expressed FcR [31, 32]. In addition, within the total B cell population, the frequency of 8-H9$^+$ B cells detected from multiple independent ferrets closely resembled the distribution of Igλ^+ B cells observed in humans [33].

While a draft sequence of the ferret genome is currently available [34], the Ig loci have not been annotated. Based on the findings presented in this report, we hypothesize that the ferret Igλ locus more closely resembles the human Igλ locus than the mouse Igλ loci due to the increase proportion of B cells that express a lambda light chain (Figure 8(c)). In laboratory mice, the Igλ locus is comprised of 3 functional Vλ gene elements that rearrange with associated Jλ-Cλ gene elements [35]. Additionally, in mice the prevalence of serum immunoglobulin utilizing a lambda light chain is severely reduced relative to immunoglobulin utilizing a kappa light chain [36]. By contrast, the human Igλ locus is more complex and encodes greater than 35 Vλ genes belonging to 10 subgroups [37]. Moreover, the distribution of kappa and lambda immunoglobulin is more balanced in human serum [38]. Collectively, these findings provide the first evidence that lambda light chain usage in ferrets closely resembles that seen in humans and further supports the use of ferrets for studying the antibody response elicited by influenza virus infection or influenza vaccination.

To more accurately mimic influenza infection and vaccination in humans, ferrets can be infected with various influenza viruses from different subtypes to establish a preimmune state in the animal. This model is useful to study both inactivated and live attenuated influenza vaccine (LAIV) candidates. In young children, an IgA recall response occurred following a second administration of LAIV [39]. However, ferrets previously infected with LAIV had a robust IgG antibody-secreting cell (ASC) response in the absence of an accompanying IgA ASC population following experimental challenge with seasonal influenza [40]. While it remains plausible that the immune response elicited by influenza infection of preimmune ferrets was distinct from that observed in young children following LAIV inoculation, it is more likely that the discrepancy between these two models originates from the use of polyclonal anti-ferret Ig detection reagents that possess an inherent lack of heavy chain specificity. This apparent lack of heavy chain specificity was observed

using a variety of polyclonal goat anti-ferret Ig reagents (Supplementary Materials and data not shown). While mAb with specificity for discrete ferret Ig heavy chain determinants were unfortunately not generated in this study, this example serves as motivation for the continued development and characterization of ferret Ig-specific reagents.

In addition, a polyclonal goat anti-ferret Ig reagent with specificity for IgA, IgG, and IgM was used to identify ferret B cells in the context of influenza infection using FCM [14]. In this study, the polyclonal goat anti-ferret Ig (A, G, and M) failed to label a substantial population of ferret B cells that exhibited intracellular staining with the anti-CD79α mAb (clone HM47). The MαF-Ig mAb reported here are an improvement over existing reagents and enable the identification of nearly all surface Ig^+ ferret B cells (Figure 5(b)) and discrimination between Igκ and Igλ-expressing cells (Figure 6(a)).

We envision that several of the MαF-Ig mAb generated in this study will have applications in basic science and veterinary medicine. Due to their strong affinity for purified ferret Ig and reactivity with denatured heavy (Igγ) or light chain proteins, *4-B10*, *8-H9*, and *11-E3* may prove to be most useful. Specifically, these MαF-Ig mAb should enable the identification of antibody-secreting cells by FCM. Additionally, these mAb are likely to have utility as secondary detection reagents in an array of assays such as ELISA, ELISPOT, and western blot. Implementation of these mAb in a variety of immunologic assays will also contribute towards the assessment of the next-generation of broadly-reactive influenza vaccines in this highly relevant model. In summary, the generation of these MαF-Ig mAb is an improvement over existing reagents available to immunologists and opens the door to more sophisticated study of ferret B cells.

Competing Interests

The authors report no conflict of interests with the results reported in this study.

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Research Article

Potential Usefulness of *Streptococcus pneumoniae* Extracellular Membrane Vesicles as Antibacterial Vaccines

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The secretion of extracellular membrane vesicles (EMVs) is a common phenomenon that occurs in archaea, bacteria, and mammalian cells. The EMVs of bacteria play important roles in their virulence, biogenesis mechanisms, and host cell interactions. Bacterial EMVs have recently become the focus of attention because of their potential as highly effective vaccines that cause few side effects. Here, we isolated the EMVs of *Streptococcus pneumoniae* and examined their potential as new vaccine candidates. Although the *S. pneumoniae* bacteria were highly pathogenic in a mouse model, the EMVs purified from these bacteria showed low pathological activity both in cell culture and in mice. When mice were injected intraperitoneally with *S. pneumoniae* EMVs and then challenged, they were protected from both the homologous strain and another pathogenic serotype of *S. pneumoniae*. We also identified a number of proteins that may have immunogenic activity and may be responsible for the immune responses by the hosts. These results suggest that *S. pneumoniae* EMVs or their individual immunogenic antigens may be useful as new vaccine agents.

1. Introduction

Streptococcus pneumonia is an alpha-hemolytic Gram-positive encapsulated aerobic diplococcus bacterium that is the main causative pathogen of community-acquired respiratory tract infections. *S. pneumoniae* is generally considered to be a human pathogen because it causes a number of human diseases, including otitis, sinusitis, bacterial meningitis, sepsis, and pneumonia [1]. The people who are most affected by this organism are children and individuals with immature or compromised immune systems, such as patients with diabetes or acquired immunodeficiency syndrome [2, 3]. Moreover, *S. pneumoniae* has been isolated from various animals, including guinea pigs, cats, horses, dogs, and gorillas. These animals all exhibit *S. pneumoniae*-related clinical symptoms [4]. These *S. pneumonia*-infected animal hosts thus may serve as an extrahuman reservoir from which the pathogen can be transmitted to humans. Therefore, to prevent *S. pneumoniae* infections, it is necessary to vaccinate both humans and animals such as pets. Pneumococcal conjugate vaccines are widely used because they are highly effective in preventing pneumococcal invasive disease. A recent review reported that failure of pneumococcal conjugate vaccines is rare, but irrespective of vaccine or schedule [5]. This may lead us to evaluate EMVs of *S. pneumoniae* for vaccine candidates.

Bacterial extracellular membrane vesicles (EMVs) are spherical vesicles that are secreted by a variety of bacteria. These vesicles measure 20–250 nm in diameter and contain various biologically active proteins that are required for bacterial nutrient acquisition, biofilm formation, and pathogenesis [6]. Since bacterial EMVs are nonviable and yet induce a host immune response, they have great potential

as acellular antibacterial vaccines. In particular, the EMV surface proteins can act as antigens that induce adaptive immune responses in the host.

Here, we isolated nonpathogenic (noncapsular) *S. pneumoniae* EMVs and examined their ability to serve as next-generation vaccines that protect against infections with pathogenic or nonpathogenic bacteria. We also identified the antigenic protein components of the EMVs.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. *Streptococcus pneumoniae* BAA-255 was purchased from the American Type Culture Collection (ATCC, http://www.atcc.org, Manassas, VA, USA). *Streptococcus pneumoniae* KCCM-41569 was obtained from the Korean Culture Center of Microorganisms (KCCM, http://www.kccm.or.kr). The bacteria were grown to an OD_{600} of approximately 1.0 in a 5% CO_2 atmosphere at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract and bacteria cells were counted by using Quantum Tx microbial cell counter (Logos Biosystems, Korea).

2.2. Purification of EMVs. EMVs of *S. pneumoniae* BAA-255 were purified from bacterial culture supernatants by using the method of Choi et al. [7], with some modifications. Briefly, the bacterial cells were removed from the bacterial culture by centrifugation at 16,000 ×g for 20 min. The supernatants were then filtered through a 0.2 μm hollow fiber membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to further remove residual cells and debris. The EMVs were concentrated and ultrafiltrated by using a QuixStand Benchtop System fitted with a 500 kDa hollow fiber membrane (GE Healthcare). The resulting EMVs were precipitated by ultracentrifugation at 150,000 ×g for 3 h at 4°C and the EMV-containing pellets were suspended in 1.0–2.0 mL of phosphate-buffered saline (PBS). Finally, the EMV solution was layered over a sucrose gradient (2.5, 1.6, and 0.6 M sucrose) to remove contaminating proteins. Each fraction was centrifuged at 200,000 ×g for 20 h at 4°C and the sucrose was removed by ultracentrifugation at 150,000 ×g for 3 h at 4°C. The protein concentration was determined by using a bicinchoninic acid assay (Thermo Scientific, Waltham, MA, USA). The purified EMVs were stored at −80°C until required.

2.3. Transmission Electron Microscope (TEM) Observation of EMVs. For TEM analysis, 5 μL of the 5 mg/mL purified EMV sample was loaded onto a freshly glow-discharged holey carbon EM grid (Quantifoil R 2/2, Quantifoil Micro Tools GmbH, Germany). Semiautomated sample verification was performed by using a Vitrobot Mark IV (FEI Company, Eindhoven, Netherlands) at 4°C and 90–100% relative humidity. The vitrified sample was imaged under low dose conditions by using a Tecnai G^2 Spirit TEM (FEI Company, Eindhoven, Netherlands) that was operated at 120 kV acceleration voltage. Images were recorded by using an UltraScan 4000 charge-coupled device camera (Gatan Inc., Pleasanton, CA, USA) at a nominal magnification of ×26,000 and −1–2 μm underfocus.

2.4. Viability and Apoptosis of Human Lung Cancer Cells after Treatment with Bacteria and EMVs. Briefly, the human lung epithelial adenocarcinoma line A549 was cultured in 96-well plates (1×10^5 cells/well) with *S. pneumoniae* bacteria or EMVs for 24 h, after which cell viability and apoptosis were measured as described previously [7]. The culture medium was RPMI 1640 (Gibco, Waltham, MA, USA) supplemented with heat-inactivated 10% FBS and antibiotics (Gibco). The ranges of multiplicity of infection (MOI) of *S. pneumoniae* that were used to infect the A549 cells were 0.001, 0.1, 10, and 1,000 for *S. pneumoniae* BAA-255 and 0.001, 0.01, 0.1, and 1 for *S. pneumoniae* KCCM-41569. The concentrations of *S. pneumoniae* BAA-255 EMVs were 50, 100, and 200 μg protein in 100 μL of culture medium. To measure cell viability, the cells were stained with acridine orange and DAPI (ChemoMetec, Allerød, Denmark). To measure apoptosis, the cells were stained with FITC-conjugated Annexin V, propidium iodide, and Hoechst (ChemoMetec) according to the manufacturer's instructions. The stained cells were then analyzed in a NucleoCounter NC-3000 image cytometer (ChemoMetec).

2.5. Vaccination of Mice Followed by Bacterial Challenge. Six-week-old female C57BL6/J mice were purchased from DBL (Korea) and housed under specific pathogen-free conditions. At the age of 8 weeks, the mice received an intraperitoneal injection (200 μg in PBS) of *S. pneumoniae* BAA-255 EMVs. This was repeated twice at intervals of 2 weeks. Two weeks after the third immunization, the mice were infected intraperitoneally with a lethal dose of nonpathogenic *S. pneumoniae* BAA-255 (1×10^8 cfu) or pathogenic *S. pneumoniae* KCCM-41569 (1×10^3 cfu). Survival was monitored for 7 days. Control mice were immunized with equivalent volumes of PBS and then challenged. All animal experiments were reviewed and approved by the Animal Ethics Committee at the Korea Basic Science Institute (approval number KBSI-AEC 1314).

2.6. Identification of the Proteins in EMVs. The proteins in *S. pneumoniae* EMVs were identified by using one-dimensional electrophoresis-liquid chromatography tandem mass spectrometry (1-DE-LC-MS/MS) as described previously [7]. In brief, purified EMVs were lysed and the protein lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by tryptic in-gel digestion. The digested peptide fractions were then loaded onto a 10 cm × 75 μm inner diameter C18 reverse-phase column (PROXEON, Odense, Denmark; Aqua; particle size, 5 μm) and subjected to a flow rate of 120 nL/min. The peptides were eluted with a gradient of 0–80% acetonitrile containing 0.1% formic acid for 80 min. All MS and MS/MS spectra were acquired by using a Thermo Finnigan LTQ mass spectrometer (San Jose, CA, USA). Each full MS (m/z range of 400–2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum, with dynamic exclusion enabled. Proteins were

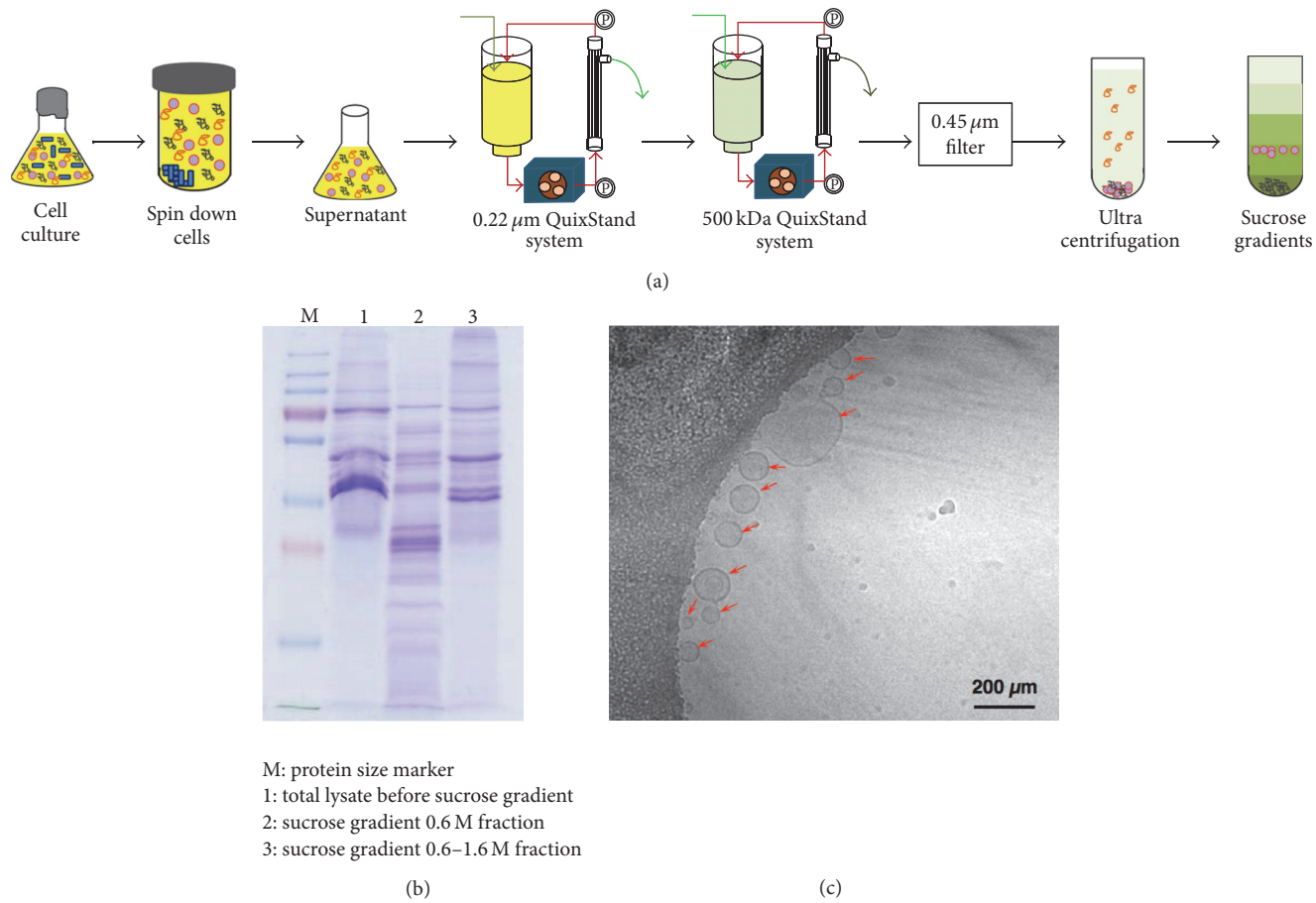

Figure 1: Purification of *Streptococcus pneumoniae* BAA-255 extracellular membrane vesicles (EMVs). (a) Summary of the method used to prepare *S. pneumoniae* BAA-255 EMVs. (b) SDS-PAGE of the EMVs before and after sucrose gradient fractionation. (c) Transmission electron microscopy of the EMVs. Red arrows indicate EMVs.

identified by using MASCOT software (ver. 2.4; Matrix Science Inc., USA). A *S. pneumoniae* R6 protein database (https://www.ncbi.nlm.nih.gov/) was used to analyze the MS/MS data. Carbamidomethylation of cysteine (+57 Da), oxidation of methionine (+16 Da), and propionamide of cysteine (+71 Da) were considered to be variable protein modifications. The exponentially modified protein abundance index (emPAI) was generated by using MASCOT software and the mol% was calculated according to emPAI values. Each sample underwent the MS/MS analysis three times.

3. Results

3.1. Production of EMVs from S. pneumoniae. We first examined whether *S. pneumoniae* produced EMVs. Thus, *S. pneumoniae* BAA-255 was grown in Todd-Hewitt broth that was supplemented with 0.5% yeast extract until the late exponential phase (OD_{600} ~ 1.0). The bacterial cells were precipitated by centrifugation and the supernatant was prepared. The supernatant was ultrafiltered to remove cells and cellular debris and sucrose gradient centrifugation was performed to remove contamination with other protein complexes (Figure 1(a)). The EMVs were enriched between 0.6 and 1.6 M sucrose (Figure 1(b)). TEM examination confirmed the presence of *S. pneumoniae* BAA-255 EMVs. The diameter of the EMVs ranged from 40 nm to 200 nm (Figure 1(c)). This is similar to the diameters of other bacterial EMVs.

3.2. Cytotoxic Effects of S. pneumonia EMVs. The endotoxic activity of virulence factors in EMVs significantly hampers their usefulness as vaccines. Therefore, we assessed whether nonpathogenic *S. pneumoniae* BAA-255 and its EMVs induce host cell damage by treating A549 cells with various concentrations of *S. pneumoniae* BAA-255 bacteria and EMVs. Cell viability and apoptosis assays showed that high concentrations (1×10^8 cfu, MOI 1,000) of *S. pneumoniae* BAA-255 cells slightly reduced cell viability and induced apoptosis (Figures 2(a) and 2(b)). However, when the cells were treated with EMVs from *S. pneumoniae* BAA-255, cell viability and apoptosis rates were unchanged, even in the presence of high concentrations (200 μg) of EMVs (Figures 2(c) and 2(d)). By contrast, a pathogenic strain of *S. pneumoniae,* namely, KCCM-41569, killed A549 cells even at very low concentrations (1×10^3 cfu, MOI 0.01) (Figure S1) (see Supplementary Material available online at https://doi.org/10.1155/2017/7931982). These results suggest that EMVs from nonpathogenic *S. pneumoniae* BAA-255 are not cytotoxic.

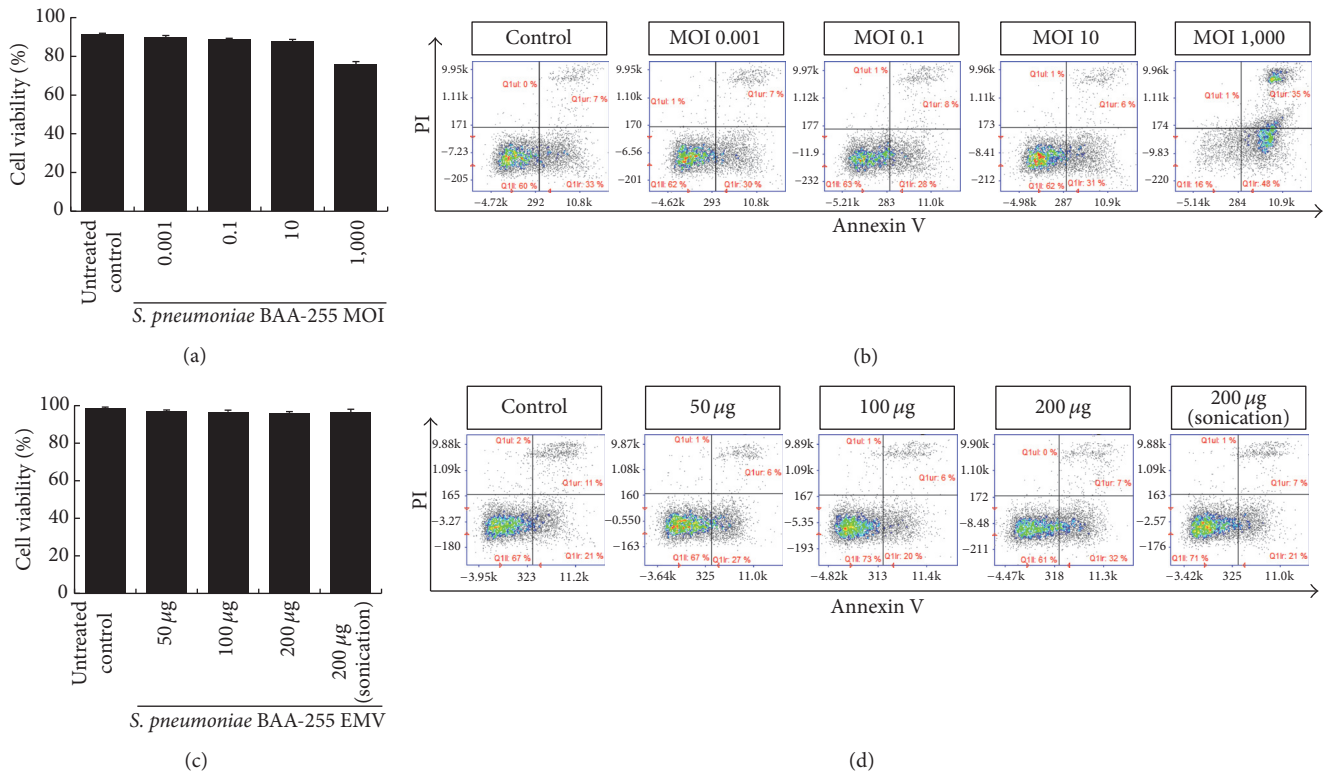

Figure 2: Cytotoxicity of intact *S. pneumoniae* BAA-255 and its EMVs. A549 cells were treated with various concentrations of intact *S. pneumoniae* BAA-255 and cell viability (a) and apoptosis (b) were analyzed. A549 cells treated with various concentrations of *S. pneumoniae* BAA-255 EMVs and cell viability (c) and apoptosis (d) were analyzed.

3.3. Immunization with S. pneumoniae EMVs. To evaluate the potential and efficacy of EMVs as a new vaccine against *S. pneumoniae* infection, mice were vaccinated with *S. pneumoniae* BAA-255 EMVs and then challenged with live *S. pneumoniae* BAA-255 bacteria. After bacterial challenge, 10% of the control mice survived. By contrast, 60% of the EMV-vaccinated and challenged mice survived (Figure 3(a)). This suggests that vaccination with *S. pneumoniae* EMVs protects mice against *S. pneumoniae*. Control and EMV-vaccinated mice were also challenged with KCCM-41569, a pathogenic strain of *S. pneumoniae*. None of the control mice survived infection with 1×10^3 cfu of *S. pneumoniae* KCCM-41569. However, some of the vaccinated mice did survive: 40% survived after three immunizations and 20% survived after a single or double immunization (Figure 3(b)). These results show that immunization with EMVs isolated from a nonpathogenic *S. pneumoniae* BAA-255 strain not only protected against homologous challenge but also provided cross-protection against challenge with a pathogenic heterologous strain.

3.4. Identification of S. pneumoniae EMV Proteins. LC-based proteomic analysis was performed to identify the protein components of *S. pneumoniae* EMVs. 1D-LC-MS/MS analysis identified a total of 104 proteins in *S. pneumoniae* BAA-255 EMVs (Supplementary Table S1). A cell location prediction program was then used to determine the subcellular localization of the identified proteins. Of the 104 proteins identified in *S. pneumoniae* EMVs, 32 were extracellular, 28 were from the membrane, one was from the cell wall, and 43 were cytoplasmic (Figure 4(a)). Of the 104 identified proteins, the 32 extracellular proteins accounted for more than half (67.2%) of the total protein in *S. pneumoniae* EMVs (Figure 4(b)).

The identified proteins were then analyzed by using a bioinformatics tool (Clusters of Orthologous Groups (COGs)) to determine the putative function of *S. pneumoniae* EMVs. The proteins in *S. pneumoniae* EMVs were mainly involved in the transport and metabolism of biomaterials such as amino acids and carbohydrates, as well as inorganic ions (Figure 5).

4. Discussion

Vaccines that prevent viral and bacterial diseases significantly improve public health. There are three types of vaccines against these microorganisms, namely, attenuated live vaccines, inactivated vaccines, and subunit vaccines. Although attenuated live and inactivated vaccines provide high levels of protection against viral and bacterial disease, there are concerns that they also elicit toxic side effects due to the presence of virulence factors. While this problem is overcome by subunit vaccines, they in turn are less effective and more expensive to produce than attenuated live or inactivated vaccines [8]. One way to sidestep these problems with existing antibacterial vaccine approaches is to use bacterial EMVs, as these are safe, cheap to produce, and provide

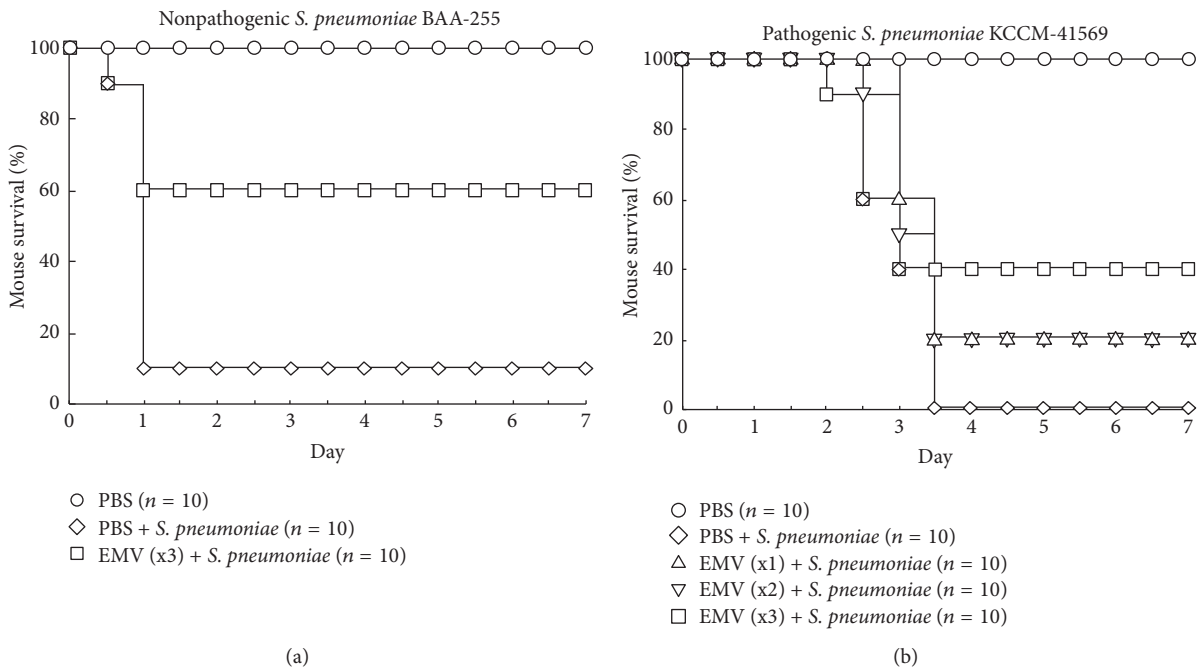

Figure 3: Survival of vaccinated mice after infection with *Streptococcus pneumoniae*. Mice (8 weeks old) were vaccinated intraperitoneally with *S. pneumoniae* BAA-255 extracellular membrane vesicles. After intraperitoneal inoculation with 1×10^8 cfu of nonpathogenic *S. pneumoniae* BAA-255 or 1×10^3 cfu of pathogenic *S. pneumoniae* KCCM-41569, survival over 7 days was assessed. Equivalent volumes of phosphate-buffered saline served as a vaccine control.

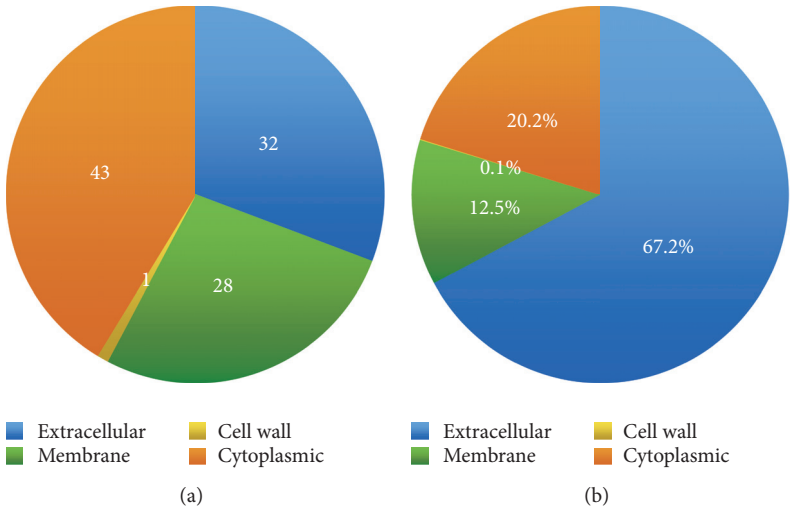

Figure 4: Subcellular localization of proteins identified in *Streptococcus pneumoniae* BAA-255 extracellular membrane vesicles (EMVs). The subcellular localization of the different proteins identified in the EMVs according to the number of proteins (a) or the total amount of protein (b) is shown. The subcellular localization of the proteins was determined by using CELLO (http://cello.life.nctu.edu.tw).

high levels of protection [9]. Indeed, several EMV vaccines against serogroup B *Neisseria meningitides* (MenB) have been licensed for human use in Norway, Cuba, Chile, and New Zealand [10–13]. Here, we showed that *S. pneumoniae* EMVs are both effective against *S. pneumoniae* infection and safe in an animal model (Figures 2 and 3). This supports the notion that bacterial EMVs have great potential as next-generation vaccines that protect humans from bacterial infections.

We identified 61 putative antigenic proteins (extracellular, membrane, and cell wall proteins) that may be recognized by the host immune system and induce adaptive immunity. Previous reports identified the immunogenic proteins in exoproteomes of *S. pneumoniae* [14, 15]. We also found some of these immunogenic proteins in *S. pneumoniae* EMVs, namely, MalX, AliA, Ami, PspA, Eno, ABC-SBP, Sphra, and ZamB (Supplementary Table S1). These results suggest that

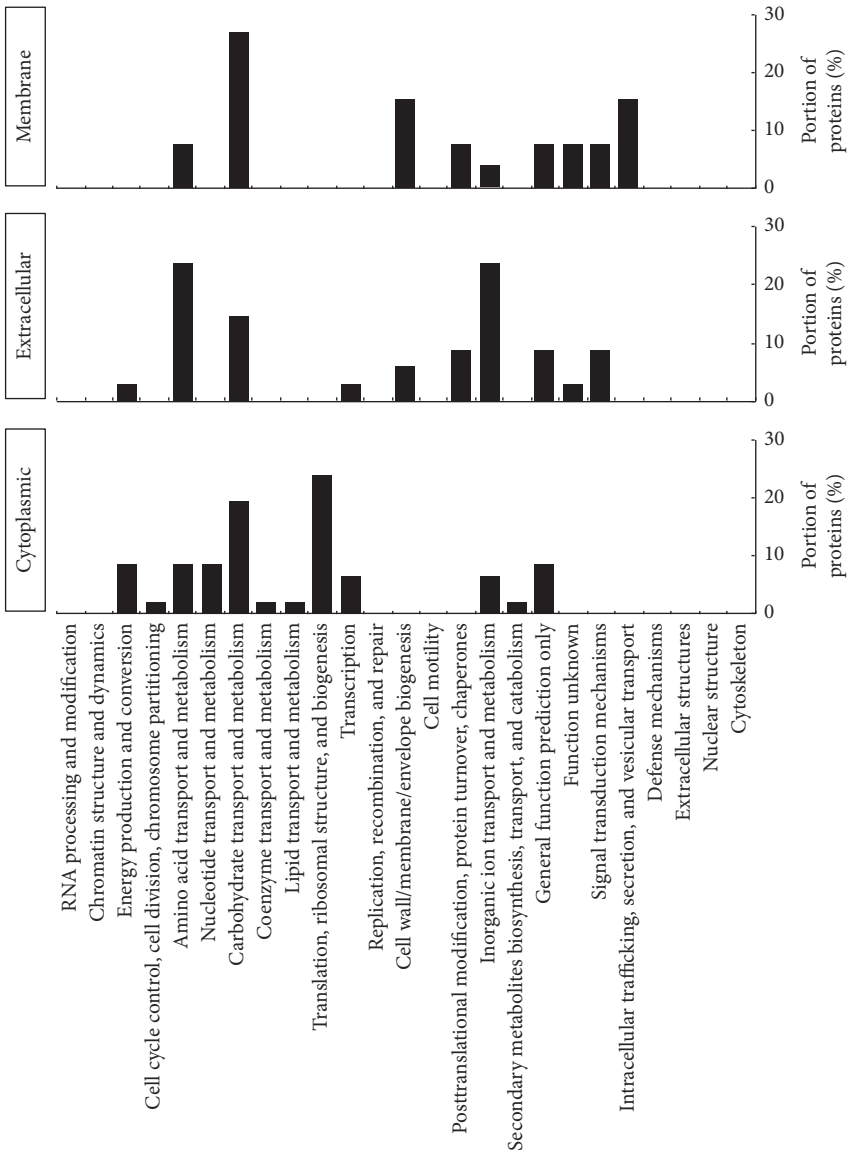

Figure 5: Functional annotation of the *Streptococcus pneumoniae* extracellular membrane vesicle proteins. The proteins were clustered according to their putative functions, which were determined by COGs (Clusters of Orthologous Groups).

the proteins in the EMVs may be immunogenic antigens that induce the production of specific antibodies by the host. Previously, existence of EMVs from other Gram-positive bacteria and their protein composition was reported. 90 proteins from *Staphylococcus aureus* EMVs [16], 104 proteins from *Bacillus anthracis* EMVs [17], and 426 proteins were identified from *Clostridium perfringens* EMVs [18]. Among the immunogenic proteins identified in *S. pneumoniae*, only MalX, Eno, and Sphra were discovered in *C. perfringens*. Therefore, these results suggest that the other immunogenic proteins may be specific for *S. pneumonia* and each bacterium has different immunogenic protein sets. These proteins can be used to develop diagnostic kits and subunit vaccines for *S. pneumoniae*.

In this study, we isolated the EMVs from a nonpathogenic *S. pneumoniae* strain (BAA-255), which has no polysaccharide capsule. The main difference between pathogenic and nonpathogenic *S. pneumoniae* is the presence of a polysaccharide capsule in the former. This polysaccharide capsule is the most potent virulence factor [19]. Capsular polysaccharide is also used as pneumococcal vaccines. Our results showed that immunization with the EMVs from the nonpathogenic *S. pneumoniae* strain BAA-255 protected mice from infection with a pathogenic *S. pneumoniae* strain (KCCM-41569), especially when multiple vaccinations were given (Figure 3(b)). This suggests that EMV proteins are responsible for acquired immunity against bacterial infection. For immunization, we inoculated EMVs only. Therefore, less EMVs would be sufficient for immunization when they are inoculated with adjuvants.

S. pneumoniae BAA-255 is a nonpathogenic noncapsulated strain and we found that its EMVs had no cytotoxic effect on A549 lung cancer cells, even at high concentrations (200 μg) (Figure 2). In fact, the EMVs from nonpathogenic

S. pneumoniae BAA-255 was even safer than the EMVs from the environmental soil bacterium *Pseudomonas putida* KT2440: a previous study showed that 25 μg of *P. putida* KT2440 EMVs induced early apoptosis in A549 cells [7]. The difference between *S. pneumoniae* and *P. putida* EMVs with respect to cytotoxicity may be due to the fact that *P. putida* is Gram-negative: the EMVs from Gram-negative bacteria often express endotoxic lipopolysaccharide (LPS) on their outer membrane (these EMVs are commonly known as outer membrane vesicles) [20]. Indeed, EMVs from many Gram-negative bacteria are cytotoxic [21]. Since *S. pneumoniae* BAA-255 is Gram-positive, its EMVs do not carry LPS. Thus, the EMVs of Gram-positive bacteria may be safer and more suitable for vaccines than EMVs derived from Gram-negative bacteria.

5. Conclusions

The present study aimed to examine the potential of bacterial EMVs as vaccines. The results showed that EMVs isolated from *S. pneumoniae* BAA-255 protected mice with no notable side effects. In addition, we identified the immunogenic proteins that are expressed on *S. pneumoniae* BAA-255 EMVs. Since EMVs are more immunogenic than an equivalent amount of bacterial cell extract [15], these findings suggest that EMVs are highly promising as potential vaccine antigens.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Chi-Won Choi and Edmond Changkyun Park contributed equally to this work.

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Research Article

Passive Immunoprophylaxis for the Protection of the Mother and Her Baby: Insights from In Vivo Models of Antibody Transport

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Pregnant women are at high risk for infection by pathogens. Vertical transmission of infectious agents, such as Zika, hepatitis B, and cytomegalovirus during pregnancy, remains a public health problem, associated with dire outcomes for the neonate. Thus, a safe prophylactic and therapeutic approach for protecting the mother and the neonate from infections remains a high priority. Our work is focused on better understanding the safety and efficacy determinants of IgG antibody preparations when used during pregnancy to benefit the mother and her baby. Using pregnant guinea pigs, we demonstrated that biodistribution of administered IgG to the fetus increases with gestation and results in lower maternal and higher fetal antibody concentrations as pregnancy progresses. Data suggests that partition of antibody immunotherapy to the fetal compartment may contribute to a lower maternal exposure (as measured by the AUC) and a shorter mean residence time of the IgG therapeutic at the end of pregnancy compared to nonpregnant age-matched controls, irrespective of the administered dose. Our studies provide insights on the importance of selecting an efficacious dose in pregnancy that takes into account IgG biodistribution to the fetus. The use of appropriate animal models of placental transfer and infectious disease during pregnancy would facilitate pharmacokinetic modeling to derive a starting dose in clinical trials.

1. Introduction

Infectious diseases are a significant contributor to pregnancy related maternal morbidity and mortality [1] accounting for more than 10% of pregnancy related deaths in the US [2]. Changes in immune status during pregnancy render women more susceptible to infections and, when infected, prone to more severe disease [3, 4]. Infections in pregnancy are associated with poor outcomes for the newborn, ranging from premature birth to congenital abnormalities and death [4–8]. Maternal immunity to pathogens improves outcomes; thus a significant emphasis has recently been placed on immunization of pregnant women in the US [9]. For vaccines that are contraindicated or not recommended during pregnancy, and for pathogens for which there are no approved vaccines, passive immunization with hyperimmune antibody preparations can be an alternative during pregnancy as there are no known risks to the fetus from such preparations [10]. However, in the few clinical studies where IgG was administered during pregnancy time-concentration data have often not been collected [11, 12]. Such information is critical, as the efficacy of IgG preparations has been shown to correlate with the dose [13] and the elevation of IgG trough levels is associated with reduced incidence of infections such as pneumonia [14].

Because intact IgG molecules can pass the placenta in a receptor-mediated fashion [15], passive immunization of the pregnant woman during pregnancy is believed to benefit not only the mother but also her baby [16] and it has been proposed or is being studied for CMV [12], HBV [17, 18],

rubella [19], and other infections, with mixed results. Gaps remain in our knowledge of the efficacious dose, frequency of administration during pregnancy, and the determinants of protection in preventing mother-to-child transmission. In addition, not all IgG subclasses traverse the placenta at the same rate [20], and the magnitude of the clinical benefit may depend on the isotype of the neutralizing antibodies for a specific pathogen.

It is clear there is a need for more data, and, until such gaps are bridged, animal studies can inform decisions regarding starting dose and frequency of administration in clinical studies. In pregnant guinea pigs we have demonstrated that pharmacokinetic properties of IgG therapeutics administered to animals at the end of pregnancy differ from those in nonpregnant controls and that these changes may correlate with the transplacental transfer to the fetus which increases with gestation.

2. Materials and Methods

2.1. Animal Studies. All animal procedures were performed in accordance with protocols approved by the CBER Animal Care and Use Committee as previously described [21]. Briefly, Hartley Albino (Crl:HA) guinea pigs were purchased from commercial sources and mated to produce timed pregnancies. For the pharmacokinetic study, a total of ten pregnant guinea pigs (n = 5/group) on day 65 ± 2 of pregnancy were weighed and a polyclonal commercial human IgG purified from pooled plasma of healthy donors with high titers of antibodies against Hepatitis B, HepaGam® (Emergent Biosolutions, 549 IU/mL and 41 mg/mL) was administered intravenously at a dose 50 or 100 IU/kg (~3.5 or ~7 mg/kg). Dose was chosen to correspond with the approved dose for infants born to mothers testing positive for hepatitis B [22]. Maternal blood samples for pharmacokinetic (PK) study were collected at 10, 30, and 60 minutes and then every day until delivery. All pregnant guinea pigs gave birth 2–6 days after test article administration. An additional ten age-matched nonpregnant controls (n = 5/group) received the same IgG doses; blood samples for the PK study were collected 10, 30, and 60 minutes after administration and then daily for 5 days. Blood was stored overnight at 4°C to coagulate and then spun in a benchtop centrifuge at 1500 ×g for 5 minutes. Serum was collected, transferred into fresh tubes, and then frozen at −80°C for storage.

For the IgG trough levels at different gestation ages study, five groups of pregnant sows, one for each gestation age, n = 4–7/group, were used. On gestation days (GD) 22 ± 1 (n = 6), 30 ± 1 (n = 6), 40 ± 1 (n = 7), 50 ± 1 (n = 7), and 60 ± 1 (n = 4), approximately corresponding to the end of first trimester, middle and end of second trimester, and middle and end of the third trimester, the animals were weighed and HepaGam (Emergent Biosolutions, 549 IU/mL and 41 mg/mL) was administered intravenously at a dose 100 IU/kg (0.182 mL/kg or ~7 mg/kg). Five days after injection, blood samples were collected from all dams, five of the litters on GD45, and all the litters of GD55 and 65 *via* cardio- or cordocentesis; whole fetuses were collected from all the remaining animals. Five days after injection was used as the sampling point for multiple reasons that have been addressed before [21] and included lack of anti-human antibody response. In addition, results from this and previous pharmacokinetic studies [23] indicated that five days following HepaGam administration in guinea pigs is approximately 1.5 times the half-life of human IgG in this species and thus can be considered equivalent to the time point when C_{min} or trough antibody levels are achieved during IGIV therapy.

Fetuses were carefully separated from the placenta, cleaned with cold PBS, weighed, flash-frozen individually, and then homogenized by placing 50% tissue : PBS w : v mixture on ice with an OMNI TH apparatus (Omni International, Kennesaw, GA). The mixture was centrifuged at 10,000 ×g for 10 minutes at 4°C and the supernate frozen at −80°C for storage until use. Human IgG and anti-HBsAg neutralizing activity in the serum and tissue homogenates were determined with a human IgG ELISA kit (Assaypro, St. Charles, MO) and ETI-AB-AUK PLUS (DiaSorin, Saluggia, Italy), respectively. IgG subclasses were measured with a human IgG subclasses kit (Cell Sciences, Newburyport, MA). All samples were measured in duplicates; data points out of data fitting range or with CV > 15% were excluded from analysis and repeated measurements taken, if possible. The kits did not cross-react with guinea pig serum or homogenates from controls that did not receive human IgG.

2.2. Data Transformation and Analysis. Absorbance values from ELISA were transformed into maternal and fetal human IgG concentration or anti-HBs international units by fitting them to an equation derived from a five-parameter fit of the standard curve (SoftMax Pro, Molecular Devices). The assumption was made that IgG was distributed equally in fetal tissue and serum, and no adjustment was made for the concentration measured in total body homogenates versus serum. Human IgG concentrations (μg/mL) from all litter-mates were averaged to obtain a litter average; the fetal : maternal concentration ratios were calculated by dividing the litter averages by human IgG concentration from the respective dam. The litter was used as the unit for statistical analysis. One-way ANOVA with Bonferroni post hoc analysis was used to compare gestation dependent human IgG concentrations or fetal : maternal concentration ratios (GraphPad Software, San Diego, CA). Two-way ANOVA was used to compare the concentrations or fetal : maternal concentration ratios of IgG subclasses in different gestations; p values < 0.05 were considered significant.

Maternal and fetal human IgG concentrations were tested for correlation; Pearson two-tailed test was used to look for presence of a linear relationship; p value < 0.05 was considered significant.

2.3. Pharmacokinetic (PK) Analysis. PK parameters from serum concentration-time data in pregnant and nonpregnant guinea pigs were estimated by noncompartmental analysis. These PK parameters were estimated as follows.

Table 1: Pharmacokinetic (PK) parameters of a hepatitis B immune globulin (HBIG) product in pregnant and nonpregnant guinea pigs.

PK parameters (Mean ± SD)	3.5 mg/kg dose		7.0 mg/kg dose	
	Control (n = 5)	Pregnant (n = 5)	Control (n = 5)	Pregnant (n = 5)
AUC (μg∗hr/mL)	8690 ± 2418	3563 ± 2500	12522 ± 3309	8100 ± 2393
CL (mL/hr per kg)	0.4 ± 0.2	1.4 ± 0.9	0.6 ± 0.2	1 ± 0.4
Half-life (hours)	107 ± 24	108 ± 40	114 ± 58	60 ± 23
MRT (hours)	53 ± 4	35 ± 15	46 ± 4	35 ± 14
V_{ss} (mL/kg)	23 ± 9	41 ± 11	27 ± 6	30 ± 5

AUC: area under the curve; CL: clearance; MRT: mean residence time; V_{ss}: volume of distribution at steady state.

Half-life was calculated by regression analysis on the terminal phase of concentration-time data according to

$$\text{Half-life} = \frac{0.693}{k}, \quad (1)$$

where k is elimination rate constant

$$\text{Clearance CL} = \frac{\text{Dose}}{\text{AUC}}, \quad (2)$$

where AUC is the area under the curve calculated by the trapezoidal rule

$$\text{Mean residence time MRT} = \frac{\text{AUMC}}{\text{AUC}}, \quad (3)$$

where AUMC is the area under the moment curve calculated by the trapezoidal rule

$$\text{Volume of distribution at steady state } V_{ss} = \text{CL} \times \text{MRT}. \quad (4)$$

Statistical differences in PK parameters for each group were analyzed using two-way ANOVA with dose and pregnancy status as variables (GraphPad Software, San Diego, CA). For any parameter, if dose was not a significant source of variance, individual values were pooled in two groups according to pregnancy status and reanalyzed using Student's t-test; $p < 0.05$ was considered significant.

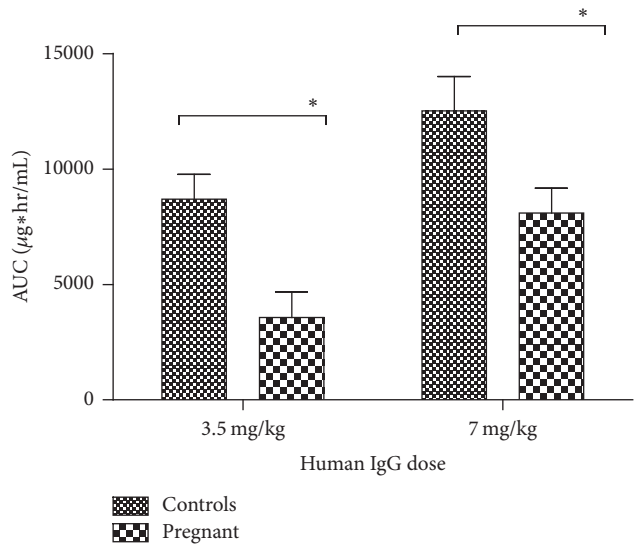

Figure 1: Area under the curve (AUC) of a hepatitis B immune globulin (HBIG) product in pregnant and nonpregnant guinea pigs. HBIG was administered intravenously at a dose 50 or 100 IU/kg (~3.5 and 7 mg/kg) in pregnant guinea pigs at the end of gestation (GD65 ± 2); age-matched nonpregnant animals served as controls. Human IgG exhibits lower AUC when it is administered at the end of pregnancy compared to nonpregnant animals, independent of dose; AUC shown as mean ± SEM, $^*p < 0.05$ (Bonferroni post hoc analysis).

3. Results and Discussion

We administered HepaGam, a commercial hepatitis B specific human IgG (HBIG) preparation, intravenously to pregnant guinea pigs at the end of gestation and nonpregnant controls and then measured the concentration-time dependence of the administered antibodies until the time of parturition (2–6 days after administration). The average concentrations and a summary of pharmacokinetic parameters for pregnant and nonpregnant age-matched controls are shown in supplemental Figure 1S in Supplementary Material available online at https://doi.org/10.1155/2017/7373196 and Table 1, respectively.

Statistical analysis of the PK parameters revealed that, irrespective of the administered dose, systemic exposure to the antibody was dependent on the pregnancy status. Thus, pregnant animals had a statistically significant lower AUC compared to nonpregnant controls (Figure 1). Both dose and pregnancy status contributed significantly (p = 0.0030 and 0.0011, resp.) to the variation in AUC values. There was no evidence of a synergistic effect between the administered dose and pregnancy status on AUC as no significant interaction between them was found (p = 0.7727). Additionally, pregnancy status, but not the dose, significantly affected clearance (p = 0.0065), mean residence time (MRT, p = 0.0075), and volume of distribution at steady state (V_{ss}, p = 0.0171). The PK parameters that were not affected by the administered dose were pooled according to pregnancy status and reanalyzed using one-tailed Student's t-test (Figures 2(a)–2(d)). Only MRT remained significantly shorter in pregnant animals (p = 0.0274), whereas the other parameters showed nonsignificant trends towards a shorter half-life, faster clearance, and larger volume of distribution during pregnancy compared to nonpregnant controls.

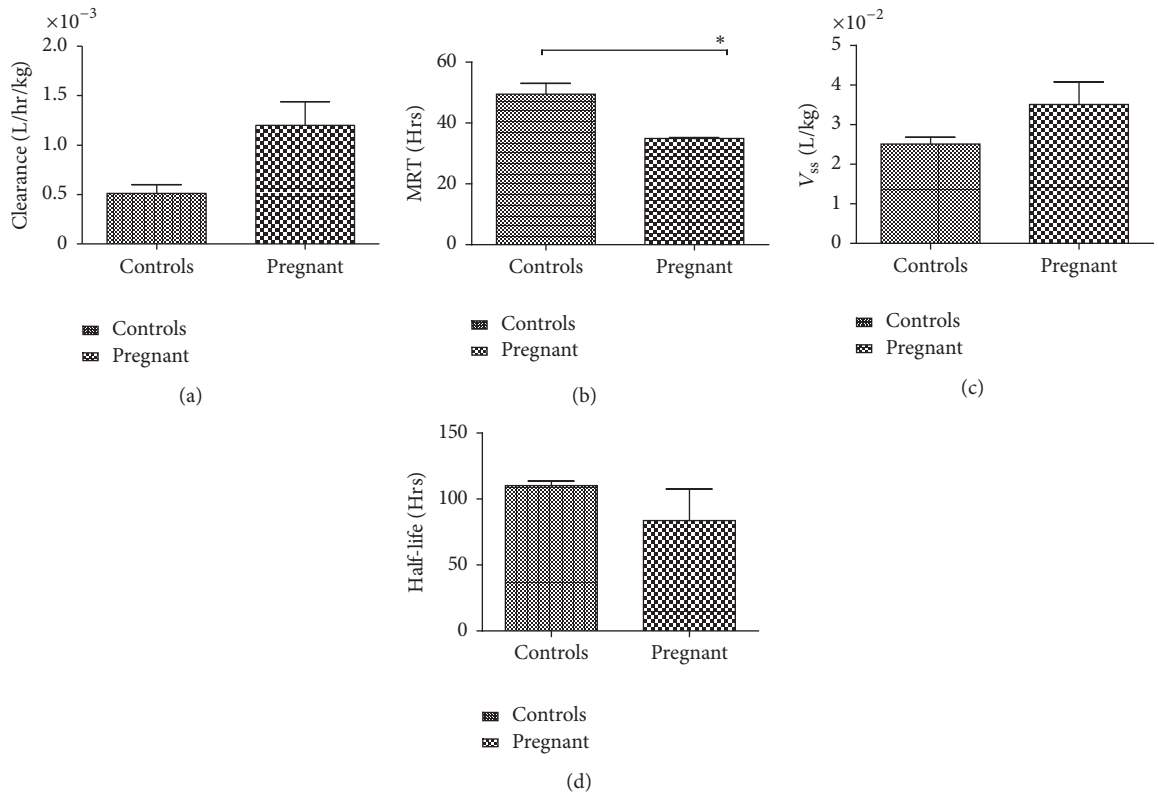

Figure 2: Pharmacokinetic (PK) parameters of a hepatitis B immune globulin (HBIG) product in pregnant and nonpregnant guinea pigs. Human IgG has larger clearance (a), lower mean residence time (MRT, (b)), larger volume of distribution at steady state (V_{ss}, (c)), and lower half-life (d) when it is administered at the end of gestation (GD65 ± 2) compared to nonpregnant animals; data shown as mean ± SEM, $^{*}p < 0.05$ (Student's t-test).

Similar PK data from clinical studies during pregnancy are not readily available. In the few studies where the decrease of maternally administered IgG concentration with time has been followed, data interpretation is complicated. For example, the half-life of CMV IGIV administered to a pregnant woman with primary CMV infection was 11 days [24] compared to the 21-22 days in healthy volunteers [25], a reduction analogous to the trend in average half-life values calculated from guinea pigs (Figure 2(d)). It should be noted that the role the primary infection as well as individual differences may play in the shorter half-life in this case cannot be ruled out and should be further investigated. In another study, AUC values derived from pregnant women receiving IGIV products were not significantly different during prepregnancy period and in the first and second trimesters [26]; women in the third trimester, however, were not included in this study.

The lower AUC and higher clearance of IGIV in pregnant guinea pigs suggest that IGIV may be distributed into the fetus in pregnant animals. Indeed, in agreement with our previous studies [23] we observed a dose dependent increase of human antibody concentration in full term babies from the pregnant animals that received human IGIV product perinatally (data not shown). To ascertain that changes in maternal PK parameters correlated with placental distribution of the administered antibodies, we injected HepaGam in pregnant guinea pigs at five gestation ages roughly corresponding to end of first trimester, middle and end of second trimester, and middle and the end of the third trimester. Then we measured the IgG concentration in maternal blood and fetal blood or total body homogenates five days after injection, a time point roughly corresponding to the minimum concentration (C_{min}) often termed trough level in an IGIV therapy regimen. We made the assumption that, on GD26, 35, and 45, the distribution of IgG in fetal tissues and serum is the same and made no adjustments to convert concentration in total body homogenates to fetal serum concentrations. As demonstrated by the individual data (supplemental Table 1S), the average IgG concentration in the serum of $n = 5$ GD45 litters is ~2.5-fold higher than that in whole body homogenate preparations of $n = 2$ litters from the same gestation age (Table 1S, italics). Thus, although our assumption may result in an underestimation of fetal serum concentrations, the data from GD45 indicates that the effect is of the same magnitude as individual variations in each litter.

We found that the mean maternal concentrations trend progressively lower with increased gestation (Figure 3(a) and supplemental Table S1) whereas the corresponding mean fetal concentrations progressively increase (Figure 3(b), and [21]). These two variables were negatively correlated (Figure 3(f)) and, after log transformation, the relationship was linear (Pearson correlation $r = -0.60$, $p = 0.0008$,

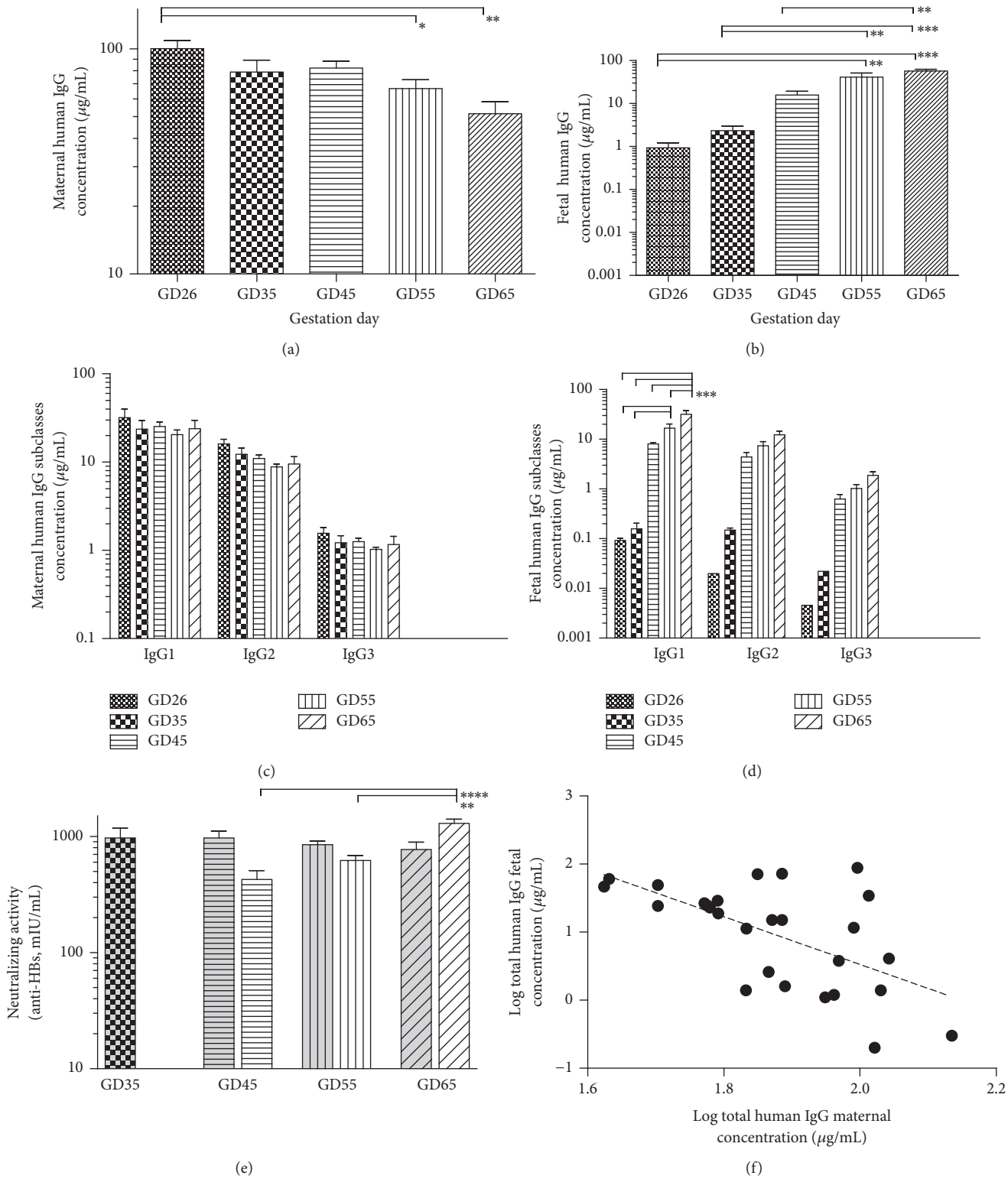

FIGURE 3: Maternal and fetal concentrations of human IgG following hepatitis B immune globulin (HBIG) administration at different gestation ages. HBIG was administered intravenously at a dose 100 IU/kg (~7 mg/kg) at different gestation ages in timed-pregnant guinea pigs and total human IgG concentrations ((a), (b), (e), and (f)), human IgG subclasses ((c), (d)), and neutralizing activity (anti-HBs, (e)) five days after administration were measured. Shown gestation days (GD, x-axis, or legend) roughly correspond to the end of first trimester (GD26), middle and end of second trimester (GD35 and GD45), and middle and end of third trimester (GD55 and 65). Maternal total human IgG concentrations decreased (a) and the corresponding fetal concentrations increased (b) with gestation. Similar trends were seen for human IgG subclasses in the maternal (c) and fetal (d) samples as well as the anti-HBs neutralizing activity in the mother ((e), shaded bars) and fetus ((e), clear bars). The maternal and fetal total IgG concentrations were negatively correlated (f) and, after log transformation, the relationship was linear (dotted line, Pearson $r = -0.60$, $p = 0.0008$). Data shown as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

confidence interval −0.80 to −0.29). One data point from GD35 was excluded from this calculation (supplemental Table 1S, gray font and italics) due to maternal concentration being a clear outlier compared to all the other points.

While the negative correlation does not prove causality, we suggest that transplacental transfer of antibody from the mother to the baby may be a significant contributor to the decreased AUC and mean residence time we observed at the end of gestation. We further noted that not only are maternal-fetal concentration changes with increased gestation correlated, but the magnitude of changes follows the same trend. Thus, trough maternal human IgG levels are significantly lower at the middle and the end of third trimester (GD54 and 65, respectively, Figure 3(a)), the same time points where statistical significant concentration increases were seen in the fetal samples (Figure 3(b)).

We also measured concentration of all human IgG subclasses and neutralizing activity (anti-HBs levels) in both maternal and fetal samples. Given that IgG4 constitutes small percentage of HepaGam (and all other plasma derived polyclonal IgG products as well as human serum) both maternal and fetal concentrations for this subclass were below the detection limit in the majority of the collected samples. Nevertheless, we were able to detect IgG4 in some of the fetal samples in the third trimester, but most of the values were below the level of quantitation (data not shown). For the other subclasses, we observed the same general trend of decreasing maternal concentrations with increased gestation (two-way ANOVA $p = 0.0636$, Figure 3(c)), a significant increase of fetal concentrations with progression of pregnancy (two-way ANOVA $p < 0.0001$, Figure 3(d)), and a significant interaction between subclass concentrations in the fetus and gestation age ($p = 0.0004$). Post hoc analyses revealed that only IgG1 increases in GD55 and GD65 are statistically significant; the concentration increases in other subclasses do not reach significance.

We obtained similar results for the neutralizing activity (Figure 3(e)), with fetal anti-HBs levels on GD45, 55, and 65 one to two orders of magnitude higher than 10 mIU/mL, the accepted serological level of protection [27], and neutralizing antibody levels GD35 fetal blood below quantification limit.

Previously we showed that pregnant guinea pigs are an appropriate animal model for studying human antibody transfer during pregnancy [21, 23]. The additional experimental data we present here enabled us to more precisely measure placental transfer and further demonstrate that this model recapitulates well the time course of the placental transfer of IgG in pregnant women. Thus, 17–22-week human fetuses have circulating concentrations of IgG that are only 5–10% of maternal levels [28] but they significantly increase during the third trimester [20, 29], often surpassing levels found in their mother. Our results show that, in the pregnant guinea pig, fetal : maternal ratios for administered human antibodies are ~3 and 20% in the middle and end of second trimester (GD35 and 45), respectively, but increase to ~60 and 110% in the middle and end of the third trimester (Figure 4(a)). Similarly to what we previously found, the fetal : maternal IgG concentration ratios with increased gestation fit an exponential growth curve ($R^2 = 0.87$, not shown). Thus, the pharmacokinetic changes we observe in the guinea pig animal model may closely match what can be observed in women receiving IGIV during pregnancy. We should note that even though the mean fetal : maternal values for each gestation differ somewhat from what we previously calculated [21], the differences are due to an increased sample size and do not change any of the previous conclusions. All fetal : placental ratios lie within one standard deviation of previously calculated means [21] and the new group averages (Figure 4(a)) can be considered more precise point estimates of the gestation dependent placental transfer.

In a similar fashion to the total human IgG, the fetal : maternal concentration ratios for IgG subclasses 1–3 increased exponentially with gestation age (two-way ANOVA $p < 0.0001$) but, unlike the concentration changes, were similar to each other at all time points (Figure 4(b)). Post hoc analyses revealed that fetal : maternal ratios for all three IgG subclasses analyzed increased significantly in the third trimester (GD55 and 65) compared to earlier in gestation. Unlike the increases in the fetal IgG subclass concentrations (Figure 3(d)), an interaction between gestation age and subclass effects in the variance of fetal : maternal concentration ratios was not observed ($p = 0.85$).

Under our experimental conditions, we did not find any difference in placental transfer propensity for human IgG subclasses 1–3 in the pregnant guinea pig. The same has not been reported in human pregnancy [28, 30] where there is a clear difference between the fetal : maternal ratios for IgG subclasses. Some of these differences may be related to population level polymorphism in the sequence of Fc [31], but other factors have also been proposed [16]. More studies are needed to better understand the differences in placental transfer of IgG subclasses aiming to optimize the efficacy of antibody therapeutics during pregnancy.

4. Conclusions

Our studies in an animal model of human pregnancy show that intact human IgG molecules of all subclasses traverse the placenta at increasing levels with progression of pregnancy. This transplacental distribution can have dual implications: it may contribute to a reduction of maternal exposure to the administered antibodies compared to nonpregnant controls (Figures 1 and 3) and it can expose the fetus to progressively higher levels of therapeutic IgG with increased gestation (Figure 3). Fetal partition of the IgG, at least for HBIG and depending on the dose, may result in fetal neutralizing activity (anti-HBs levels) at time points starting with the end of second trimester (GD45) that reach and surpass the accepted serological level of protection for children and adults (Figure 3(e), [23, 27]). However, it is unknown if and at what levels neutralizing antibodies in the fetus can prevent fetal viral infections and what the effects of reduced maternal exposure to administered antibody therapy would be, especially in the presence of maternal infection. The clinical scenario may be further complicated by changes in immunity and other pregnancy related changes [7]. Thus, well-designed clinical studies and careful dosing considerations, especially in light of changes in biodistribution to the fetus at different

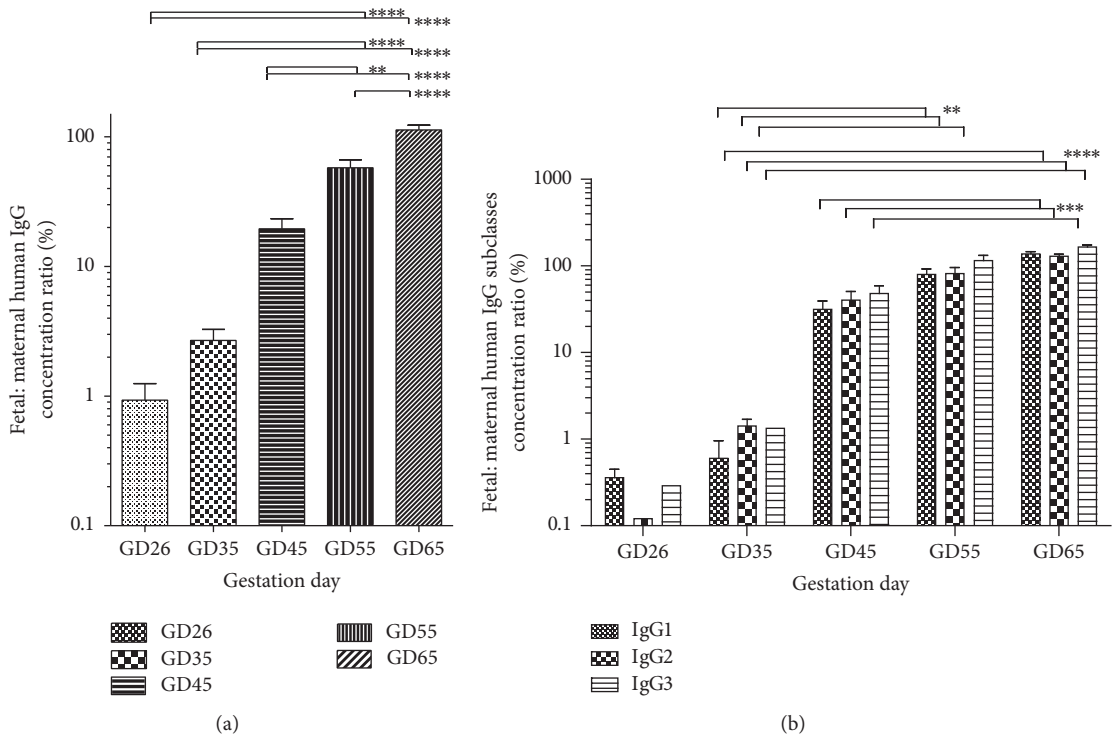

Figure 4: Fetal : maternal concentration ratio for total human IgG (a) and IgG subclasses (b) following hepatitis B immune globulin (HBIG) administration at different gestation ages. HBIG was administered intravenously at a dose of 100 IU/kg (~7 mg/kg) in pregnant guinea pigs at different gestation ages (GD, x-axis). Fetal : maternal concentration ratios (shown as mean ± SEM) for the total IgG and IgG subclasses 1–3 increase exponentially with gestation age. Shown time points roughly correspond to the end of first trimester (GD26), middle and end of second trimester (GD35 and 45), and middle and end of third trimester (GD55 and 65). One- and two-way ANOVA with Bonferroni post hoc analyses were used to compare total human IgG and human IgG subclasses fetal : maternal concentration ratios in each gestation age; $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

gestation ages, are needed to assess the efficacy of therapeutic antibody treatments during pregnancy.

Disclosure

This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

Competing Interests

The authors have no conflict of interests to disclose.

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Review Article

An Introduction to B-Cell Epitope Mapping and In Silico Epitope Prediction

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Identification of B-cell epitopes is a fundamental step for development of epitope-based vaccines, therapeutic antibodies, and diagnostic tools. Epitope-based antibodies are currently the most promising class of biopharmaceuticals. In the last decade, in-depth in silico analysis and categorization of the experimentally identified epitopes stimulated development of algorithms for epitope prediction. Recently, various in silico tools are employed in attempts to predict B-cell epitopes based on sequence and/or structural data. The main objective of epitope identification is to replace an antigen in the immunization, antibody production, and serodiagnosis. The accurate identification of B-cell epitopes still presents major challenges for immunologists. Advances in B-cell epitope mapping and computational prediction have yielded molecular insights into the process of biorecognition and formation of antigen-antibody complex, which may help to localize B-cell epitopes more precisely. In this paper, we have comprehensively reviewed state-of-the-art experimental methods for B-cell epitope identification, existing databases for epitopes, and novel in silico resources and prediction tools available online. We have also elaborated new trends in the antibody-based epitope prediction. The aim of this review is to assist researchers in identification of B-cell epitopes.

1. Introduction

Antigen-antibody interaction is a key event in humoral immune response to invading pathogen. A specific antibody (Ab) recognizes antigen (Ag) at discrete regions known as antigenic determinants or B-cell epitopes. B-cell epitopes can be defined as a surface accessible clusters of amino acids, which are recognized by secreted antibodies or B-cell receptors and are able to elicit cellular or humoral immune response [1].

Most of the Ag surface may become part of epitopes after recognition with antibodies and the exact selection mechanism why certain antigen regions become B-cell epitopes is not fully understood [2]. The classification of antigenic determinants into epitopes and nonepitopes ignoring the antigen reconfiguration in Ag-Ab complex may not accurately reflect biological reality [3]. The accurate identification of B-cell epitopes constitutes a basis for development of antibody therapeutics [4], peptide-based vaccines [4, 5], and immunodiagnostic tool [6].

Based on the spatial structure B-cell epitopes can be categorized as a continuous (linear or sequential) and discontinuous (nonlinear or conformational) epitopes; in the latter case amino acid residues are in close contact due to the three-dimensional conformation [7]. The minimal amino acid sequence (contact residue span) required for proper folding of the discontinuous epitope in native proteins may range from 20 to 400 amino acids. It is generally believed that most of identified linear antigenic determinants are parts of the conformational B-cell epitopes [8–10]. Using a less stringent definition for continuity, it was found that the majority of discontinuous epitopes (over 70%) are composed of 1–5 linear segments of lengths of 1–6 amino acids [10].

The experimental methods developed to identify the epitopes can roughly be divided into structural and functional studies. The X-ray crystallography can exactly locate

the position of epitope within the protein structure but is laborious, time consuming, costly, technically difficult, and not applicable for all antigens [11]. Some of the commonly used methods for functional B-cell epitope mapping are screening of antigen-derived proteolytic fragments or peptides for antibody binding and testing the Ag-Ab reactivity of mutants (site-directed or randomly mutated) [11]. Other techniques like display technologies and mimotope analysis have also become acceptable alternative choices for epitope mapping thanks to their relative cheapness, flexibility, and speed [12, 13].

Rubinstein and colleagues proposed a null hypothesis that the surface of the antigen is homogeneously antigenic. With the large-scale statistical analysis of Ag-Ab cocrystals derived from the protein databases, they were able to define physicochemical, structural, and geometrical aspects of epitopes and concluded that epitopes are clearly distinguishable from the remaining antigen surface [10]. In another study, Kringelum and coworkers described B-cell epitope as a flat, elongated, oval shaped bundle with unorganized secondary structure [14]. Thanks to the comprehensive experimental studies and in silico analyses conducted hitherto, it is possible to defined the features distinguishing epitope from nonepitope. The majority of epitopes span 15–25 residues and an area of 600–1000 $Å^2$ organized in loops. The epitope surface accessibility is common feature. Sequence of the epitopes is enriched with Y, W, charged, and polar amino acids (amino acids with exposed side chains) and with specific amino acid pairs. The Ag-Ab interaction occurs without preference for a specific CDR loop and involves epitope compression [10]. In recent years, it was shown that the differences between residues within epitopes and other residues are not substantial and amino acid composition is not sufficient for differentiating between epitopes and nonepitopes (reviewed in [2]).

Advancement in the epitope mapping technologies hand in hand with bioinformatics has greatly contributed to developing immunoinformatics, which involves application of computational methods in immunology to unveil structures of antibody, B-cell, T-cell, and allergen, prediction of MHC binding, modelling of epitopes, and analysis of immune networks. Several algorithms have been developed to predict B-cell epitopes from their sequence or structure [15–18]. The early prediction methods were focused on the identification of linear epitopes through propensity scale. To improve prediction performance, methods based on machine learning such as Hidden Markov Model [19], recurrent neural network [20], and support vector machine [21] were developed. Despite this advancements, there are still a limited number of methods that predict discontinuous epitopes, and they need combination of the information, for example, amino acid statistics, spatial information, and surface exposure [22].

Identification B-cell epitopes is extensively employed in the development of diagnostic tests, therapeutics and vaccines [23–26]. Use of epitope mapping in the drug development is reviewed earlier [27]. In spite of advances in B-cell epitope mapping, it is important to note that antibodies raised against peptides often lack the ability to bind native proteins due to unstructured nature of the peptide [28].

The main purpose of this review is to provide researcher with the general knowledge about existing methods of B-cell epitope mapping and short overview of epitope databases, recently used prediction methods, and publicly available tools.

2. B-Cell Epitope Mapping

Most of the existing methods for epitope mapping (structural and functional approach) are expensive, laborious, time consuming, and often fail to identify all epitopes. Structural epitope mapping methods interpret the protein structure comprising residues in direct contact with an antibody but often fail to reveal contribution of amino acids in binding strength. The identification and characterization of residues important for binding within structurally defined antigenic determinant are the aim of functional epitope mapping tools.

The most accurate method for structural epitope mapping is X-ray crystallography of Ag-Ab complexes and is often regarded as the only method to define a structural epitope [29]. Among "wet" lab methods this technique is a guarantee of precise identification of both continuous and discontinuous epitopes and provide information about strength of binding [30–32]. Bacterial or viral antigens, especially small soluble proteins, are ideal for crystallography. However, the X-ray crystallography is limited by quality of cocrystals and electron density of the antibody [33]. Recently developed freely available program FTProd can be used as computational alternative to expensive and time consuming X-ray crystallography [34]. Nuclear magnetic resonance (NMR) has also potential to replace traditional X-ray crystallography. This approach provides data about the structure, dynamics, and binding energy of Ag-Ab complex and is performed in solution where no crystals are needed. However, NMR is limited to small proteins and peptides (<25 kDa) [35]. Saturation transfer difference NMR (reviewed in [36]) and antibody inhibition of hydrogen-deuterium exchange in the antigen are other two methods capable of mapping epitope regions with moderate resolution [37]. The electron microscopy (EM) can also be used for epitope localization; however it is a low-resolution structural method that is utilized on larger antigens (e.g., whole viral particles) [38]. Unfortunately, this method is unable to detect contact residues and can be used for confirmation of surface accessibility of the epitope [39]. An alternative, cryoelectron microscopy allows observations of rapidly frozen Ag-Ab complexes in physiological buffers avoiding the need for stains and fixatives [40, 41].

The methods for functional epitope mapping can be divided into four main groups: competition methods, antigen fragmentation methods, modification methods, and methods using synthetic peptides or peptide libraries [42]. Competition methods have low-resolution degree of mapping and are commonly used to determine whether two different monoclonal antibodies (mAbs) can bind to antigen at the same time or whether they compete with each other for the same epitope [11]. Most of the functional methods are based on the ability to detect binding of antibody to antigen fragments, synthetic peptides, or recombinant antigens

(including mutated variants, antigens arrayed by in situ cell-free translation, and/or expressed using selectable systems such as phage display). In the binding assays, peptides are immobilized on solid support and binding of antibody is detected by western blot, dot blot, and/or ELISA. This approach does not require expensive equipment and is able to quantify the immune response towards a specific epitope. The dot blot requires the purified molecule to be spotted on the membrane and is mainly used for qualitative detection [43]. The peptides can be synthesized on pins (PEPSCAN®), on a cellulose membrane support (SPOT method®), or on peptide microarrays [44, 45]. Such techniques simplify the handling of large numbers of peptides and eliminate the need for identification of positive peptides by sequencing or by mass spectrometry. Binding assays were successfully used in identification of epitopes in several viruses, bacteria, fungi, parasites, and human diseases (reviewed in [27]).

2.1. Mutagenesis. Mutagenesis is a rapid epitope mapping method that relies on the fact that substitution of individual residue/s (hot-spot/s) that constitutes a functional epitope causes loss of antibody binding. Hot-spots (most frequently Tyr, Arg, and Trp) are energetically important residues and comprise only a fraction of the complete protein-protein interface area [39]. The protein library can be generated by either random or site-directed mutations. The combination of mutagenesis approach with display techniques enables screening of many hundreds or thousands of mutated proteins (reviewed in [46]). The saturation mutagenesis, another versatile tool, replaces amino acid residue at specific position with all 20 naturally occurring residues. However, in some cases the loss of immunoreactivity due to the disruption of antigenic structure complicates the interpretation of the results.

The majority of epitope contacts in Ag-Ab complex occur through amino acid side chains [10]. Alanine scanning mutagenesis provides a controlled method to define the contributions of each residue's side chain to Ab binding by alanine sequential substitution (causing truncation of side chains to β-carbon without additional flexibility of protein backbone) for each nonalanine residue one at a time. Although this mapping strategy may not identify every residue in contact with an antibody, the critical residues identified using this approach represent amino acids whose side chains make the highest energetic contributions to the paratope-epitope interaction [47]. The generation of combinatorial libraries of displayed alanine mutations significantly accelerates the functional mapping of epitopes. Computational alanine scanning can also rapidly calculate the effect of alanine mutation on a binding free energy in protein-protein complex using a simple free energy function (available at http://robetta.bakerlab.org/alaninescan) [48].

The combinatorial mutagenesis enables identifying residues, which are not critical for binding but contribute to the formation of epitope or establish multiple individually weak interactions with paratope. This strategy is based on combinatorial randomization of a discrete antigenic region and grouping of mutated residues (primary sequence proximity) to maximize the chances of underscoring combined effects mediated by neighboring residues [49].

Another technique in mutagenesis, a shotgun mutagenesis, enables identification of both linear and conformational epitopes with mapping rates of over 20 epitopes/month. This high-throughput strategy is based on large-scale mutagenesis, where each clone bears a defined amino acid mutation (such as an alanine substitution) and direct cellular testing for mAb reactivity of natively folded proteins (proper oligomerization, disulphide bonds, glycosylation, and other posttranslational modifications). Shotgun mutagenesis has been used to map over 250 mAbs targeting dengue, chikungunya, and hepatitis C viruses, with additional mAb epitopes mapped on hepatitis B virus, respiratory syncytial virus, and HIV (reviewed in [50]).

2.2. Display Techniques. Display technologies, best exemplified by phage and yeast display, provide a powerful technique for epitope mapping. Display techniques have become acceptable alternative for epitope mapping due to their relative cheapness and quickness [12]. The principle of display methods is based on testing the binding capacity of a variety of peptides displayed on the display platforms (tethering of proteins to ribosomes-mRNA complex, or to the surface of phage, bacteria, mammalian, insect, or yeast cells) to the monoclonal antibody of interest through the affinity selection method of biopanning.

One of the most frequent and popular display methods for epitope mapping is phage display. Construction of phage display peptide libraries (displaying $>10^9$ of peptides) represents popular way of generation of antigenic fragments which are screened for antibody binding [12]. This powerful approach involves fusion of the foreign DNA fragments with the filamentous phage gene coding coat protein (e.g., pIII, pVI, pVII, pVIII, and pIX). The bacteriophage M13 or lytic alternatives such as T4, T7, and P4 bacteriophages or lambda phage are usually used as model viruses for phage display. Random peptide phage libraries (combinatorial libraries) and gene or genome fragment phage libraries are commonly used techniques for epitope identification (reviewed in [29]).

3. B-Cell Epitope Databases

Thanks to the technological advances in genomics, proteomics, and epitope mapping techniques, huge amounts of data are being generated and are necessary to organize in a searchable form. B-cell epitope databases provide a training set for evaluation of existing epitope prediction methods and constitute platform for development of novel and better algorithms for prediction. The B-cell epitope databases can be classified as multifaceted database such as IEDB and AntiJen, B-cell oriented database such as BciPep, Epitome, and SDAP, and single pathogenic organism oriented database such as the HIV Molecular Immunology Database, FLAVIdB, and Influenza Sequence and Epitope Database. It has to be mentioned that most of the available databases include peptide/s recognized by the receptors of the adaptive immune system and/or amino acid residues of antigen that are in

Table 1: List of B-cell epitope databases.

Database	Source (URL)	Ref.
IEDB IEDB-3D	http://www.iedb.org/	—
AntiJen	http://www.ddg-pharmfac.net/antijen/AntiJen/antijenhomepage.htm	[56, 57]
CED	http://immunet.cn/ced/	[59]
Epitome	http://www.rostlab.org/services/epitome/	[60]
BciPep	http://www.imtech.res.in/raghava/bcipep/info.html	[61]
SEDB	http://sedb.bicpu.edu.in	[62]
SDAP	https://fermi.utmb.edu/	[63]
HIV Molecular Immunology Database	http://www.hiv.lanl.gov/content/immunology/index.html	—
FLAVIdB	http://cvc.dfci.harvard.edu/flavi/	[64]

close contact with antibody (structural epitopes) and lack important epitope information such as a detailed molecular characterization of epitopes and the mention of contact residues that make energetic contributions to binding. The databases that collect B-cell epitope are listed in Table 1.

The Immune Epitope Database (IEDB) is a comprehensive resource aimed to catalogue experimentally determined B-cell and T-cell epitopes from human, nonhuman primates, and other animal species along with the experimental contexts. It captures epitopes related to category A-C pathogens, emerging and reemerging pathogens, allergens, and autoantigens. IEDB contains epitopes derived from the peer-reviewed literature, patent applications, direct submission, and other publicly available databases, for example, FIMM [51], HLA Ligand database [52], and MHC binding database [53]. IEDB also provides tools for the prediction of linear B-cell epitopes from protein sequence including amino acid scales and HMMs, DiscoTope, ElliPro, Paratome, and PIGS. The database houses epitope conservancy analysis tool for determination of the degree of epitope conservation or variability, tool for analysis of population coverage, or tool for localization of epitope in 3D structure of antigen. The IEDB-3D catalogues T-cell and B-cell epitopes and MHC ligands with accompanied functional assays and immunologically relevant information derived from PDB and provides calculation of intermolecular contacts and interface areas [54]. An application, EpitopeViewer, allows visualization of the antigen structures and is fully embedded in IEDB-3D [55].

AntiJen v2.0 (developed from JenPep) contains quantitative binding data for peptides binding to MHC ligand, TCR-MHC complexes, T-cell epitopes, TAP (a transporter associated with the MHC class I restricted antigen processing), and B-cell epitopes. It also contains immunological protein-protein interactions and biophysical data such as diffusion coefficient and cellular data [56, 57]. AntiJen is linked to protein database Swiss-Prot, NCBI, MPID, PDB, and PubMed, which enables further in-depth cross referencing. The aim of AntiJen is to integrate quantitative kinetic, thermodynamic, and biophysical data, with functional and cellular information, which can be used in immunology and immunovaccinology [58]. AntiJen does not allow downloading of the data.

Conformational Epitope Database (CED) provides a manually curated dataset of conformational epitopes that can be used to evaluate existing epitope prediction methods and develop new and better algorithms for prediction [59]. This database has limited size and contains only high quality clearly defined conformational epitopes collected from published peer-reviewed articles. The database implies additional information, such as residues dispatching, localization, immunological properties, source antigen, and corresponding antibody of the epitope. CED is hyperlinked to other databases (e.g., Swiss-Prot, PDB, KEGG, or PubMed). Conformational epitopes with corresponding PDB structures can be viewed interactively in the context of the Ag-Ab complex, antigen structure, or known theoretical model that can help to identify important structural features. The semiautomatic database Epitome collects structure-inferred antigenic residues in proteins that are involved in interaction with residues on antibody CDRs, and it provides information of corresponding paratope [60]. It serves for detailed description of residues with and enables visualization of three-dimensional structure of Ag-Ab complex derived from PDB through Jmol tool [60].

The comprehensive database BciPep provides dataset of experimentally validated linear B-cell epitopes derived from literature and other publicly available databases [61].

In BciPep, B-cell epitopes are categorized into three classes: immunodominant (2-3-fold enhancement of anti-peptide antibody synthesis compared to reference protein or control, e.g., BSA or KLH), immunogenic (onefold enhancement of anti-peptide antibody synthesis compared to reference protein or control, e.g., BSA or KLH), and null-immunogenic (no difference observed when compared to reference protein or control, e.g., BSA or KLH). The database provides information (isotype and name/number) about anti-peptide antibodies produced against an epitope and their neutralization potential. The database is linked with Swiss-Prot, PDB, MHCBN, and PubMed [61].

Table 2: List of web available tools for continuous B-cell epitope prediction.

Tool	Source (URL)	Input data	Ref.
ABCPred	http://www.imtech.res.in/raghava/abcpred/	FASTA	[20]
APCPred	http://ccb.bmi.ac.cn/APCpred/	FASTA	—
BCPREDS	http://ailab.ist.psu.edu/bcpred/	FASTA	[92, 93]
BepiPred	http://www.cbs.dtu.dk/services/BepiPred	FASTA or FASTA file	[19]
LBtope	http://crdd.osdd.net/raghava/lbtope/	FASTA or FASTA file	[94]
Bcepred	http://www.imtech.res.in/raghava/bcepred/	FASTA or FASTA file	[76]
SVMTriP	http://sysbio.unl.edu/SVMTriP/	FASTA	[78]

Table 3: List of web available tools for discontinuous/conformational B-cell epitope prediction.

Tool	Source (URL)	Input data	Ref.
DiscoTope	http://www.cbs.dtu.dk/services/DiscoTope-2.0/	PDB ID or PDB file	[22, 71]
BePro (PEPITO)	http://pepito.proteomics.ics.uci.edu/	PDB ID or PDB file	[79]
ElliPro	http://tools.immuneepitope.org/ellipro/	FASTA or Swiss-Prot ID	[83]
SEPPA	http://badd.tongji.edu.cn/seppa/	PDB ID or PDB file	[81]
EPITOPIA	http://epitopia.tau.ac.il/	FASTA/PDB ID or PDB file	[95]
CBTOPE	http://www.imtech.res.in/raghava/cbtope/	FASTA or FASTA file	[85]
EPCES	http://sysbio.unl.edu/EPCES/	PDB ID or PDB file	[96]
EPSVR	http://sysbio.unl.edu/EPSVR/	PDB ID or PDB file	[97]
PEASE	http://www.ofranlab.org/PEASE	Ag PDB ID or PDB file Ab FASTA or FASTA file	[88]
EpiPred	http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/EpiPred.php	PDB ID or PDB file	[72]

Structural Epitope Database (SEDB) contains 3D complexes of B-cell, T-cell, and MHC bound molecules and shows Ag-Ab interaction plot. SEDB collects related information of epitopes, like gene-ontology information, Ag-Ab interaction graph, and epitopes location in protein with interaction data, which are missing in currently available epitope databases [62].

Structural Database of Allergenic Proteins (SDAP) contains sequences, structures, and IgE epitopes of allergenic proteins and offers additional computational tools for structural studies. SDAP enables allergen-peptide matching for the detection of novel allergens and the cross-reactivity between known allergens [63].

Databases oriented on single pathogenic organism have been developed to target vaccine design. The HIV Molecular Immunology Database collects cytotoxic, helper T-cell epitopes and B-cell epitopes in annotated and searchable form and offers several generic data analysis tools. FLAVIdB is a comprehensive database of antigens from *Flavivirus* spp. derived from external databases (GenPept, UniProt, IEDB, and PDB) and corresponding literature. It contains flavivirus antigen sequences, T-cell epitopes, B-cell epitopes, and molecular structures of the dengue virus envelope protein. Database is equipped with tools for block entropy analysis and flavivirus species classification [64].

4. In Silico B-Cell Epitope Prediction

Correlation between B-cell epitope localization and physicochemical properties (e.g., hydrophilicity, solvent accessibility, flexibility, turns, polarity, antigenicity, and surface exposure), has been demonstrated in several studies (reviewed in [65]). Earlier prediction methods were monoparametric (based on single residue property or propensity scale) calculating average propensity value along a sliding window [66–68]. It was demonstrated that methods based on propensity profiling yield poor results in the practice [69]. To improve the performance of prediction of both continuous and discontinuous epitopes, machine learning methods were evolved. Most of these methods were developed based on very small datasets and used randomly selected peptides instead of experimentally verified nonepitopes as a negative training set [70]. Currently used methods for continuous epitope prediction combine two or more residue properties with machine learning approaches (summarized in Tables 2 and 3). In general, prediction methods can be divided based on the level of input information to methods based on antigen sequence and methods based on 3D structure of antigen. Structure-based methods significantly outperform sequence-based methods [71]. Unfortunately, existing prediction methods are not accurate enough and annotate general immunogenic/epitope-like regions on the antigen [69, 72]. It was demonstrated that consensus of various B-cell epitope prediction methods ensures greater accuracy of the results [73]. Here we offer a short overview of publicly available methods and servers for prediction of continuous as well as discontinuous B-cell epitopes (summarized in Tables 2 and 3).

4.1. Prediction of Continuous B-Cell Epitopes. The first prediction method using recurrent neural network, ABCPred,

has been trained on B-cell epitopes obtained from BciPep database and nonepitopes obtained randomly from Swiss-Prot database. The ABCPred is a neural network based method for prediction of continuous B-cell epitopes using fixed length pattern [20]. The ABCPred dataset contains data of epitopes from viruses, bacteria, parasites, and fungi that are stored in BciPep database with the prediction accuracy of 65.9%. ABCPred, AAP method and BCPred, and BayesB predict only short peptide fragments. The B-cell epitopes of the Emy162 protein of *Echinococcus multilocularis* (the causative agent of zoonotic helminthosis) were predicted using BCPred and ABCPred [74].

APCPred combines amino acid anchoring pair composition (APC) and support vector machine (SVM) methods, which significantly improved the prediction accuracy. APCPred achieved an improved area under curve (AUC) of 0.794 [75]. BCPred server allows choosing prediction method among amino acids pair scaling method (AAP), BCPred, and FBCPred. AAP approach is based on the finding that particular amino acid pairs occur more frequently in epitope than nonepitope sequence. Combination of AAP propensity scale with turns, accessibility, antigenicity, hydrophilicity, and flexibility propensity scales improved the accuracy (72.5%).

BCPred method employs subsequence kernel-based SVM classifier and was trained on homology-reduced dataset of linear B-cell epitopes (with <80% sequence identity) derived from dataset previously used to test ABCPred. The performance of BCPred (AUC 0.758) outperforms implementation of AAP (AUC 0.7).

FBCPred is a novel method developed for prediction of B-cell epitopes with flexible length. Homology-reduced dataset is publicly available for comparing existing linear B-cell epitope prediction methods and testing of new prediction software.

BepiPred predicts continuous epitopes by combining two residues properties with Hidden Markov Model. BepiPred was evaluated on dataset of epitopes extracted from the literature, AntiJen, and HIV databases. This method has a quite low sensitivity [19].

The server BcePred is used for prediction of continuous B-cell epitopes based on physicochemical properties and allows user to select any residue property or combination of two or more properties employed in prediction. The performance of BcePred was evaluated on dataset containing epitopes obtained from BciPep database and dataset of randomly chosen nonepitopes from Swiss-Prot. The accuracy of BcePred combining four amino acid properties (hydrophilicity, flexibility, polarity, and exposed surface) is 58.70% [76].

A novel continuous B-cell epitope prediction method EPMRL was developed using multiple linear regression. EPMLR was tested on BEOD dataset containing only experimentally verified epitopes and nonepitopes and achieves overall sensitivity of 81.8% and precision of 64.1% and area under the receiver operating characteristic curve (AUC) of 0.728 [77].

B-cell epitope prediction using support vector machine tool (BEST) is sequence-based tool designed for prediction of both linear and conformational epitopes from full antigen sequence. Prediction is based on averaging of selected scores (sequence conservation, similarity to experimentally validated B-cell epitopes, predicted secondary structure, and relative solvent accessibility) generated from 20-mers. BEST achieves AUC at 0.81 and 0.85 for the fragment-based prediction and 0.57 and 0.6 for full antigen. BEST outperforms several modern sequence-based B-cell epitope predictors including ABCPred, BCPred, COBEpro, and CBTOPE [16].

SVMTriP employs support vector machine to combine the tripeptide similarity and propensity scores to predict linear epitopes. SVMTriP achieves a sensitivity of 80.1% and a precision of 55.2% and the AUC value 0.702, when tested on nonredundant epitopes extracted from IEDB [78]. A comparative study concluded that the methods based on sequence analysis do not predict epitopes better than chance. Since the majority of epitopes are discontinuous, prediction methods taking into account structural data could increase the accuracy of epitope prediction [69].

4.2. Prediction of Discontinuous B-Cell Epitopes. Although the majority (~90%) of the B-cell epitopes are discontinuous (conformational), to date much effort was concentrated on identification of continuous epitopes [22]. However, with the advance of proteomics and increasing number of Ag-Ab crystal structures available in databases, it is now easier to perform deeper analyses of conformational epitopes. These epitopes comprise linear stretches of residues brought into close proximity upon protein folding and the reconfiguration of epitope residues when an antigen is in complex with specific antibody. Most of the prediction methods are antibody-ignored methods. One must also take into account the fact that predicted epitopes are frequently short sequences of residues that represent the part of discontinuous epitope. The tools currently used for prediction of discontinuous epitopes are summarized in Table 3.

The first attempts at epitope prediction based on 3D structure began with development of CEP server, which is based on accessibility of amino acid residues and requires the 3D data in PDB format [17]. Hitherto, this tool is deprecated and is not available. Subsequent server, DiscoTope, predicts discontinuous B-cell epitopes by combining the surface accessibility and spatial and amino acid statistics to differentiate between epitopes and nonepitope sites. It generates one residue propensity score in the sphere of 10 Å which is the result of combination of the hydrophilicity scale and the epitope log-odds ratios [22]. DiscoTope has been recently updated to 2.0 version by Kringelum and coworkers with several improvements for proper benchmark definitions and use and achieves an AUC of 0.731 [71].

BEpro server (formerly known as PEPITO) uses a combination of amino acid propensity scores along with side chain orientation and solvent accessibility information using half sphere exposure values at multiple distances to predict discontinuous B-cell epitopes. It achieves AUC of 75.4 on the DiscoTope dataset [79]. BEpro and CEP prediction is based on the detection of exposed residues ignoring the residues buried in the spatial structure, which may affect the reliability of predictions.

PEPOP is structure-based method, which identifies clusters of accessible surface residues and segments that might form putative discontinuous epitopes and can be used to design immunogenic peptides. The anti-peptides antibodies showed reactivity with the cognate antigens in 80% of the cases (four cases from five) and were used in sandwich capture assay. Compared to CEP and DiscoTope, PEPOP showed comparable specificity and slightly better sensitivity [80]. Hitherto, this tool is deprecated and is not available.

Improved Spatial Epitope Prediction of Protein Antigens server (SEPPA) focusing on single residue propensity scales and continual segment clustering was developed in 2009 by Sun and colleagues [81]. SEPPA employs a novel concept of unit patch of residue triangle and spatial clustering coefficient to describe local spatial context in protein antigen surface and 3D characteristic of epitopes. A parameter of 4 Å was chosen in the definition of unit patch of residue triangle. Curated data of nonredundant spatial epitopes from PDB database was used for method testing. SEPPA outperforms popular prediction tools, CEP, DiscoTope, and BEpro, and achieves an average AUC over 0.742 [81].

Server ElliPro (derived from Ellipsoid and Protrusion) implements modified method for identifying continuous epitopes in the protein regions protruding from the globular surface of antigen [82] in combination with a residue clustering algorithm for prediction of discontinuous epitopes from primary antigen sequence or structure [83]. ElliPro performs BLAST search of PDB for antigen sequences homologues or use MODELLER [84] to predict 3D structure. ElliPro (AUC value of 0.732) outperforms structure-based methods CEP and DiscoTope. ElliPro enables visualization of linear and discontinuous epitopes on the protein 3D structure [83].

Computational prediction tool EPITOPIA employs Naïve Bayes classifier to predict epitopes in linear sequence or 3D structure. It distinguishes the nonepitope and epitope regions by computing an immunogenicity score (reflecting the immunogenic potential of a certain residue relative to all residues in the antigen) for each solvent accessible residue or a score for every amino acid. EPITOPIA yields higher success rate of 89.4% (mean AUC value of 0.60) when compared to ElliPro and DiscoTope [18].

CBTOPE was proposed for the prediction of discontinuous epitopes from antigen primary structure. This SVM-based predictor combines traditional features of physicochemical profiles and sequence-derived inputs including composition and collocation of amino acids. It outperformed other structure methods using binary profile of pattern and physicochemical profile of patterns with better sensitivity and AUC on the same benchmark dataset [85].

Epitope prediction method, which uses Consensus Scoring (EPCES) combines scores from residue epitope propensity, residue conservation, side-chain energy, contact number, surface planarity, and secondary structure composition. EPCES predicts discontinuous epitopes with 47.8% sensitivity, 69.5% specificity, and an AUC value of 0.632, which is statistically similar to other published methods.

The Antigenic Epitopes Prediction with Support Vector Regression server (EPSVR) employs vector regression to integrate same scores as are combined in EPCES and achieves AUC value of 0.597. EPSVR is integrated in metaserver EPMeta together with five existing prediction servers (EPCES, EPITOPIA, SEPPA, PEPITO, and DiscoTope 1.2) and provides consensus prediction results. The performance of EPMeta is AUC value of 0.6, which is higher than performance of any other existing single server. Unfortunately, this server met unsolvable technical difficulties and is no more available.

Evaluation of performance of prediction tools is often difficult, especially when each of them has their own testing dataset. To solve this problem and help users to choose the tool, the recent web servers, CEP [17], DiscoTope [22], PEPOP [80], ElliPro [83], BEpro [79], and SEPPA [81], were tested with an independent dataset created by collection of the experimentally confirmed discontinuous epitopes. SEPPA gave the best performance among the six tools (the averaged AUC value of 0.62, sensitivity of 0.49) followed by DiscoTope and BEpro (the averaged AUC value of 0.58 and 0.55, sensitivity of 0.36 and 0.18). The performance of CEP, PEPOP, and ElliPro did not exceed averaged AUC values of 0.55 [86]. The detection based on exposed residues ignoring the residues buried in the structure can account for low performance of CEP tool. The best performance achieved by SEPPA could be attributed to growing number of available structural data and new spatial features incorporated in its algorithm [86].

4.3. Antibody-Specific Epitope Prediction. The traditional antibody-ignored epitope prediction methods do not take into account the reconfiguration of epitope residues when an antigen is in complex with a specific antibody [3]. Reconfiguration of Ag takes place when Ab binds both short peptide or whole antigen. To reflect this biological reality, several prediction methods based on sequence or structure of interacting Ab and Ag have been introduced in the last few years. The performance of antibody-based prediction of epitopes is competitive, or even better, when compared with structure-based predictors (rigid-body docking algorithms) [3].

A method using Antibody-Specific Epitope Prediction (ASEP) index developed by Soga and coworkers, represents the first benchmark in epitope prediction for individual antibody and has been used to narrow down candidate epitopes previously predicted by the conventional methods [87].

The EpiPred combines conformational matching of the Ab-Ag structures and knowledge based asymmetric Ab-Ag scoring to annotate the likely epitope regions specific to the given antibody [72]. This global docking pipeline requires the sequence of Ab and structure of unbound Ag. Compared to rigid-body docking algorithms, EpiPred significantly enriches the number of close-to-native decoys when adjusting the Ab sequence against the Ag [72].

Predicting Epitopes Using Antibody Sequences (PEASE) evaluates a pair score for all combinations of one residue from the complementarity determining regions (CDR) of antibody and one residue from the surface exposed region of antigen. A residue score of antigen surface residue is its highest pair

Table 4: List of B-cell epitope prediction tools based on mimotope analysis.

Tool	Source	Ref.
MIMOX	http://immunet.cn/mimox/	[91]
MimoPro	http://informatics.nenu.edu.cn/MimoPro http://informatics.nenu.edu.cn/PepMapper/	[98]
Pep-3D-Search	http://informatics.nenu.edu.cn/PepMapper/	[99]
MIMOP	upon request	[13]
LocaPep	http://atenea.montes.upm.es/#soft	[100]
PepSurf	http://pepitope.tau.ac.il/sources.html	[101]

score. A higher residue score means that contact between antibody and antigen residue is more strongly predicted and that this residue constitutes a part of B-cell epitope. PEASE also identifies surface patches on the antigen, which contain multiple residues with high residue scores [88]. PEASE was successfully used to predict the vaccinia virus epitopes [89].

B-cell epitope prediction through association rules (Bepar) is predicting epitopes based on antibody-antigen (paratope-epitope) association patterns which can be applied to any antibody-antigen sequence pair. Residue cooperativity and relative composition have been used to enhance the performance of this method. Bepar shows competitive performance on epitope prediction and outperforms CEP even without antigen 3D structure information [90].

4.4. Mimotope-Based Epitope Prediction. In recent years, the epitope prediction methods employing mimotopes derived from phage display experiments were developed. In general, these methods can be classified as methods that map mimotopes to the overlapping location patches on the antigen surface using statistical features of mimotopes and methods using mimotope mapping back to the antigen sequence through alignment. Mimotope has similar physicochemical properties and spatial organization but however rarely shows sequence similarity to the native antigen. In some cases, mimotope mapping back to the antigen can indicate B-cell epitope location [91]. B-cell epitope prediction tools based on mimotope analysis are summarized in Table 4.

5. Conclusion

Antibodies are currently the most promising class of biopharmaceuticals. The main objective of epitope identification is to replace an antigen in the immunization, antibody production, and serodiagnosis. The accurate identification of B-cell epitopes and large-scale data integration still presents major challenges for immunologists. Advances in B-cell epitope mapping and computational prediction have yielded molecular insights into the process of biorecognition and formation of Ag-Ab complex, which may help to formulate even more precise algorithms to predict their localization in the antigen. However, based on statistics it is not possible to precisely determine the epitope characteristics, which allow biorecognition. One has to keep in mind that the epitopes are not intrinsic feature of proteins and antibody-ignored prediction methods predict only putative epitope to which an undefined Ab might bind. The real epitopes cannot be predicted ignoring the structural effect upon Ag-Ab complex formation. This fact opens new space for all algorithms to improve further.

Competing Interests

The authors declare no conflict of interests.

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Review Article

Mucosal Vaccine Development Based on Liposome Technology

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Immune protection against infectious diseases is most effective if located at the portal of entry of the pathogen. Hence, there is an increasing demand for vaccine formulations that can induce strong protective immunity following oral, respiratory, or genital tract administration. At present, only few mucosal vaccines are found on the market, but recent technological advancements and a better understanding of the principles that govern priming of mucosal immune responses have contributed to a more optimistic view on the future of mucosal vaccines. Compared to live attenuated vaccines, subcomponent vaccines, most often protein-based, are considered safer, more stable, and less complicated to manufacture, but they require the addition of nontoxic and clinically safe adjuvants to be effective. In addition, another limiting factor is the large antigen dose that usually is required for mucosal vaccines. Therefore, the combination of mucosal adjuvants with the recent progress in nanoparticle technology provides an attractive solution to these problems. In particular, the liposome technology is ideal for combining protein antigen and adjuvant into an effective mucosal vaccine. Here, we describe and discuss recent progress in nanoparticle formulations using various types of liposomes that convey strong promise for the successful development of the next generation of mucosal vaccines.

1. Introduction

Most pathogens enter the body through mucosal surfaces and, therefore, vaccines that target the respiratory, gastrointestinal, or urogenital tracts are attractive as they stimulate local protection against infections. However, because of the requirements for strong mucosal adjuvants and usually relatively large amounts of antigen, only few such vaccines have been developed and most of these are live attenuated vaccines. Whereas live attenuated vaccines can be effective, subcomponent vaccines are usually safer and with less manufacturing and regulatory complications. Therefore, efforts are focused on developing mucosal vaccines based on subcomponents, but this also requires identifying appropriate and effective mucosal adjuvants to enhance the immune response. Subcomponent vaccines can consist of bacterial whole cell components, virus-like particles or other particles, polysaccharides, complete protein structures, or peptides that delivered at mucosal membranes together with an adjuvant can stimulate strong immune responses and protection against infection. Such mucosal vaccines are much warranted, as they carry several advantages over injectable vaccines. In particular, mucosal vaccines can elicit both local and systemic immune responses and they are safer as they do not require needles and may allow for mass vaccination, when pandemic spread of infection is a threat [1, 2]. Mucosal vaccination could also lead to increased compliance and reduce the risk of spreading transmissible diseases, as has been experienced with spread of hepatitis C and HIV infections following the use of injectable vaccines [3]. Most importantly, mucosal immunization elicits antigen-specific local IgA and systemic IgG antibodies, as well as strong systemic and tissue resident $CD4^+$ and $CD8^+$ T cell immunity (Figure 1). Despite these advantages, only few mucosal vaccines are commercially available. The reason for this is the need for safe and effective mucosal adjuvants and the fact that many vaccine formulations require protection from degradation of the antigens as seen, for example, after oral administration [4]. Consequently, the development of novel combinations of antigen and adjuvant into nanoparticles for

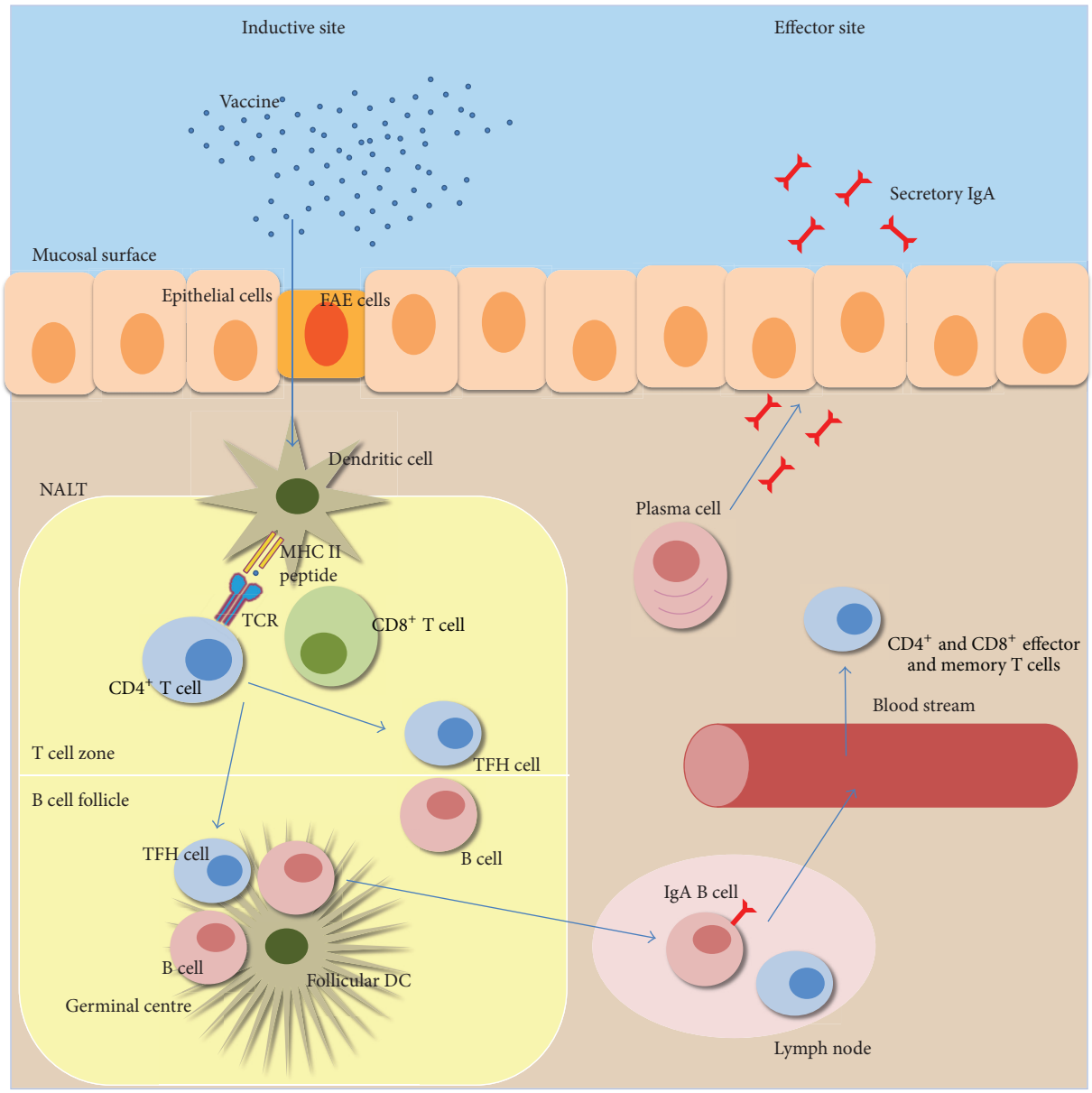

Figure 1: Principles for induction of mucosal immune responses after intranasal vaccination. The respiratory mucosal immune system consists of clusters of lymphoid cells beneath the mucosal epithelium, hosting both innate and adaptive immune cells [29]. There is a clear distinction between inductive and effector sites and these are also physically separated. Inductive sites are organized lymphoid tissues where antigen is taken up by DCs and other APCs. The effector sites, on the other hand, are tissues that provide protection against infection where specific antibodies and $CD4^+$ and $CD8^+$ effector and memory T cells reside [30]. The main inductive sites for mucosal immune responses after intranasal vaccination are known as nasopharynx-associated lymphoid tissue (NALT), which harbors B cell follicles and T cell zones in well demarked microanatomical areas [31]. Antigens are taken up by DCs that get access to the luminal content either through direct uptake through the epithelium or via the follicle associated epithelium (FAE) that overlay the NALT. After antigen uptake, the immature DCs undergo maturation and subsequently leave the mucosal tissue for the draining lymph nodes, alternatively, if already in the NALT, the DCs will directly prime naive $CD4^+$ or $CD8^+$ T cells. Activated $CD4^+$ T cells differentiate into various subsets: T helper 1 (Th1), Th2, or Th17 cells, regulatory T cells (Tregs), or follicular helper T cells (T_{FH}). The latter are critically needed for the expansion and differentiation of the activated B cells in the germinal center (GC), which is formed in the B cell follicle in the lymph node after vaccination. T_{FH} cells are involved in the development of long-lived plasma cells and memory B cells in the GC.

the next generation of effective mucosal vaccines is much needed.

Liposomes have been extensively used as delivery vehicles for vaccine antigens; some of the advantages of these formulations are (a) protection against antigen degradation, (b) tissue depot effects or slow release of antigen, and (c) facilitated uptake of antigen by antigen presenting cells (APC) [5, 6]. Phosphatidylcholines are the most common lipids

employed for liposome manufacturing. However, nanoparticles can be developed from a wide range of lipids and proteins, which have been found to also alter their physicochemical and biological properties. Classical liposomes are now also gradually being replaced by more advanced technologies with the new generation of lipid-based nanovesicles (L-NVs), which have more elaborate functions and less weaknesses. Niosomes, transfersomes, sphingosomes, and other nonliposomal lipid-based nanoparticles are excellently reviewed by Grimaldi et al. [7]. For example, the virus-like particle (VLP) or virosome L-NV incorporates virus-derived or recombinant proteins that in this way are effectively delivered to the immune system [8]. This technology is used in two commercial vaccines, Inflexal (against influenza) and Epaxal (against hepatitis A) [9, 10]. Currently, several liposome-based vaccine delivery systems against infectious diseases are undergoing clinical testing (Table 1).

Mucosal vaccines were initially designed to be administered orally. Later, also intranasal vaccines were developed and today several different routes of administration are being explored for mucosal vaccination, including pulmonary, genital tract, rectal, and sublingual routes. Whereas preclinical examples in animal models have shown that in principle all of these routes work well, only the oral and intranasal routes have been used for licensed human vaccines [11]. The reason for this may simply be attributed to that a majority of these vaccines are against gastroenteric infections (requiring oral vaccines). Importantly, the adjuvant choice is critical because it enhances and modulates the immune response to the vaccine. For example, the breadth, the quality, and the long-term protective effect of the vaccine may be directly dependent on the adjuvant [12]. Liposomes can also function as adjuvants in their own right, as they have been shown to enhance immune responses even after oral administration [13]. A particular type of liposomes, that is, layersomes, which are liposomes coated with single or multiple layers of biocompatible polyelectrolytes, has been found to stimulate significant serum IgG and mucosal IgA antibodies and T cell responses producing IL-2 and IFN-γ [14]. Noteworthy, though, the oral route most often requires high amounts of antigen and protection against enzymatic degradation. Moreover, an effective oral liposome vaccine should be effective at breaching the mucus barrier to facilitate uptake of antigen by gut mucosal antigen presenting cells (APCs).

Whereas oral vaccination provides a real challenge to vaccine developers, the intranasal (i.n.) route is more permissive. In fact, i.n. vaccination has several advantages compared to oral vaccination. These include the need for less antigen and a substantially reduced risk of antigen degradation [15]. Interestingly, IgG-coupled liposomes with an enhanced transmucosal transport were more immunogenic than plain liposomes given i.n. [16]. Because of the compartmentalization of the mucosal immune response, due to the acquisition of tissue-specific homing receptors on activated lymphocytes, nasal immunization also promotes a much stronger specific immune response in the respiratory tract compared to oral immunization. This also results in that excellent genital tract immunity can be achieved after i.n, immunizations, while this is not the case after oral vaccination. Thus, local secretory IgA (sIgA) antibodies and genital tract cytotoxic T cells were more effectively stimulated after i.n. immunization than through oral immunizations [17–20].

In this review we will describe and discuss liposomes as vaccine delivery vehicles for efficient mucosal immunization. In the first section, the impact of liposome composition and structure for vaccine efficacy will be discussed and in the second section the nature and qualities of the resulting immune responses following liposome vaccination will be described. Finally, in the last section, we have attempted to summarize the current standing of the field of liposome-based vaccines and future perspectives towards the development of the next generation of effective mucosal vaccines.

2. Liposomes as Vaccine Delivery Vehicles

Liposomes are spherical lipid bilayer structures with an aqueous core ranging in size from tens of nanometers to several micrometers in diameter. Liposome technology was first explored in the 1960s by Bangham et al. as a model system for diffusion of ions across biological membranes, and already in the 1970s there was an interest in using them for drug delivery [39, 40]. At that time, some researchers also tested them for adjuvant functions and ever since they have been used in various vaccine formulations owing to their inherent structural and chemical properties [41]. Phospholipids are most commonly the main constituents of the shell that delimit the aqueous core of the liposome. These molecules are amphiphilic and contain a hydrophobic tail consisting of two fatty acids linked by a glycerol backbone to a hydrophilic headgroup made up of phosphate and potentially another organic molecule that together determine their chemical properties, as shown in Figure 2. In an aqueous environment, this polarized structure facilitates self-assembly into arrangements with the fatty acids facing each other and forming an oil-like compartment between the outwards-facing phosphate groups. In liposomes, the arrangement is a hollow sphere, a vesicle consisting of either a single or multiple phospholipid bilayers forming unilamellar or multilamellar particles. Other types of lipids, all with the amphiphilic structure in common, may be incorporated in liposomes. Examples of other interesting lipid categories in the context of vaccine carrier formulations are sterols, most notably cholesterol (Chol); sphingolipids, with a sphingosine backbone linked to a headgroup and a single acyl chain tail; and fat-soluble vitamins such as tocopherols (vitamin E).

The fact that liposomes have both a hydrophobic and a hydrophilic region makes them versatile and useful as carriers for antigens: hydrophobic peptides or proteins can be inserted into the inner hydrophobic center of the bilayer while hydrophilic molecules can be either encapsulated in the core of vesicles or bound to their surface (Figure 3(c)). Surface binding of the antigen can be achieved by covalent attachment or it can occur through adsorption or electrostatic interactions. Alternatively, an antigen-bound hydrophobic anchor can be inserted into the bilayer. Another advantage of liposomes in vaccine formulations is that their physicochemical properties are highly adaptable and their size, charge, and lamellarity can be tailored to meet the requirements

Table 1: Examples of liposome adjuvant vaccines against infectious diseases tested in clinical trials. This compilation of completed or ongoing studies involving liposomes for vaccination of humans was generated with data from ClinicalTrials.gov. Here we have indicated the target disease, the vaccine composition, the route of administration, the clinical testing stage, and the reference number.

Name	Disease	Description	Route of administration	Sponsor	Status	ClinicalTrials.gov Identifier
FMP012 with AS01B	Malaria	Falciparum malaria protein (FMP012) in a formulation based on liposomes mixed with the immunostimulants monophosphoryl lipid (MPL) and *Quillaja saponaria* Molina, fraction 21	Intramuscular injection	US Army Medical Research and GlaxoSmithKline	Phase 1	NCT02174978
TVDV with Vaxfectin [21]	Dengue fever	Tetravalent dengue vaccine (TVDV) with Vaxfectin® cationic lipid-based adjuvant	Intramuscular injection	US Army Medical Research and Materiel Command	Phase 1	NCT01502358
Ag85B-ESAT-6 with CAF01 [22]	Tuberculosis	Subunit protein antigen Ag85B-ESAT-6 with two-component liposomal adjuvant system composed of a cationic liposome vehicle (dimethyldioctadecylammonium (DDA) stabilized with a glycolipid immunomodulator (trehalose 6,6-dibehenate (TDB))	Intramuscular injection	Statens Serum Institut	Phase 1	NCT00922363
Biocine with lipid A [23]	HIV	Recombinant envelope protein rgp120/HIV-1SF2 combined with lipid A	Intradermal	National Institute of Allergy and Infectious Diseases	Phase 1	NCT00001042
PAMVAC with GLA-SE or GLA-LSQ	Malaria	Placental malaria vaccine candidate adjuvant with alhydrogel, glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), or glucopyranosyl lipid adjuvant-liposome-QS-21 formulation (GLA-LSQ)	Intramuscular injection	Tuebingen University Hospital	Phase 1	NCT02647489
ID93 with GLA-SE [24]	Tuberculosis	Recombinant fusion protein incorporating four *M. tuberculosis* antigens (ID93) formulated with glucopyranosyl lipid adjuvant- (GLA-) stable emulsion (SE)	Intramuscular injection	National Institute of Allergy and Infectious Diseases	Phase 1	NCT02508376
Novel liposomal based intranasal influenza vaccine	Influenza	Liposomal-based influenza vaccine	Intranasal	Hadassah Medical Organization	Phase 2	NCT00197301
VaxiSome with CCS/C [25]	Influenza	Commercial split influenza virus and polycationic liposome as adjuvant (CCS/C)	Intramuscular injection	NasVax Ltd.	Phase 2	NCT00915187
Fluzone with JVRS-100 [26]	Influenza	Inactivated trivalent influenza virus vaccine administered with cationic lipid-DNA complex adjuvant JVRS-100	Intradermal	Colby Pharmaceutical Company and Juvaris BioTherapeutics	Phase 2	NCT00936468
RTS/S with AS01 [27]	Malaria	Repeat sequences of the *Plasmodium falciparum* circumsporozoite protein (RTS/S) fused to the hepatitis B surface antigen with a liposome-based adjuvant system that also contains monophosphoryl lipid A (MPL) and *Quillaja saponaria* Molina, fraction 21	Intramuscular injection	KEMRI-Wellcome Trust Collaborative Research Program and GlaxoSmithKline	Phase 3	NCT00872963

Table 1: Continued.

Name	Disease	Description	Route of administration	Sponsor	Status	ClinicalTrials.gov Identifier
Amphomul [28]	Visceral leishmaniasis	Amphotericin B lipid emulsion	Intramuscular injection	Bharat Serums and Vaccines Limited	Phase 3	NCT00876824

of the vaccine. In particular, the charge and membrane fluidity of the liposome can be fine-tuned by altering the lipid composition [42].

The properties of different phospholipids depend on both their polar headgroup and the nature of their fatty acid tails. The characteristics of the lipid headgroup play a key role in determining the surface properties and in particular the surface charge of the vesicles. Naturally occurring phospholipids can be categorized into 6 types according to their headgroup: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), or phosphatidic acid (PA). Whereas PS, PI, PG, and PA are negatively charged, PC and PE are neutral but zwitterionic. Further, both the fluidity and permeability of the membrane and, in extension, its resistance to degradation, here referred to as stability, depend both on the length and degree of saturation of the acyl chains of the tail as well as the charge of the headgroup. All of these factors influence the transition temperature of the lipid, which is in turn decisive for whether the lipid membrane exists in gel- or fluid-phase at a certain temperature. These factors also determine the tendency of multicomponent membranes to undergo small-scale phase separations resulting in heterogeneous distribution of different lipids. A common modulator of the membrane permeability and fluidity is cholesterol, which also influences the liquid-to-gel phase temperature [43].

The possibility to chemically modify both the headgroup and the tail region gives rise to the option of producing synthetic phospholipids tailored to specific requirements. For example, positively charged liposomes have been made using synthetic cationic lipids, such as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dimyristoyl-trimethylammonium propane (DMTAP), and dimethyldioctadecylammonium bromide (DDA). From this, it follows that the choice of lipid composition will greatly impact the biophysical and chemical properties of the liposome. Also, by choosing lipids that naturally exist in cell membranes, liposomes can be completely biodegradable, nontoxic, and nonimmunogenic in themselves [44, 45]. On the other hand, if components with an origin in archaeal, bacterial, or viral membranes are chosen, they could enhance the immunogenicity of the formulation [35, 37, 46–50]. Accordingly, PS is naturally exposed on the surface of cells undergoing apoptosis, and in this way liposomes containing PS may effectively trigger phagocytosis by macrophages [51–53]. Finally, many other modifications can be made to liposomes that further extend the high versatility of these nanoparticles. Such modification includes the attachment of targeting agents such as galactose or APC-specific antibodies, of polymers, for example, poly(ethyleneglycol), or the addition of different kinds of coatings such as chitosan [38, 54–58]. Furthermore, when introduced into a physiological fluid, nanoparticles such as liposomes acquire a dynamic layer of adsorbed proteins in a corona, the nature of which is determined by both the particle's size and surface properties as well as the shear stress the particle is exposed to [59–62]. In practice this means that carefully engineered surface properties may be altered by the adsorption of a complex protein mixture. This can have many diverse consequences; for example, the protein corona may mask surface bound ligands and in this way prevent receptor binding, or it can cause complement activation [63–65].

3. Physiochemical Properties of Liposomal Vaccines

3.1. Surface Charge. Although liposomes with different characteristics have been extensively used for vaccine delivery, it still remains to explain why the immune response is modulated differently by different liposomal formulations. It is inherently difficult to dissect the contribution of different properties, as changing one property usually influences one or several others. Surface charge can, for instance, be changed by altering the lipid composition; however, by changing the lipid composition also other properties might change, such as membrane fluidity, rigidity, and stability. Hence, it may be difficult to directly assess the influence of changing different physicochemical properties of liposomes on the immune response. Nevertheless, many attempts have been made to describe the effects on immune responses after altering liposomal characteristics. One of these parameters is the charge of the liposome, which is assessed by the zeta potential, a measure of the electrostatic potential at the limit of what is called the diffuse electric double layer that surrounds the particle (Figure 3(a)). The double layer is a diffuse layer of differently charged ions spatially distributed at the surface of the particle, which in this way becomes shielded. The magnitude of the zeta potential, thus, depends on the concentration of ions within the double layer, but also other factors, such as the ionic strength and pH of the dispersion medium. This must be kept in mind when comparing zeta potential values reported in different studies and under different conditions.

Because the cell surface as well as the mucus coating of the mucosal membrane is negatively charged, it is frequently hypothesized that positively charged liposomes will exhibit stronger interactions with the cell membrane as well as an increased mucoadhesion. The latter leads to reduced clearance rate, that is, slower removal from the mucosal membranes. Noteworthy, both increased interactions with the cell membrane and prolonged exposure time of the antigen at the mucosal surface are thought to lead to increased cellular

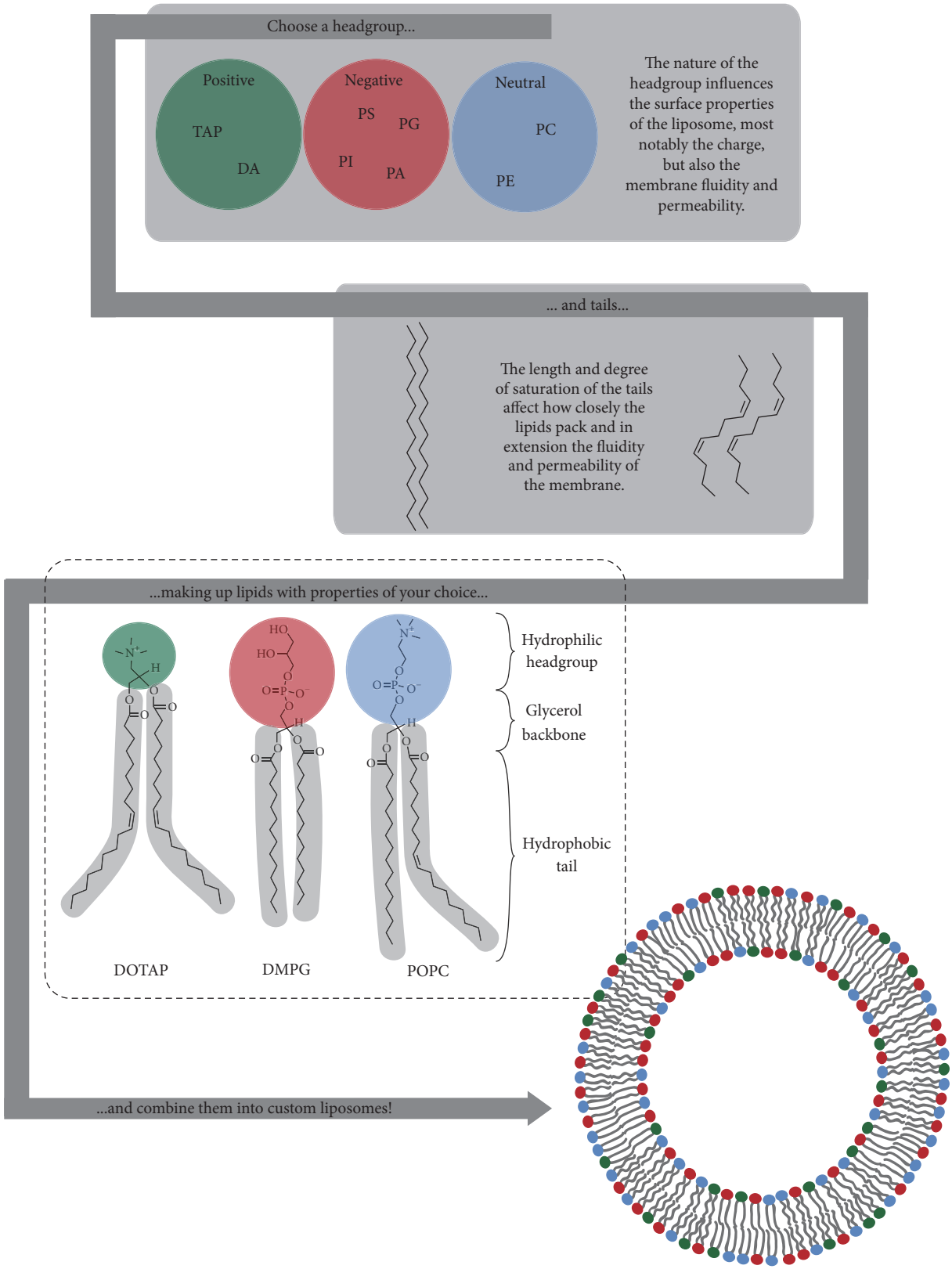

Figure 2: Generation of customized liposomes. Lipids are polar molecules consisting of a hydrophilic headgroup and hydrophobic fatty acid tails. Examples of positively charged headgroups are trimethylammonium propane (TAP) and dioctadecyl ammonium bromide (DA), while negatively charged headgroups are phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), or phosphatidic acid (PA), and finally neutral headgroups are phosphatidylcholine (PC) or phosphatidylethanolamine (PE). A headgroup can be combined with tails of different nature to create lipids with the desired properties; the examples shown are the lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and the phospholipids dimyristoylphosphatidylglycerol (DMPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). Different lipids can then be combined into liposomes with different functional features, which provide the basis for this highly diverse and versatile technology that is so excellently suited for vaccine development.

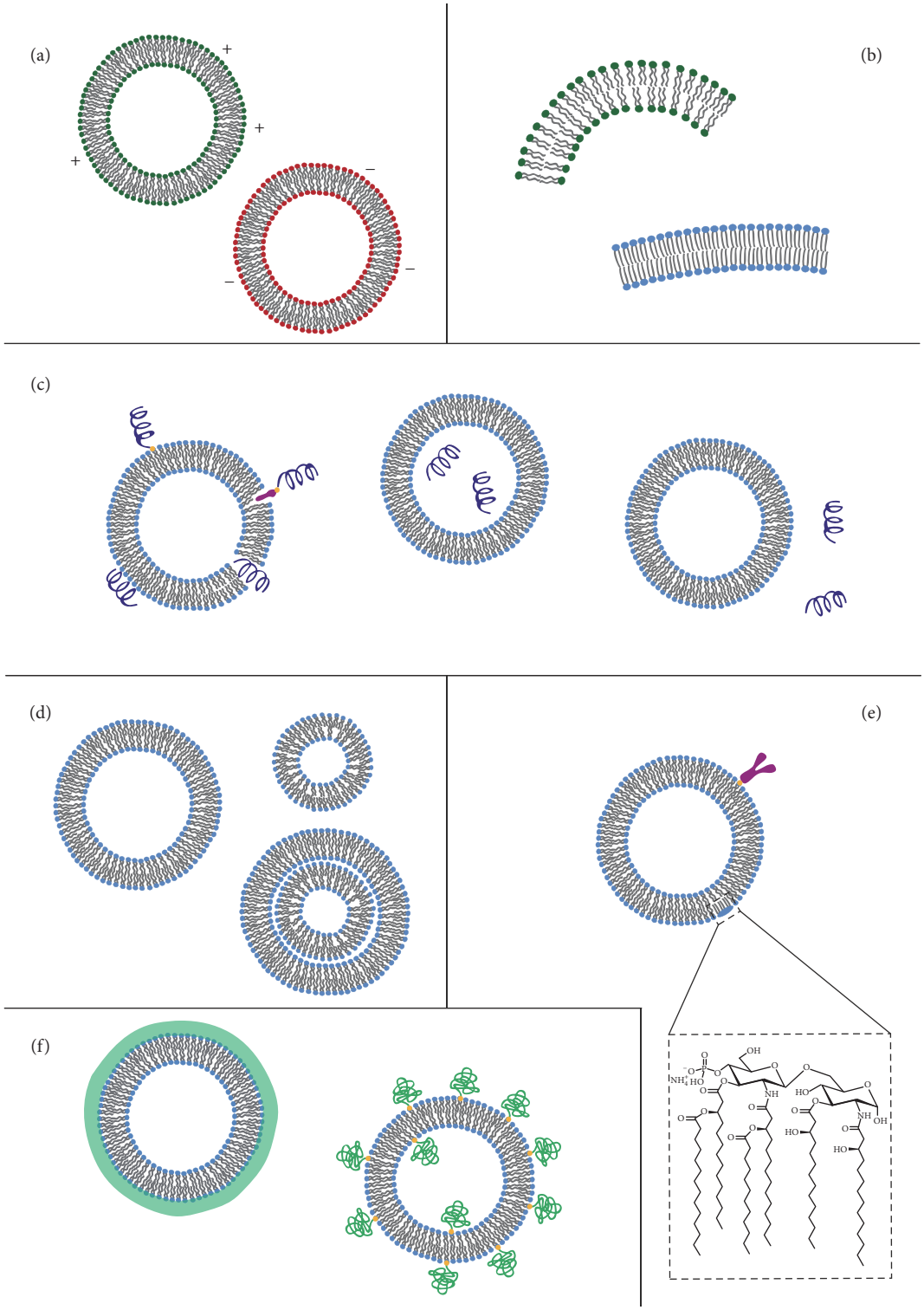

Figure 3: Various properties of liposome-based vaccines. (a) The effect of altered surface charge on liposome function has been extensively examined [32, 33]. (b) The lipid composition is critically influencing the immune response [34]. (c) Also the localization of the antigen on or inside the liposome plays an important role in shaping the immune response to the vaccine. There are several modes of antigen association to liposomes. Firstly, antigens may be encapsulated in the aqueous core or they could be linked to the surface via covalent attachment. Alternatively, a hydrophobic anchor can be used to attach the antigen to the surface via adsorption or through electrostatic interactions with lipids of opposite charge. For proteins with a hydrophobic region one may even successfully insert these in the liposome membrane. The liposome may also be used as an immunoenhancer simply by admixing the antigen and the liposomes. (d) Only few studies have addressed the impact of size or lamellarity [35, 36]. (e) Modifications of liposomes to increase their immunoenhancing effect can be done through attaching PAMPs, such as lipid A (LPS), or through specific targeting strategies using cell-specific antibodies (anti-CD103 or -DEC205) [16, 37]. (f) Other modifications, including addition of poly(ethylene glycol) (PEG) or different polymer coatings that increase the liposome penetration of the mucosal barrier or to increase liposome resistance in biological fluids, have also been developed [38].

uptake of antigen and stronger immune responses. However, this may not always be the case. In general, positively cationic charged liposomes have been shown to be better retained and more immunogenic at mucosal membranes than negatively charged or neutral liposomes [66, 67]. Furthermore, cationic liposomes were found to effectively deliver antigen to both mucus and antigen presenting cells (APCs) as shown in an in vitro model of the airway epithelium with liposomes made with distearoylphosphatidylcholine (DSPC)/trehalose 6,6-dibehenate (TDB) (neutral) and DSPC/TDB/DDA (positive) with varying amounts of DDA [68]. Moreover, cationic liposomes consisting of DOTAP/Chol, DMTAP/Chol, or, most prominently, the polycationic sphingolipid ceramide carbamoyl-spermine (CCS) and cholesterol were shown to effectively stimulate systemic and mucosal humoral and cellular immune responses after i.n. immunizations in mice [32]. By contrast, neutral liposomes with dimyristoylphosphatidylcholine (DMPC) or anionic liposomes with DMPC/dimyristoylphosphatidylglycerol (DMPG) were comparably ineffective as immunogens [32]. While a positive charge appears to increase the immunogenicity of liposomes, it still remains to be investigated in greater detail. In fact, negatively charged liposomes have been shown to be more immunogenic than both zwitterionic and positively charged liposomes and it has even been postulated that anionic liposomes could exert an immunosuppressive effect on alveolar macrophages and in this way promote an enhanced humoral immune response [33, 69–72]. Hence, it can be hypothesized that several mechanisms are modulated by the charge of the liposome. It is also important to point out that altered charge of the liposomes by necessity involves modifying the lipid composition, which most likely will change also other properties, such as membrane heterogeneity, fluidity, and stability [73]. Naturally, also the charge of the liposome may dramatically influence the immunogenicity when given by different routes, as this may provide differentially charged microenvironments.

3.2. Lipid Composition. The lipid composition (Figure 3(b)) is known to influence the stability of the liposome; a more stable formulation might lead to a larger amount of bioavailable antigen and potentially also a depot effect. Han et al. made liposomes from various combinations of Chol, dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), and distearoylphosphatidylcholine (DSPC) and found that certain combinations impacted on their stability. Liposomes with DSPC, having a higher transition temperature, were more stable in vitro and likely protected antigen better from degradation in the gastrointestinal tract [49]. It was also found that stable liposomes containing DPPS induced stronger IgA responses compared to formulations without DPPS [34]. Combinations of both DPPC/DMPG and DPPC/PS have been found effective at targeting liposomes to macrophages, though DPPC/DMPG were found more immunogenic than liposomes with DPPC/palmitoyl phosphatidylethanolamine (DPPE) or DPPC/PS. Noteworthy, changing the lipid composition also resulted in an altered charge, with the DMPG and PS types being more negatively charged [73]. Furthermore, cationic liposome-hyaluronic acid (HA) hybrid nanoparticle systems have recently been developed and tested for DC maturation. It was found that primarily an upregulation of costimulatory molecules, including CD40, CD86, and MHC class II, were responsible for an enhanced effect, which greatly contributed to an enhanced specific T cell and antibody response following i.n. vaccination [74].

As previously mentioned, using archaeal lipids, liposomes can be made more immunogenic and liposomes comprising archaeal membrane lipids (archaeosomes) were found considerably more potent than liposomes made with Egg phosphatidylcholine (EPC)/Chol at inducing ovalbumin-(OVA-) specific IgG and IgA antibodies following oral administration in a mouse model [46]. This was likely due to increased stability in the gastrointestinal tract and to the fact that the archaeosomes were better retained in the intestine [46]. However, the difference may also partly reflect the fact that the archaeosomes are negatively charged while the EPC/Chol-liposomes are neutral.

3.3. Antigen Localization in Liposomal Formulations. There are many ways of incorporating antigens into liposomes. This raises the question of whether some strategies are more effective than others in the context of optimizing the immunogenicity of the liposome. Antigens can be hosted in the aqueous core of the liposome, inserted into the membrane leaflet or bound to the surface by covalent bonds or intermolecular forces (Figure 3(c)). Hence, a plethora of combinations exist and those could be used to enhance resistance against antigen degradation or facilitate antigen uptake. Thus, the liposome formulation may be tailored for specific needs and purposes. If an oral vaccine is to be designed, one may hypothesize that encapsulating the antigen inside the liposomes should be an effective strategy to prevent enzymatic degradation. However, by hiding the antigen in the liposome, the immunogenicity may be compromised because the antigen will not be immediately accessible for APCs. Therefore, choosing how to physically incorporate the antigen in the liposome may have critical consequences and could dramatically change the immune response. Unfortunately, until now such aspects have not been addressed in a systematic manner. When administered orally, encapsulated antigen may more effectively stimulate local IgA and serum IgG antibody responses compared to when soluble antigen is admixed with the liposomes [73, 75]. On the other hand, following i.n. administration, admixed antigen and liposome have been quite effective even compared to liposome-encapsulated antigen [32, 70]. Interestingly, liposomes have been found to exert an immunoenhancing effect even when administered 48 hours prior to the antigen [70]. Furthermore, even liposome surface bound antigens, rather than fully encapsulated antigens, have been found to be more immunogenic following i.n. immunization [71]. Probably, these observations underscore that the i.n route is less sensitive to antigen degradation compared to the oral route. Thus, depending on the route of administration, it seems clear that antigens may or may not be immunogenic when exposed on the surface of the liposome and for many formulations it may, in fact, be advantageous to have surface

bound as well as encapsulated antigens. Indeed, this may also apply to the adjuvant. It was found that cholera toxin B-subunit (CTB) adjuvant bound to the surface of the liposome was more effective compared to when encapsulated in the liposome [76]. In fact, a challenging question is what the relationship and localization should be between the antigen and the adjuvant in the liposome. Theoretically, it can be argued that since the adjuvant is included primarily to promote dendritic cells- (DC-) priming of the T cells it should be encapsulated, while the antigen should be both encapsulated and surface bound to secure sufficient stimulation also of naïve B cells. Of note, B cells normally recognize 3D structures with their receptors, while T cell receptors react to degraded linear peptides. However, this interesting question has been poorly investigated and only few studies have been published on this topic. For example, it has been observed that by altering the lipid-to-antigen ratio, the humoral and cellular immune responses can be differentially induced [32, 77]. Thus, it is likely that the immune response following liposome administration is susceptible not only to the choice of antigen and lipid components but also to their relative proportions and localization in the liposome.

3.4. Size and Lamellarity. A broad range of unilamellar and multilamellar liposomes with varying sizes have been found to have variable effects following mucosal immunization (Figure 3(d)). While, unfortunately, the degree of multilamellarity is not routinely reported, the influence of size and/or lamellarity on the immunogenicity of the liposome is yet to be determined. For example, a comparative study between unilamellar archaeosomes, 100 nm in diameter, or large multilamellar aggregates of these clearly identified better immunogenicity of the multilamellar aggregates [35]. Noteworthy, not only the size but also the lipid assembly was different between the unilamellar and multilamellar constructs, in this example. On the other hand, another study reported that a "double liposome," consisting of small (~250 nm) liposomes made from SoyPC, DPPC, Chol, and SA encapsulated into a bigger (1 to 10 μm) outer liposome made from DMPC and DMPG, was found only marginally more immunogenic than the small liposomes given alone by oral administration [36]. Taken together, constructing homogeneous monodisperse and unilamellar liposomes is highly challenging and various degrees of multilamellar constructs may coexist, making interpretations of experimental results difficult, but recent advancements in this technology may allow for more accurate comparisons of the influence of size, lamellarity, and overall structure in the future [78].

3.5. Modifications Increasing the Bioavailability of Liposomal Antigens. The microenvironment at mucosal surfaces often promotes a high clearance rate of liposomes. Therefore, various strategies have been tested to enhance mucus penetration or to increase membrane adhesion to facilitate bioavailability of the vaccine antigens (Figure 3(f)). Layer-by-layer deposition of polyelectrolytes onto the liposome, for example, has been used as a liposome-stabilizing approach which resulted in higher specific IgA and IgG antibody levels as well as an increased T cell response [79]. Polyvinyl alcohol or chitosan has been tested to enhance bioadhesive properties of the liposome and it has been observed that chitosan-loaded liposomes, indeed, stimulated enhanced IgG antibody responses [58]. Chitosan is a positively charged polysaccharide that can form strong electrostatic interactions with cell surfaces and mucus and, therefore, increase retention time and facilitate interactions between the liposome and APCs in the mucosal membrane. Alternatively, such modifications can also transiently open tight junctions between epithelial cells to allow for transmucosal transport of the liposomes [80–82]. In fact, chitosan-coated liposomes have been shown to give better serum IgG antibody levels compared to other bioadhesive polymers, such as hyaluronic acid or carbopol coated liposomes, and host much better immunogenicity than uncoated negative, neutral, or positively charged liposomes [38, 56–58].

Considerable attention has been given to studying how liposomes are retained by and/or taken up across the mucosal membranes. Liposome interactions with the intestinal mucosa have been studied in vivo and ex vivo using various in vitro models [46, 79, 83, 84]. The latter models have addressed whether passage of liposomes between the tight junctions of epithelial cells can be achieved. Indeed, tight junctions were reported to be open when using PC/Chol-liposomes or *Tremella*-coated liposomes [84]. Enhanced immune responses were also observed with mucus-penetrating liposomes made with poly(ethylene glycol) (PEG) or the PEG-copolymer pluronic [38]. Significantly higher specific IgA and IgG antibody levels were found with PEGylated than un-PEGylated liposomes. Charge-shielding modifications with PEG or Pluronic F127 also proved useful in preventing liposome aggregation to obtain small (<200 nm) chitosan-coated liposomes. In fact, these shielded chitosan-coated and PEGylated liposomes yielded the highest functional serum antibody titers of all the formulations tested and the strongest IgA responses [38].

3.6. Cell-Targeting Modifications of Liposomes. Modifications aimed at increasing liposome stability and/or uptake have indeed proven effective. One of the most explored modifications is aimed at targeting the delivery of liposomes to subsets of cells. Liposomes can be equipped with various targeting elements, aiming at enhancing their immunogenicity (Figure 3(e)). For example, additional targeting components may enhance the uptake by APCs or the penetration of the liposome through the mucus layer. The strongly GM1-ganglioside-binding molecule CTB has been reported to enhance liposome immunogenicity. Moreover, when monophosphoryl lipid A, acting through the TLR4 receptors, was added to liposomes their ability to stimulate the innate immune response was dramatically improved [34, 37, 49, 85, 86]. Other Toll-like receptor agonists or *Escherichia coli* heat-labile toxin (LT) have also been used in combination with liposomes as adjuvants [47, 87]. Furthermore, linking CpG, which acts through TLR9 signaling, or *Bordetella pertussis* filamentous hemagglutinin to the liposome has all been found to enhance immunogenicity [88, 89].

In fact, many different liposome cell-targeting approaches have been investigated. To this end, specific antibodies have been found to enhance binding to M cells, thereby targeting the liposome to the follicle associated epithelium (FAE). This is the thin epithelial cell layer that is responsible for antigen uptake from the luminal side, such as the epithelium that overlays Peyer's patches (PP) in the small intestine [16]. Similarly, lectin Agglutinin I from *Ulex europaeus* coated liposomes were shown to improve M cell-targeting and antigen uptake [83, 90, 91]. Also, galactosylation of liposomes resulted in higher specific IgA and IgG antibody levels compared to unmodified liposomes [54]. Moreover, liposomes coated with the influenza virus protein hemagglutinin were more immunogenic than uncoated liposomes [50]. In addition, mannosylated lipids or anti-CD40 antibody-coated liposomes were found to host an enhanced ability to target DCs and, thereby, greatly promoted a stronger immune response [55, 87]. Furthermore, the identification of Mincle, a receptor for the mycobacterial cord factor trehalose 6,6′-dimycolate (TDM), on innate immune cells, led to that TDM analogs were found to be effective stimulants of the production of G-CSF in macrophages. Indeed, immunizations in mice with cationic liposomes containing the analogues TDM demonstrated a superior adjuvant activity [92].

Many strategies have, indeed, been applied to achieve cell-targeting of liposomes, with varying degrees of improved function. Needless to say, there exist a plethora of possibilities to explore when it comes to targeting liposomes to the cells of the mucosal immune system. If analytical tools are combined with suitable in vitro and in vivo assay systems it will greatly help identifying the relative importance of liposome targeting and how composition, such as size, lamellarity, surface charge, and fluidity of the membrane, can influence the immune response.

4. Mucosal Immune Responses to Liposomes

Prior to an adaptive immune response, innate immune activation must have occurred, leading to the production of proinflammatory molecules and the expression by APC of costimulatory and immunomodulating molecules, that is, chemokines, cytokines, and the costimulatory molecules CD80, CD86, CD40, and others. Innate immune activators can be classified into several categories, including the dominant ones, Toll-like receptors (TLRs), C-type lectin receptors (C-LRs), and non-Toll-like receptors (NLRs) [93, 94]. These receptors recognize pathogen-associated molecular patterns (PAMPs), such as bacterial cell-wall components (e.g., peptidoglycan, lipoteichoic acid, and flagellin) and different forms of microbial nucleic acids (e.g., double-stranded RNA, high-CpG-content DNA). The role of an adjuvant in a vaccine formulation is, thus, to activate innate immunity and, therefore, most vaccine adjuvants are derived from PAMPs. Also for mucosal vaccines it is critical to induce a sufficient and appropriate innate immune response, preferentially, without causing unwanted side effects, such as tissue damage. Thus, a successful mucosal vaccine must be capable of inducing not only an adaptive immune response, but also a strong innate immune response [13]. Fortunately, liposomes can do both. They can both serve as delivery vehicles for vaccine antigens and act as immunomodulators, triggering both innate and adaptive immune responses. Indeed, many modifications of the liposome itself can dramatically influence the innate immune response and, thereby, augment or qualitatively modify also the adaptive immune response. To this end, altered lipid composition, charge, particle size, or added targeting elements can all be utilized to tailor the immune response to the liposome and stimulate the required anti-infectious immune response (Figure 3). In addition, to get an even stronger activation of innate immunity, liposomes can be equipped with specific adjuvants/PAMPs, such as flagellin or CpG, as we have already discussed.

Mucosal immunizations as opposed to systemic immunizations effectively support IgA class switch recombination (CSR) and production of sIgA at mucosal sites [95]. In the intestine, this occurs in the organized gut associated lymphoid tissues (GALT) and, in particular, in Peyer's patches (PP), which are the dominant sites for IgA CSR in the gut. In the upper respiratory tract the most active inductive site is the nasal associated lymphoid tissues (NALT), but also cervical lymph nodes and the mediastinal lymph node (mLN) are central to the mucosal immune response. Ultimately, targeting of the liposome to these sites could be a much preferred strategy in future vaccine formulations, as already discussed. Following antigen recognition and activation of specific B cells in GALT and NALT, these cells undergo strong expansion in the germinal centers (GC) in the B cell follicles. Most immune responses are T cell dependent and, thus, the expanding B cells require the participation of follicular helper T cells (T_{FH}) in the GC to differentiate into long-lived plasma cells and memory B cells. These CD4+ T cells are generated through the peptide-priming event that the DCs orchestrate in the T cell zone in the lymph node. In this way, the DC is a key player in the immune response and it impacts also the ability of the activated lymphocytes to migrate to the effector tissue from where the DC originated. The plasma cells, thus, eventually migrate from the inductive site via the lymph and blood back to the lamina propria in the mucosal membrane, where they produce sIgA. Thus, there is a complex series of events that need to be completed before a productive sIgA response can be found in the lamina propria of the mucosal membrane (Figure 1). Hence, liposomes can be made to impact or modulate many different steps in this series of events. A critical question is how we can assess and compare different liposome formulations for their efficacy and characteristic impact when multiple steps are involved. We would advocate a reductionist approach where different liposomes are evaluated for their effect in different stages of the buildup of an immune response. Therefore, in the next section we focus on the interaction of liposomes with DCs and APCs in general.

4.1. Innate and Adaptive Immunity against Liposome Vaccination. Although significant progress has been made in understanding antigen uptake and processing of liposome delivered antigens, the fine details of these processes are still poorly known. Liposomes that enhance cell membrane fusion, that is, fusogenic liposomes, deliver their antigenic

content to the cytoplasm of the APC, which enables MHC class II presentation to $CD4^+$ T cells and in some DC subsets also allows for cross-presentation to MHC class I restricted $CD8^+$ T cells. Of note, liposomes that are taken up via scavenger receptors (CD68, CD36, and Clec LOX-1) or other innate immune receptors are usually restricted to prime $CD4^+$ T cells through MHC class II presentation. Thus, targeting of liposomes to different DC subsets or uptake mechanisms can provide a means to specifically tailor the immune response to a certain antigen or facilitate the development of a distinct type of immune response [96, 97]. For instance, whereas zwitterionic or anionic liposomes have not been reported to drive inflammation, cationic liposomes have been shown to stimulate proinflammatory responses in DCs, leading to an upregulation of costimulatory molecules, CD80 and CD86, and proinflammatory cytokines [98]. Furthermore, Yan et al. reported that DC stimulation by cationic liposomes composed of DOTAP (1,2-dioleoyl-3-trimethylammonium propane) also stimulated reactive oxygen species (ROS), which activated extracellular signal-regulated kinase (ERK) and p38, and downstream proinflammatory cytokines/chemokines, interleukin-12 (IL-12), and chemokine (C-C motif) ligand 2 (CCL2) [99]. In addition, DOTAP liposomes were shown to induce transcription of monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), and macrophage inflammatory protein-1 beta (MIP-1β/CCL4) [100]. Also DiC14-amidine cationic liposomes can induce the secretion of IL-1β, IL-6, IL-12p40, interferon-β (IFN-β), interferon-γ-inducible protein 10 (IP-10), and TNF-α by human and mouse myeloid DCs [101]. While anionic liposomes in general are poorly proinflammatory, modifications such as using mannosylated lipids could make these liposomes much more proinflammatory and effective at stimulating DCs [102, 103]. With regard to macrophages it has been reported that galactose-modified liposomes can stimulate TNF-α and IL-6 production, which was associated with significantly higher specific sIgA antibody levels in the nasal and lung tissues and increased serum IgG antibodies [54].

Carefully analyzing the literature, it appears unclear how different liposomes stimulate strong innate immune responses. A high density of positive charges on liposomes is considered beneficial, while negatively charged or neutral lipids are likely to lower this capacity [104, 105]. As mentioned previously, liposomes effectively stimulate both T and B cell responses, but it is their direct impact on the DCs that matters for the adaptive immune response. In fact, the uptake of liposomes by DCs has an important effect on the development of different CD4+ T cell subsets. These $CD4^+$ T cell subsets have both distinct and overlapping functions, but their individual impact on the immune response is critical. For example, if protection against a pathogen requires IFN-γ production (Th1 cells), then the development of exclusive Th2-dominated responses can be detrimental. Therefore, in this context, monophosphoryl lipid A (MPL) (a TLR4 ligand) or monomycoloyl glycerol (MMG) combined with DDA in a liposome will consistently promote IFN-γ production, that is, a Th1-biased immune response [106]. Moreover, trehalose dibehenate (TDB) liposomes, together with the combined Ag85B-ESAT-6 vaccine antigen, enhanced antituberculosis specific IFN-γ and IL-17 production, as well as increased specific serum IgG2 antibody levels [107]. While the composition of lipids matters for the subset of $CD4^+$ T cell that the DC can prime, also a larger size of the liposome may influence the generation of Th1 $CD4^+$ T cells [108]. However, the mechanism for this effect is unclear but could relate to the fact that different subsets of DCs or other APCs like macrophages are involved in processing differently sized liposomes, such that small sized liposomes preferentially stimulate Th2 $CD4^+$ T cell responses. On the other hand, it has been claimed by many investigators that protection is best achieved with a balanced Th1/Th2 response. Liposome-based microneedle array (LiposoMAs) and a mannose-PEG-cholesterol (MPC)/lipid A-liposome (MLLs) system are both examples of liposome formulations with a balanced Th1/Th2 response [109–111]. An even more complex vector is the combination of nanoparticle technology and liposomes with biodegradable poly(DL-lactic-co-glycolic acid) (PLGA), cationic surfactant dimethyldioctadecylammonium (DDA) bromide, and the immunopotentiator TDB which promotes Th1 and Th17 $CD4^+$ T cell responses and enhanced specific serum antibodies [112]. Moreover, the mycobacterial cell-wall lipid monomycoloyl glycerol analog has been used in combination with DDA. This combination resulted in a promising vaccine delivery system that induced strong antigen-specific Th1 and Th17 responses [113].

Protection against most infections requires both antibodies and adequate T cell immunity. However, for most infectious diseases we have given strongest attention to antibodies as correlates of protection. However, this scenario may change when we will identify more and more T cell mediated parameters that correlate with protection. Recently, it was identified that in influenza the best correlate of protection in a study with healthy human volunteers was the preexisting influenza-specific CD4+T cells [114]. It has been found that high density antigen coating onto liposomes often stimulates better antibody responses than encapsulated antigens, but a combination, such as the DOTAP-PEG-mannose liposomes (LP-Man), will enhance not only antibody responses but also APC antigen uptake and strong memory $CD4^+$ T cell development [115–118]. This and other progress in the field have to be considered when developing programs for the evaluation of vaccine efficacy. For example, with liposome vaccines as novel universal, broadly protective, influenza vaccines against also heterosubtypic virus strains, it will be important to also assess and evaluate $CD4^+$ T cell immunity as a correlate of protection. Therefore, we need to better define how specific $CD4^+$ T cells, and, for that matter $CD8^+$ T cells, correlate to impaired viral replication or bacterial growth and reduced transmission of infection.

5. Concluding Remarks and Future Perspectives

Here, we have described and discussed recent progress in nanoparticle formulations using liposomes for mucosal

vaccine delivery against infectious diseases. We have underscored the complexity of the liposome formulation and pointed to many combinations and plasticity of the liposome nanoparticle as a carrier of vaccine antigens and adjuvants. Needless to say, when considering liposome-based vaccines, it is important to consider all the properties of the formulation, the route of administration, and the biological response. Thus, liposome size, lamellarity, and surface charge as well as lipid composition and fluidity of the membrane can all influence the immune response following vaccination. Importantly, the choice of antigen, with its own inherent physicochemical properties, and the position of the antigen and the adjuvant in the liposome critically affect the function of the liposome. The antigen/lipid ratio and properties of the added adjuvant are also important parameters that change the immunogenicity and stability of the liposome.

Most studies using liposome-based vaccines have focused on the end result of immunization, namely, the magnitude and the quality of the immune response. Few studies have attempted to systematically identify mechanism of action at the different stages of an immune response. For mucosal immunizations liposome vaccines have been thoroughly investigated for their stability in intestinal fluids, their mucoadhesive properties, or the efficiency in targeting and uptake by DCs and other APCs [46, 49, 58, 79, 119, 120]. The site of liposomal penetration of the mucosal barrier is important to determine. If occurring at the duodenal site, the outcome may be significantly different from penetration that takes place in the ileum or colon of the gut. Similarly, antigen uptake in the NALT may be more effective than if the liposome reaches deeper into the respiratory tract. This is not just because of the stability of the liposomal antigen at a distinct location, but also the DC subsets that are exposed to the antigen may differ dramatically and, hence, the outcome of the vaccination may vary. At the cellular level, we largely lack studies that investigate how liposome delivered antigens are processed and presented by DCs, the kinetics of these processes, and whether the formulation will affect the migration and function of the DC in the draining lymph node. More work is needed to generalize the principles for an optimal design of the liposome in this regard. For example, it still remains unclear whether liposomes that rapidly penetrate the mucosal barrier are also good inducers of a mucosal immune response or, alternatively, should mucoadhesive liposomes be used to provide a depot of antigen for an extended loading of DCs with antigen. Simple screening systems should be developed to address these questions. To assess whether a liposome efficiently delivers peptide for CD8+ or CD4+ T cell priming, one could focus on surface expression on the DC of complexes with MHC class I and II molecules plus peptide using specific antibodies that detect these complexes and flow cytometry analysis [121]. In this way, screening of liposome-antigen formulations could be evaluated on the basis of effective expression of such complexes on the surface of a target DC population in vitro and in vivo. The density of such complexes most likely will relate to the ability of the DC to effectively prime the T cells in the lymph node as the T cell receptor does not recognize the peptide, but the complex.

Identifying and standardizing liposome-stimulated immune responses would greatly aid in the prospects of comparing different liposome vaccine formulations for their efficacy. This would also contribute to a more rational design of effective liposome-based mucosal vaccines. Currently, there is no agreed protocol or procedure on how to evaluate and characterize the immune response to liposomes. Hence, most studies are performed without relevant comparisons and the evaluation of whether the liposome formulation is more or less effective compared to other types of formulations is impossible to assess. Therefore, it would be an advantage if investigators could agree on using some standardization protocol, perhaps using cholera toxin or some other strong soluble adjuvant, to admix with antigen and compare the immune response to those of the liposomes. Thus, it is fair to say that we still lack comprehensive comparisons to other modes of formulating antigens for mucosal vaccination.

The liposome technology applied to the development of the next generation of mucosal vaccines holds much promise. It is conceivable that better targeted liposomes with added adjuvant capacity will prove to be effective in stimulating mucosal immune responses that are protective against multiple infectious diseases. It is likely that these liposomes have both surface anchored antigen and encapsulated antigen to optimize B cell as well as T cell priming. The lipid compositions that yield higher gel-liquid crystal transition temperatures will be preferred as they have been found to stimulate stronger immune responses. Cationic rather than neutral liposomes, being more proinflammatory, will be selected for mucosal immunization. It will be important in the future to apply the better understanding of the liposome manufacturing technology and the principles for induction of mucosal immune responses to the design and development of the next generation of mucosal vaccines.

Competing Interests

The authors have no conflict of interests.

Authors' Contributions

Valentina Bernasconi and Karin Norling contributed equally to this work.

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Research Article

Altered Intracellular ATP Production by Activated CD4+ T-Cells in Very Preterm Infants

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Background. The neonatal immune system is not fully developed at birth; newborns have adequate lymphocytes counts but these cells lack function. *Objective*. To assess the activity of T-cells and the influence of the main perinatal factors in very preterm infants (birth weight < 1500 g). *Design*. Blood samples from 59 preterm infants (21/59 were dizygotic twins) were collected at birth and at 30 days of life to measure CD4+ T-cell activity using the ImmuKnow™ assay. Fifteen healthy adults were included as a control group. *Results*. CD4+ T-cell activity was lower in VLBW infants compared with adults ($p < 0.001$). Twins showed lower immune activity compared to singletons ($p = 0.005$). Infants born vaginally showed higher CD4+ T-cell activity compared to those born by C-section ($p = 0.031$); infants born after prolonged Premature Rupture of Membranes (pPROM) showed higher CD4+ T-cell activity at birth ($p = 0.002$) compared to infants born without pPROM. Low CD4+ T-cell activity at birth is associated with necrotizing enterocolitis (NEC) in the first week of life ($p = 0.049$). *Conclusions*. Preterm infants show a lack in CD4+ T-cell activity at birth. Perinatal factors such as intrauterine inflammation, mode of delivery, and zygosity can influence the adaptive immune activation capacity at birth and can contribute to exposing these infants to serious complications such as NEC.

1. Introduction

Although the immune system development begins early during fetal life, its maturation is not completed at birth as confirmed by the increased susceptibility of newborns and preterm infants to infectious diseases. The immune system of the fetus/newborn protects the infant against infection at the maternal-fetal interface but must also avoid the potentially harmful proinflammatory/Th1-cell response that can induce a dangerous reaction between mother and fetus. Thus, the suppression of proinflammatory response helps the infant in the transition from the intrauterine environment to the foreign antigen-rich environment of the outside world. Therefore this ineffectiveness that has been interpreted for a long time as a deficiency of the immune system could represent a biologically advanced response.

The faults of the neonatal immune system are mostly related to a functional deficit of their components. Newborns and especially preterm infants have higher leukocyte and lymphocyte counts compared to adults [1]; however these cells show poor function at birth as a consequence of the adaptive immune system not having had strong antigenic exposure *in utero*. This is confirmed by the decreased number of memory T- and B-cells and the increased number of naïve T- and B-cells in the neonatal bloodstream [2].

In order to measure the immune cell function during the third trimester of gestation, the immune status in a group of preterm infants was evaluated at birth and after 30 days of

life using the ImmuKnow assay (Viracor-IBT Laboratories, Lee's Summit, MO). This immunological test measures the levels of intracellular adenosine triphosphate (iATP) after *in vitro* nonspecific stimulation with phytohemoagglutinin (PHA) as marker of CD4+ T-cell activity; therefore it is one of the methods for non-pathogen-specific immune monitoring. Exposing T-cells to a mitogenic stimulus such as PHA leads to their metabolic activation and polyclonal expansion, a process in which the ATP synthesis and release precede surface receptor expression, cytokine production, and other subsequent events. Therefore, the iATP levels offer a proxy for the degree of functionality of the cell-mediated immune response [3]. The ImmuKnow assay has been cleared by the US Food and Drug Administration in 2002 for measuring changes in cell-mediated immunity in solid organ transplant recipients undergoing immunosuppressive therapy [4] and now represents one of the few well-established strategies for functional immune monitoring in the above-mentioned patient population [3].

Despite that, there are limited reports on the use of ImmuKnow assay in a pediatric population. Hooper et al. tested ImmuKnow assay in healthy children aged 1–17 years. The authors found that children aged 12 years and older have immune function levels equal to adults, while children aged less than 12 years present lower immune function levels compared to adults and older children [5].

However, to the best of our knowledge, ImmuKnow assay has never been tested in newborns and preterm infants.

The aim of this study was to estimate the peripheral blood CD4+ T-cell activation rate in response to *in vitro* stimulation with PHA in order to assess the basal condition of the adaptive immune system at birth, its development in the first month of life, and the influence of the main perinatal factors on the immune response in a population of very preterm infants.

2. Methods

2.1. Population. A prospective longitudinal study was carried out between November 2013 and July 2015 at the Neonatal Intensive Care Unit (NICU) of St. Orsola-Malpighi University Hospital in Bologna, Italy.

All the infants with gestational age (GA) ≤ 30 weeks and birth weight (BW) < 1500 g admitted at birth to the NICU were considered eligible to the study. Infants with congenital malformations or congenital infections or born to a mother with pregnancy complications (immunosuppressive disorders, diabetes mellitus, or infections during or preexisting the pregnancy) were excluded. Before enrollment in the study, written informed consent was obtained from each of the infants parents.

Fifteen healthy adults were also included as a control group.

2.2. Study Design. Peripheral blood samples were collected in the first day of life and at 30 days of life from each patient to evaluate the pattern of lymphocytes subpopulations and the levels of iATP in activated CD4+ T-cells *in vitro.* Anamnestic and clinical data were prospectively collected during the hospital stay.

Sepsis was defined as presence of clinical signs of infection (worsening of respiratory dynamics, apnea and increased oxygen requirement, cardiovascular instability with tachycardia or bradycardia, poor perfusion, hypotonia, and shock), elevation of infections markers (white cell count, CRP, and procalcitonin), and ≥1 positive blood cultures.

Necrotizing enterocolitis (NEC) was defined according to Bells modified criteria [6].

The study was approved by the St. Orsola-Malpighi University Hospital Research Ethics Committee (CIMPre study, 114/2012/U/Oss).

2.3. Assessment of CD4+ T-Cell Activity and Lymphocyte Subsets. CD4+ T-cell immune response was measured using the ImmuKnow assay (Viracor-IBT Laboratories, Lee's Summit, MO). The test was performed at the Operative Unit of Clinical Microbiology—Laboratory of Virology—of St. Orsola-Malpighi University Hospital in Bologna, Italy, according to the package insert.

Peripheral blood was collected in sodium heparin tubes and processed on the same day. Briefly, 250 μL of whole blood was diluted with sample diluent, added to wells of a 96-well microtiter plate (12 × 8-well separable strips; one 8-well strip/each specimen), and incubated for 15–18 h with PHA in 37°C in 5% CO_2. Magnetic particles coated with anti-human CD4 antibodies were introduced to the wells, and using a strong magnet, CD4+ T-cells were positively selected and separated. Then, the cells were lysed to release intracellular ATP. The released iATP was measured using luciferin/luciferase reagent and a luminometer.

The patient's CD4+ T-cell immune response was expressed as the amount of iATP (ng/mL).

In order to check each assay run, a control specimen collected from a healthy adult individual (quality control sample) was processed with patient specimen. Quality control samples were not included in the adult control group.

The lymphocyte subsets were evaluated using the BD FACSCalibur Flow Cytometr (BD FACSCaliburTM system, Becton & Dickson, Mountain View, CA, USA) according to the manufacturer's instructions.

2.4. Statistical Analysis. Statistical analysis was performed by IBM SPSS (Statistical Package for Social Sciences, version 20). Data distribution was checked for normality with the Shapiro-Wilk test. As the data is not normally distributed, nonparametric tests were used. Univariate analysis was performed in order to evaluate which clinical variables were related to iATP values at birth and at one month of life: Mann–Whitney and Kruskall-Wallis tests were used for categorical variables and Spearman correlation test for continuous variables. Regression multivariate analysis was performed using as independent variables all those variables that proved to be significant in the univariate analysis. Statistical significance was defined as a p value < 0.05.

Table 1: Lymphocytes subpopulations at birth and at 30 days of age of infants enrolled in the study (WBC: white blood cells, N: neutrophils, L: lymphocytes, and NK: natural killer lymphocytes).

	Birth: 59 infants Median (range)	30 days: 39 infants Median (range)
WBC (n)	7350 (1170–119200)	10700 (4030–34650)
N (n)	1814 (283–97744)	4267 (908–23562)
N (%)	24.8 (4.3–88)	42 (8.9–75)
L (n)	3775 (903–10775)	4141 (557–10098)
L (%)	60 (6–86.8)	40 (4.4–74)
Pan T (CD3+)	2875 (668–8404.5)	2865 (701–5481)
Pan T (%)	73.9 (47–88)	64.5 (36–84)
CD4+/mL	1954 (388–6680.5)	1982 (438–4725)
CD4+ (% L tot)	54 (32–73)	45.5 (20–69)
CD8+ mL	673 (172–2312)	729.5 (198–2650.5)
CD8+ (% L tot)	18 (8–32)	16 (8–45)
CD4+/CD8+	2.92 (1.37–7.38)	2.67 (0.77–6.25)
NK/mL	242.25 (35.6–1536)	442.75 (15–1371)
NK (% L tot)	7 (1–24)	9.5 (1–42)
Pan B/mL	501 (60.56–3770)	829.35 (210–3635.3)
Pan B (%)	15 (2–30.6)	22.9 (9–41)

3. Results

Seventy-three eligible infants were admitted to NICU during the study period. Fourteen infants (19,1%) were excluded because they either fulfilled the exclusion criteria (4 cases of congenital heart disease and 4 cases of polymalformations) or peripheral blood samples could not be collected within the first day of life (4 infants died and 2 were transferred from our unit in the first days of life). The study was carried out in a population of 59 preterm infants with GA ≤ 30 weeks and BW < 1500 g; 21/59 were dizygotic twins (the study population does not include monozygotic twins).

Samples were obtained at 30 days of life from 39/59 (66.1%) of the recruited newborns; the remaining 20/59 (33.8%) encountered death (4 infants) or were transferred to other hospitals before 30 days of life.

3.1. Lymphocyte Subsets. The pattern of lymphocytes subpopulations at birth and at 30 days of life is reported in Table 1. We found no significant difference between the absolute cell count at birth and at the end of the first month of life. While the absolute number of CD4+ T-cells did not correlate to the median value of iATP at birth ($p = 0.831$) a significant positive correlation was observed at 30 days of life ($p = 0.011$).

3.2. CD4+ Activity in Preterm Newborns at Birth and 30 Days of Age Compared to Adult Controls. While there were no significant differences in CD4+ T-cells ATP levels at birth versus 30 days of life (median: 100 ng/mL [range: 6–733 ng/mL] versus 168 ng/mL [range: 3–383 ng/mL]; $p = .142$), both these values were significantly lower compared to adults controls ($p < 0.001$) as shown in Figure 1.

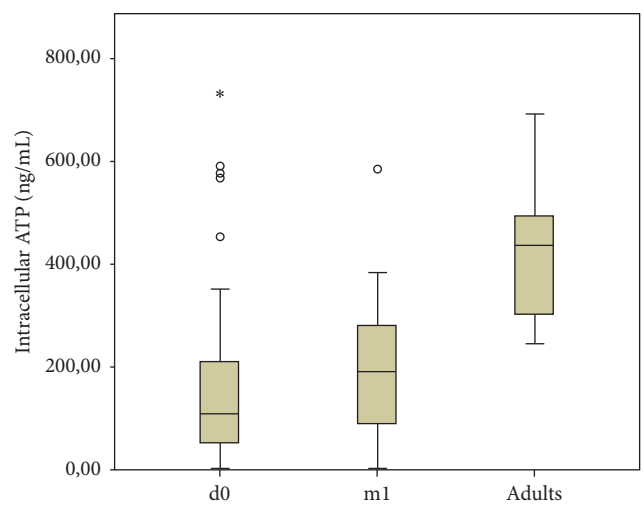

Figure 1: CD4+ T-cell activity (median iATP values) in preterm infants and in adult controls (d0: evaluation at birth; m1: evaluation at 30 days of life). Outliers are marked as ∘ ("out values": 1,5–3 × interquartile range, IQR) and ∗ ("extreme values": > 3 × IQR).

3.3. Perinatal Factors and CD4+ Activity. Univariate analysis showed no significant correlation between median ImmuKnow iATP levels at birth and GA, BW, gender, intrauterine growth retardation (SGA, small for gestational age), and use of prenatal steroids (Table 2).

Table 2: Characteristics at birth of infants enrolled in the study: median (range); GA: gestational age; SGA: small for gestational age; AGA: appropriate for gestational age; ***pPROM: prolonged Premature Rupture of Membranes: a case of premature rupture of membranes in which more than 18 hours has passed between the rupture and the onset of labor/delivery.

	Birth (59 infants)	p value	30 days of life (39 infants)	p value
Gestational age, weeks, mean ± DS	27.7 ± 2.4		27.5 ± 2.2	
Birth weight, mean ± DS (g)	992 ± 297		981 ± 281	
Gender male, infants number (%)	33 (55.9)		23 (59.0)	
Male sex, mean ± DS (iATP ng/mL)	182 ± 157	$p = 0.091$	170 ± 123	$p = 0.326$
Female sex, mean ± DS (iATP ng/mL)	110 ± 140.6		187 ± 105	
Singleton, infants number (%)	38 (64.4)		25 (64.1)	
Singleton: iATP ng/mL^	163 (6–733)	$p = 0.005$	178 (3–383)	$p = 0.731$
Twins: iATP ng/mL^	84 (26–153)		168 (10–365)	
Small for GA, infants number (%)	10 (16.9)		6 (15.4)	
SGA, mean ± DS (iATP ng/mL)	106 ± 121.1	$p = 0.255$	175 ± 96.0	$p = 0.770$
AGA, mean ± DS (iATP ng/mL)	164 ± 158		177 ± 120	
Vaginal delivery, infants number (%)	28 (47.5)		19 (48.7)	
Vaginal delivery: iATP ng/mL^	123 (15–733)	$p = 0.031$	123.5 (3–383)	$p = 0.795$
Cesarean delivery: iATP ng/mL^	83 (6–352)		215 (3–292)	
pPROM***, infants number (%)	15 (25.4)		9 (23.0)	
pPROM***: iATP ng/mL^	197 (52–336)	$p = 0.002$	185.5 (65–383)	$p = 0.343$
All others conditions: iATP ng/mL^	87 (6–733)		168.5 (3–365)	
Prenatal steroids, infants number (%)	48 (81.3)		33 (84.6)	
Prenatal steroids, mean ± DS (iATP ng/mL)	169 ± 162	$p = 0.140$	189 ± 116	$p = 0.159$
No prenatal steroids, mean ± DS (iATP ng/mL)	79 ± 52		117 ± 95	

^ means that the values are expressed as median and range (brackets) instead of mean ± standard deviation.

The twenty-one twins showed significantly lower median ImmuKnow iATP levels at birth compared to the remaining 38 singleton infants ($p = 0.005$); this difference was no longer significant at 30 days of life (Table 2 and Figure 2(a)).

Infants born to vaginal delivery had higher median ATP levels produced by activated CD4+ T-cells at birth compared to those born to C-section ($p = 0.031$); no difference was found at 30 days of life (Table 2).

Fifteen out of 59 preterm infants were born to mothers with pPROM (prolonged Premature Rupture of Membranes > 18 hrs); these infants showed a higher activity of CD4+ T-cells at birth compared to the 44 remaining infants ($p = 0.002$); this difference disappeared at 30 days of life (Table 2 and Figure 2(b)).

A multivariate analysis was performed including those variables (pPROM and twin pregnancy) which proved to be significant in the univariate analysis: both pPROM (Standardized Beta = 0.344, $t = 2.875$, and $p = 0.006$) and twin pregnancies (Standardized Beta = −0.359, $t = -3.008$, and $p = 0.004$) were found to be independently associated with ImmuKnow iATP levels at birth (higher and lower values, resp.).

3.4. Morbidity during the First 30 Days of Hospital Stay and CD4+ Activity. No correlation was found between CD4+ T-cell activity at birth or at 30 days of life and mechanical ventilation, patency of ductus arteriosus, early onset sepsis, late onset sepsis, postnatal steroids, antibiotics use, type of enteral nutrition, and death.

Five out of 59 (8.5%) preterm infants developed NEC ≥ stage 3 during the first week of life; these infants had significantly lower median iATP values at birth compared to the 55 infants without NEC ($p = 0.049$) (Figure 2(c)). We also performed a subgroup analysis dividing the infants into three subgroups according to gestational age (23-24, 25–27, and 28–30 weeks). We found that three out 5 infants who developed NEC were in the 23-24 weeks subgroup and the remaining two were in the 25–27 weeks one. In the 23-24 weeks group, there was still a significant difference in terms of immune cell function between infants who did and did not develop NEC ($p = 0.036$). On the contrary, the difference of median iATP levels between infants who did and did not develop NEC in the 25–27 weeks group was not significant, but we can not exclude the possibility that this is a consequence of the little data we had available.

No difference in CD4+ T-cell activity was found at 30 days of life in relation to NEC condition.

3.5. Short-Term Outcomes and CD4+ Activity. We found no influence of CD4+ T-cell activity on short-term outcomes (IVH: intraventricular hemorrhage; PVL: periventricular

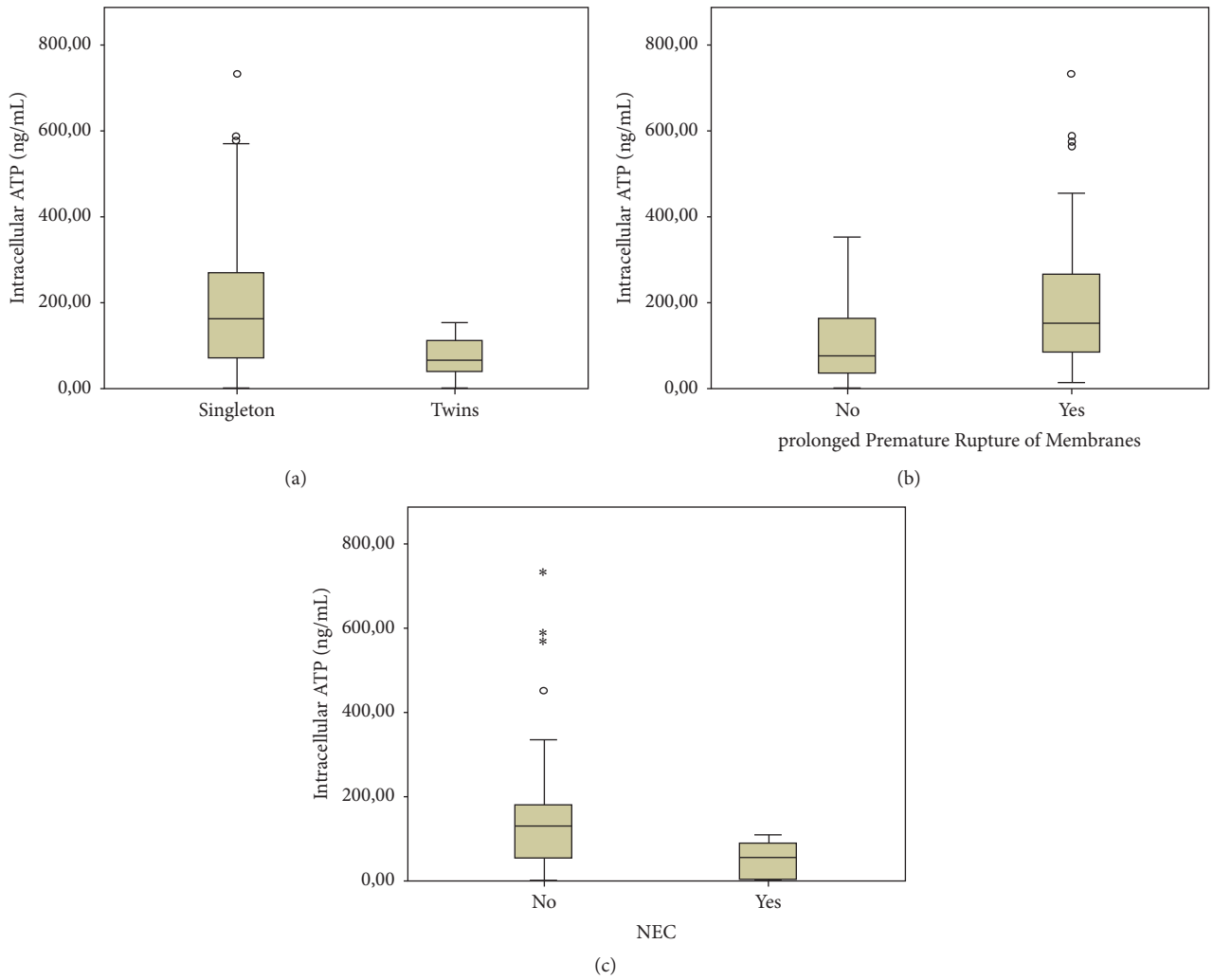

Figure 2: Levels of ATP at birth: (a) in twins and singleton; (b) pPROM versus other conditions of delivery; (c) patients with and without NEC. Outliers are marked as ∘ ("out values": 1,5–3 × interquartile range, IQR) and ∗ ("extreme values": > 3 × IQR).

leukomalacia; ROP: retinopathy of prematurity; BPD: Bronchopulmonary Dysplasia; death), at birth or at 30 days of life.

4. Discussion

The neonatal immune system is not fully developed at birth and newborns are therefore exposed to the risk of infection by a large number of viruses, bacteria, protozoa, and fungi. This weakness can be partly attributed to the lack of preexisting immunological memory and competent adaptive immunity. Newborn infants have deficiencies in T-cell activation and cytokine production, B-cell immunoglobulin production, and interactions between T- and B-cells, when compared to adults [7]. T-lymphocytes, especially CD4+ T-cells, play a crucial role in the regulation of the immune system. They are often targeted as a marker of the global immune function because they are involved in the modulation of the adaptive immunity, both humoral and cell-mediated, and they also play an important role in the control of innate immune response.

From 19 weeks of gestation, T-cell subpopulations gradually increase in number and continue to rise after birth to peak at about 6–9 months of life. The numbers subsequently decline, to reach adult levels at 6-7 years of age [8]. In term neonates, CD4+ T-cells constitute a higher proportion of T-cells than adults. CD8+ T-cells on the other hand are fewer both in terms of absolute number and as a percentage of total T-cells. Preterm infants have a significantly higher number of CD4+ T-cells while the number of CD8+ T-cells does not seem to change with gestational age [9]. Reference values for T-lymphocyte count are established for all ages but they do not reflect cell function.

In this study, we used the ImmuKnow assay in order to investigate the function of CD4+ T-cells at birth in a population of preterm infants. This test is one of the methods for non-pathogen-specific immune monitoring and measures the levels of ATP produced by CD4+ T-cells in response to *in vitro* stimulation with a nonspecific mitogen such as PHA [4]. ATP production is the final response of CD4+ cells that is common to different nonspecific factors. ATP is a key

metabolic marker; it is produced within minutes to hours of initial stimulation and is necessary for cellular function regardless of eventual effector function and therefore it is a highly suitable marker for T-lymphocyte activation and a clinical evaluation of global T-lymphocyte function [10]. At this moment there are no certainties regarding if the low ATP production by premature CD4 cells is due to inherent immaturity of ATP production, signaling, immature proliferation mechanism, or something else. However, some authors [11–15] suggest that the inability of neonatal CD4+ T-cells to go towards an efficient activation may be related/caused by an impaired process of signaling. A limit of the ImmuKnow assay is the lack of specificity, because PHA cause a nonspecific mitogenic stimulation that can not define any specific aspect of the immune response, like cytokine expression that marks Th1 or Th2 activation; but its major advantage is that it is clinically available unlike most other immunological assays. ImmuKnow assay has previously only been tested in adults and children and this is the first study that uses this assay to assess T-cell-mediated immunity in preterm infants. This test seemed quite suitable for preterm infants since it requires a very small amount of peripheral blood to be performed (250 μL).

Our findings show similar leukocyte and CD4+ T-cells counts to previously reported studies in preterm population [2, 16].

We documented a lack of association between the number of CD4+ T-cells at birth and their function (measured by intracellular ATP production) and a positive correlation between number and activity of CD4+ T-cells at 30 days of life. Whether there is or there is no correlation between the number of lymphocytes and their function is still a matter of debate in literature.

Kowalski et al. [10] demonstrated that this correlation is weak ($r = 0.24$), emphasizing the usefulness of ImmuKnow assay to provide a quantification of CD4+ T-cells activity that is independent of cells absolute number.

However, other studies performed afterwards questioned this observation since a positive association between white blood cell count and ImmuKnow levels was observed [17, 18]. Dharnidharka and Hesemann suggest that this may represent an underappreciated limitation of the ImmuKnow test since the levels of ATP are expressed as a concentration (ng/mL) without accounting for the number of white blood cells present in the volume of sample tested [19].

Because each study is heterogeneous from the other, differing in characteristics such as population, type of organ transplant, immunosuppression protocols, and timing of ImmuKnow assay it is difficult to come to conclusions.

We hypothesized that the correlation in our study is initially absent as a consequence of the immunological immaturity at birth (lymphocytes may be present in the blood without being functional) and becomes significant over time with the functional maturation of lymphocytes. Other neonatal confounding factors may also play a role and cannot be excluded and larger studies on immunocompetent subjects may validate our hypothesis.

In our study the adult control population includes healthy HIV-seronegative immunocompetent people, whose lymphocytes subpopulations related data are not available.

We found that preterm infants have a reduced CD4+ T-cell activation capacity compared to adults: the iATP values at birth are low, and despite a trend towards higher values over time, they remain significantly lower at 30 days of life. It is known that newborns, especially preterm infants, have deficiencies in both innate and adaptive immunity and many studies have demonstrated lower concentrations of cytokines such as TNF-α, IFN-α, IL-4, IL-5, IL-10, IL-15, and IFN-γ in preterm infants' blood compared to adults [20–23]. However cytokines production is an indirect estimate of cellular function. The present study sets the functional impairment of CD4+ T-cells at the initial steps of T-lymphocyte activation, when ATP is produced. Since ATP is a basic energy source within cells, its production is an essential requirement for all lymphocytes function following activation [10].

In this study twin infants showed median ImmuKnow iATP levels at birth much lower compared to singletons. This is an interesting find and literature lacks information on the immunological peculiarities of multiple pregnancies. All the twins included in the study were dizygotic and thus immunologically different. We hypothesize that, in addition to a generalized status of immunological deficiency or insufficiency, the copresence of twins in utero may induce a deeper immune tolerance that involves both fetuses and the mother in order to avoid the potentially harmful immune reaction between the three. Our hypothesis is in accordance with previous studies that documented higher levels of Th2-cytokines in the blood of mothers carrying twins compared with singleton pregnancies [24] underlining the more profound Th1-Th2 shift that occurs in twin pregnancies. Moreover, a recent study demonstrated that both dizygotic twins and their mothers are more prone to infection than monozygotic twins, singletons, and their mothers [25].

We found that pPROM significantly increases CD4+ T-cells activation at birth. This data is consistent with previous findings that reported that lymphocytes are activated during infections in utero, indicating that fetal adaptive immune response is at least partly responsive [26]. It has been shown that bacteria and proinflammatory mediators in amniotic fluid can elicit a fetal inflammatory response, documented by an increase in fetal plasma cytokines and C reactive protein [27–29]. Fetuses and neonates exposed to intrauterine inflammation have increased Th1 cells response and increased levels of IFN-γ, indicating a potential shift from Th2 to Th1 of the fetus [7, 30].

We have also demonstrated that CD4+ T-cell activity at birth is increased in infants born after a vaginal delivery. Increasing evidence suggests that parturition itself is an inflammatory event [31].

Our findings support the current knowledge that intrauterine inflammation/infection can lead to immune maturation in the fetus. But these differences disappear at 30 days of life most likely because all the categories (pPROM versus non-pPROM and vaginal delivery versus cesarean section) are exposed to the same extrauterine environment.

In our study population, the levels of iATP at birth did not correlate with the risk of sepsis. This is not the first study that fails to detect an association between low ImmuKnow iATP values and infection. Regarding this topic, previous studies performed in immunocompromised adults showed conflicting results [32]. In fact in the transplant setting, some authors reported that the ImmuKnow assay was not able to identify individuals at risk of infection, while others found that the assay was a useful tool for assessing this risk [33, 34].

Significant positive correlations were observed between low CD4+ T-cell activation capacity at birth and NEC development in the first week of life. Although the etiopathogenesis of NEC is still a matter of debate, some authors support the involvement of innate immune system [35]. We hypothesize that an impairment of the adaptive immune system may also play a role in the altered immune reaction that leads to NEC development. CD4+ T-cells impaired function may hinder infection control in the intestinal lumen. Other mechanisms may also be involved and a more detailed characterization of CD4+ T-cells is needed to clarify their role in the NEC pathogenesis.

This study has some limitations. The study sample is small and lacks control groups both at term and with gestational age > 30 weeks.

Another limitation is the absence of an immunophenotypic classification of CD4+ T-cells. The evaluation of Treg cells is crucial to understanding the immunological characteristics of the preterm infants. These CD4+ T-cells are provided with immunosuppressive functions and represent a high proportion of lymphocytes at birth with a significant inverse correlation with GA [9]. The role of Treg cells in the modulation of the immune response is an expanding field of research and this data can add precious information to our findings.

In conclusion, preterm infants show a lack in CD4+ T-cells activation and fail to show a functional maturation of lymphocytes over the first month of life. An impaired ability to respond to stimulation can contribute to expose these infants to serious complications such as NEC. However, the adaptive immune response can at least partially be elicited during the fetal life by events occurring before delivery such as pPROM or vaginal delivery. Further studies in larger populations are needed to clarify these results and to better understand the cellular mechanism that regulates neonatal adaptive immune response to pathogens.

Competing Interests

All the authors declare that they have no conflict of interests in connection with this paper.

Authors' Contributions

Giulia Aquilano and Maria Grazia Capretti contributed equally to this work.

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Review Article

Evaluation of T Cell Immunity against Human Cytomegalovirus: Impact on Patient Management and Risk Assessment of Vertical Transmission

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Cytomegalovirus (CMV) is one of the most common infectious agents, infecting the general population at an early age without causing morbidity most of the time. However, on particular occasions, it may represent a serious risk, as active infection is associated with rejection and disease after solid organ transplantation or fetal transmission during pregnancy. Several methods for CMV diagnosis are available on the market, but because infection is so common, careful selection is needed to discriminate primary infection from reactivation. This review focuses on methods based on CMV-specific T cell reactivity to help monitor the consequences of CMV infection/reactivation in specific categories of patients. This review makes an attempt at discussing the pros and cons of the methods available.

1. Introduction

Cytomegalovirus (CMV) infects roughly 50% of the population in industrialized countries by adulthood. In developing countries, infection rate is much higher, leaving few adults seronegative [1]. Most new infections do not reach clinical relevance, but in specific instances CMV may represent a risk. For instance, rejection after transplantation may be associated with CMV infection or reactivation. In addition, transmission of CMV to fetus during pregnancy is one of the most frequent causes of deafness, to name but one of the consequences possible [2]. For different reasons, in both of these situations, it is quite relevant to assess whether CMV viremia is due to new infection or reactivation of latent CMV. Several methods for CMV diagnosis are available on the market, spanning almost the whole range of possible formats [3]. Certain methods may be useful to discriminate between primary infection or reactivation. To help assess the real risk of severe disease posed by CMV, determination of T cell reactivity is being evaluated by several clinical researchers.

2. CMV Pathology

CMV belongs to Herpesviridae and is the prototype of the Betaherpesvirinae subfamily group. It is a ubiquitous virus that infects a large percentage of humans worldwide, often at an early age. CMV spreads through a variety of ways, and virus is persistently expressed in epithelial cells resulting in virus excretion in bodily fluids, with infected saliva as its preferential vehicle. Other vehicles include breast milk, urine, genital secretions, and other body fluids, to a lesser extent.

As all members of this family, CMV establishes latent infection in specific body districts after primary infection, specifically in monocytes and bone marrow $CD34^+$ myeloid progenitor cells. Viral reactivation occurs from time to time, under the influence of numerous factors. Latency is regulated by a variety of specific genes in the virus genome. However, CMV is thought to persist also due to its many ways of evading the host immune defenses [4, 5]. Several viral proteins, such as interleukin 10 homolog and others, create an immunosuppressive environment around infected cells that avoids elimination of the latently infected cell by

the immune system [6]. Virus expression is kept under the control of the immune system. $CD4^+$ T cells are considered key elements, as proven by the enormous reduction in the rate of CMV-related disease in HIV patients after the introduction of the highly active antiretroviral therapy (HAART) [7].

In the immunocompetent host, primary infection is almost always subclinical. However, symptoms resembling infectious mononucleosis by EBV can occasionally be present. Typically, most of the population gets infected during childhood and for this reason children in childcare are a population at risk of infection by exposure to saliva-contaminated objects. Primary infection is followed by viral excretion in urine and other body fluids for over 6 months [8]. Viremia drops when CMV-specific $CD4^+$ T cells peak [9].

Vertical transmission of infection from a mother with active infection to the fetus is a cause of concern. Between 0.2% and 2% of newborns are infected with CMV in utero or perinatally and, of these, approximately 10% develop clinically evident disease [1, 10]. Approximately 90% of symptomatic newborns and 15% of the asymptomatic ones experience long term sequelae [11]; nowadays, CMV is the most common cause of hearing and neurological impairment due to nongenetic causes [10].

The risk of infection for a fetus of an actively infected mother has long been considered to depend on whether the mother has primary infection or reactivation [12]. Pregnant women with primary infection have higher chances of transmission during pregnancy, with a frequency of transmission of roughly a third [11]. Because in developed countries seroprevalence for CMV ranges between 40% and 60%, many women are at risk of acquiring primary CMV infection during pregnancy. The presence of antibodies is considered a factor that decreases both the risk of transmission of CMV during reactivation to the fetus and the chances of serious long term consequences of infection. Indeed, the risk of transmission drops to 1.4% as a consequence of reactivation during pregnancy [13]. Nevertheless, in developing countries, where almost everybody is seropositive for CMV, prevalence of congenital CMV infection ranges between 1% and 5% of births. Therefore, recently, reactivation of maternal CMV infection was recognized as being responsible for the majority of congenital CMV infections [14].

Contrary to the immunocompetent host, CMV infection often has serious consequences in the immunocompromized host. For solid organ transplantation (SOT) recipients, the risk of serious disease varies according to whether recipients are seropositive or not: patients who are seronegative before transplantation have a 40 to 80% risk of acquiring primary CMV infection from the graft [15, 16]. In these patients, CMV infection increases the risk of superinfection by many pathogens, and, most importantly, they are likelier to undergo graft rejection. On the other hand, patients who are seropositive before transplant are at a lesser risk of infection by the graft, but CMV reactivation may still have serious consequences when they are under immunosuppressive drugs. For these reasons, transplanted patients routinely undergo preemptive antiviral prophylaxis for at least 3 months after transplant, although a period of 6 months is recommended [17].

HIV patients, another category of immunocompromized patients, may experience retinitis, gastrointestinal and central nervous system end organ disease, and pneumonia due to CMV reactivation [18, 19]. The frequency of such events has been greatly reduced by HAART therapy.

3. Immune Response to CMV

A very large number of viral proteins are encoded by the relatively complex CMV genome. A total of 751 translated ORFs were recently identified by several experimental approaches, including next generation sequencing and high-resolution proteomics [20].

CMV infection activates robust responses to many of these proteins by the adaptative and innate immune system, but it has been somewhat hard to define proper correlates of immune protection.

Upon primary CMV infection, the earliest antibody response is directed against tegument protein [21]. Neutralizing antibodies are mounted after at least 2 months from infection and are directed against a number of envelope proteins, such as the gH/gL/UL128-131A complex and gB, gH, and gM/gN [22].

Antibodies have a relevant protective role during infection, in terms of both disease transmission and severity. Their presence in maternal blood has long been considered to lower the chances of fetal infection [12]. However, it is not clear to what extent antibodies are protective since their presence in maternal milk does not prevent transmission of infection to babies [23, 24]. In addition, a recent clinical trial aimed at studying fetal transmission of CMV infection by pregnant women with primary infection showed that infusion of hyperimmune globulin did not seem to prevent transmission to fetus [25].

One can conclude that antibody titer is a correlate of protection, but the causative link remains elusive. The fact that some women with high neutralizing Ab titers still transmit virus to their fetus and that pregnant women can be reinfected despite vigorous neutralizing antibody titers indicates that these responses do not absolutely prevent infection, although they undoubtedly reduce the potential for infection [12]. Recently, it has been shown that kinetics of antibody responses to different CMV targets are markedly different [26, 27]. These results make it tempting to speculate that the interplay between viral replication and the development of antibodies to very specific targets might be more relevant compared to the entire anti-CMV antibody titer.

Antibody titers may reflect the entity of $CD4^+$ T cell responses, since they depend on T cell help in a stringent way. Indeed, very recent data on *Macacus rhesus* demonstrate that protection of the fetus from vertical transmission of CMV infection depends on $CD4^+$ T cells [28]. Whether this depends on $CD4^+$ T cells directly or indirectly is still to be clarified. In *Macacus*, a lower titer of neutralizing antibodies,

in spite of a normal antibody titer, was observed in the absence of adequate T cell help [28].

It has been known for more than 20 years that helper and killer T cells are also expanded at extraordinarily high frequencies during CMV infection. Although $CD4^+$ T cells seem to react preferentially against structural viral proteins and $CD8^+$ T cells react mostly against immediate early ones, many proteins are recognized by both [29, 30]. Certain viral proteins are immunodominant, especially pp65 and IE-1; however, not all antigens seem to induce protective immunity. For example, it was shown that developing high frequencies of $CD8^+$ T cells against IE-1, but not against pp65, early after transplantation is associated with protection from CMV disease [31]. These data are quite controversial, as they were not confirmed by several subsequent studies [32].

CMV-specific $CD8^+$ T cells have long been known to be key in controlling viral replication in infected hosts and their adoptive transfer has proven therapeutical in transplanted patients [33, 34]. Though underestimated in the past, such T cell-mediated response seems to be quite relevant in the infected fetus as well [23]. A very recent study performed on pregnant women with primary CMV infection who either transmitted or did not transmit CMV to their fetus showed that proliferative ability of both $CD4^+$ and $CD8^+$ T cells and IL-2 secretion by CMV-specific $CD4^+$ T cells are lower in women who transmit CMV [35].

Repeated reactivation of CMV progressively enhances the number of CMV-specific $CD8^+$ T cells, which can accumulate in time up to 20% of total $CD8^+$ T cells, in what is known as "memory inflation" [36]. $CD4^+$ T cells are also expanded with similar kinetics, though to a lesser extent [37]. Interestingly, proteins expressed during latency are also recognized by T lymphocytes but these seem to quench immune reactivity rather than having an effector role [29].

$\gamma\delta^+$ T cells are also expanded during CMV infection in the adult and in the fetus [38], where expansion of $V\delta2^-\gamma\delta^+$ T cells was shown to be a specific blood signature of CMV infection [39]. Notably, $\gamma\delta^+$ cells were proven to have a protective activity in SOT patients and in early life [23, 38].

Natural killer cells have long been known to be critical in recovery from CMV infection, both in adult and in fetal life [23]. As part of innate immunity, they have been shown to rapidly increase in primary infection until roughly 2 months after infection and then to decrease [35]. They have recently been demonstrated to be more similar to the adaptive arm of the immune system than first thought. Indeed, they seem to undergo some selection similarly to T cell development in the thymus; in addition, although they do not undergo somatic rearrangement of their receptor genes, they have been shown to expand in an antigen-specific fashion and establish a pool of "memory" cells in the mouse [40].

In humans, Rölle et al. showed that expansion of specific NK cell subsets during CMV infection occurs in an MHC-dependent way and relying on CD94 and NKG2C on NK cells and HLA-E and IL-12 derived by monocytes, although the antigen, if there is a specific one, is still unknown [41]. In turn, CMV devotes at least 6 ORFs to counteracting NK activity, although this is not their only function [42]. This underlines how important NK cells are in recovering from CMV infection.

To sum up, both humoral and cellular immune responses contribute to protection from CMV infection reactivation and to recovery. However, the specific role of each of the two arms of the immune response has not been defined, nor have the interactions of the two components. This consideration paves the way to future studies where antibody-dependent cellular cytotoxicity will have to be deeply investigated.

4. Most Commonly Used Methods Available for CMV Diagnosis: A Brief Overview

Because the consequences of CMV infection vary in different types of patients and treatment is readily available, it is important to diagnose active CMV infection. In many instances, the most important diagnostic questions are whether CMV viremia is due to primary infection or reactivation and what the chances of serious disease are. CMV infection can be diagnosed with practically all available methods in laboratory diagnostics, but different clinical questions can be solved with careful choice.

In the case of primary infection in adults, the search of serum antibodies is first choice to determine whether adults with infectious mononucleosis symptoms or pregnant women who have been exposed to CMV have been recently infected by CMV. CMV-specific antibodies can be determined by enzyme-linked immunosorbent assay (ELISA) in various formats as a first choice. ELISA can also be used to search for IgM [3].

The presence of CMV-specific IgM with low-titer and no IgG characterizes primary infection. Because IgM tends to be present also during reactivation, IgG is often found together with IgM; IgG-avidity tests may therefore be useful tools to differentiate primary infection from reactivation. As for other pathogens, avidity of IgG binding CMV increases with time from infection. When low, this index identifies recent CMV infection as opposed to reactivation [43].

In pregnant women, it is important to determine the risk of transmitting CMV infection to the fetus in utero, besides the occurrence of infection itself. Studies have shown that, surprisingly, cellular immunity is directly correlated to the risk of transmission [44, 45].

Direct determination of the presence of CMV virions is first choice in the diagnosis of CMV infection in the newborn and fetus, where maternal antibodies may render serology cumbersome. While intrauterine infection can be diagnosed by sampling amniotic fluid, newborns shed CMV in most body fluids, such as urine and saliva for months [46]. Isolation of CMV may be attempted by classical or shell-vial cell culture methods. This method has several drawbacks, above all the fact that CMV grows slowly and some clinical isolates do not easily adapt to culture conditions, thus leading to false negative results.

Molecular methods have largely replaced cell culture in many laboratories. Most of these are based on nucleic acid amplification of CMV DNA from blood, urine, bronchoalveolar lavage fluid, and other body fluids. Modern quantitative

molecular methods mostly rely on real-time PCR for clinical viral load testing. Recently, digital droplet PCR (ddPCR) was evaluated as a method allowing precise direct quantification without requiring a calibration curve [47, 48]. It relies on limiting partition of the PCR volume in a large number of droplets, each of which may be envisioned as a PCR reaction giving a positive or negative result according to whether a single target molecule was present in the droplet or not. When compared to quantitative real-time PCR, ddPCR proved to reduce quantitative variability but was not as sensitive as real-time PCR [48].

These methods are more expensive than cell culture but are much faster and more sensitive. In addition, they may be rendered quantitative and are relatively independent of sample deterioration. Determination of T cell immunity may be helpful diagnosing infection in children younger than 12 months [49, 50].

Finding CMV-specific IgM in fetal and newborn serum may also be used to diagnose infection.

Consensus guidelines have been set for the management of SOT patients [17]. Before transplantation, CMV IgG should be determined in patients and donors because CMV^- recipients transplanted with tissues from CMV^+ donors are at high risk of primary CMV infection. To discriminate equivocal serology results, it may be useful to assess cellular immune status against CMV. After transplantation, active disease, whether primary or due to reactivation, should be closely monitored by determining viral load. Classically, this has been carried out by the pp65 antigenemia test: purified peripheral blood leukocytes (PBLs) from patients are enumerated at UV microscope after staining with anti-CMV nuclear protein pp65 fluorescent monoclonal antibodies. A semiquantitative result of the number of PBLs with infected nuclei out of 200,000 is obtained. However, neither inter-laboratory standard is available nor has common agreement on cutoff values been reached for this test. Most laboratories have replaced determination of antigenemia with quantitative determination of viral DNA load in whole blood or plasma.

Although a plasma DNAemia cutoff value of 10,000 copies/mL has been suggested before CMV therapy is started [51], a consensus has not been reached and cutoffs differ between laboratories. A WHO standard to calibrate diagnostic tests is now available [17]. Serology is not helpful in posttransplant patients, where negative results may be due to immune suppression.

5. Diagnostic Methods Based on T Cell Reactivity

While the presence of CMV-specific IgG has been long considered the golden standard to define infection by CMV, it has been proposed that measuring CMV-specific T cell responses by specific assays might help predict whether a patient will develop serious CMV disease after transplant. Improving criteria to treat patients for active CMV infection would avoid unnecessary treatment and related toxicity and allow saving the costs of repeated monitoring of CMV reactivation and of prophylaxis. Although at present it is not routinely performed, monitoring cell immunity to CMV, alone or in parallel to viral load determination, may help identify patients that actually require treatment for CMV disease after transplantation and/or help set personalized cutoff values for CMV DNAemia or antigenemia for patients at high or low risk of CMV disease, that is, with low or high T cell responses to CMV, respectively.

CMV-specific T cell reactivity before transplantation, and not so much seropositivity, was shown to inversely correlate to the risk of CMV viremia and disease after transplant; spontaneous clearance of CMV may occur in patients with positive CMV DNAemia or antigenemia, especially in patients where robust T cell immunity can be detected against CMV, independently of their serological status against CMV [52].

In the past years, different techniques have been developed to detect antigen-specific T cell response: flow cytometry-based multimer or intracellular cytokine staining (ICS) and ELISpot have been employed in research and in clinical studies [53, 54]. The advantages and disadvantages of each technique have been highlighted in a comparative study [55].

ICS for CMV-specific T cells followed by flow cytometry is particularly useful in basic research [54]. This assay consists of a 4–6 h stimulation of PBL or whole blood with CMV antigen, costimulatory antibodies, and a secretion inhibitor like Brefeldin A. This is followed by fixation, permeabilization, and staining with antibodies against an intracellularly retained cytokine, most commonly IFNγ, and surface markers like CD4, CD8, and others of interest, labeled with different fluorophores. FACS analysis is then performed. The assay allows enumeration of CMV-specific T cell subsets and simultaneously permits determination of the phenotype of single cells and the cytokines they produced after stimulation. ICS is a very informative test because it allows the detection of both $CD8^+$ and $CD4^+$ antigen-specific T cells in a single assay. A broad spectrum of different phenotypic markers and cytokines can also be analyzed at the same time [54]. It may be useful to detect immune responses also in samples of patients who lack response against particular dominant epitopes in their pathogen-specific T cell repertoire [56]. However, ICS requires a good level of expertise and is labour intensive, in addition to being costly.

A variation of the ICS can be carried out by the use of tetramers of MHC class I and CMV-derived peptide, used in ICS both as stimulants of specific $CD8^+$ T cells and as fluorescent labels [53]. Recently, this technique was used in an effort to define cutoff values of CMV-specific $CD8^+$ T cell in allogeneic hematopoietic stem cell transplant recipients associated with protection from recurrent or persistent CMV infection. Recovery from CMV disease was shown to be faster when these cells were ≥ 7 cells/μL of blood during the first 65 days after transplantation, whereas a value < 7 cells/μL was indicative of CMV-related complications [57].

More recently, the tetramers technique have been improved by using "multimers" which have higher affinity for the T cell receptor (TCR) compared to tetramers and guarantee more stable binding. Multimer staining allows labeling, visualization, and enumeration of peptide-specific

T cells in patient peripheral blood samples. Even if this test requires a small amount of blood sample and the results are available within 3 hours, it is not suitable as a diagnostic tool, because several multimers are required to obtain a full overview of the immune response for each patient. Furthermore, due to MHC polymorphism, a multimer for each single HLA allele would be necessary [58].

Because of their complexity, both methods are limited to research activities.

An ELISPOT-based method to determine cellular immune reactivity against CMV was developed by Oxford Immunotec. It uses a mixture of peptides derived from CMV antigens IE-1 and pp65 that stimulate IFNγ secretion by both $CD4^+$ and $CD8^+$ T cells from purified PBL. Individual IFNγ-producing cells are enumerated out of a total of 200,000 PBLs placed in each ELISPOT well coated with antibodies capturing secreted IFNγ. Controls include unstimulated (negative) and phytohemagglutinin- (PHA-) stimulated (positive) cells.

ELISPOT allows discriminating between low responders (20–50 spots/10^6 PBLs) and high responders (>100 spots/10^6 PBLs) in 2 days from blood sampling [59]. This assay was shown to be useful to predict the risk of CMV reactivation and infection in hematopoietic and kidney transplant recipients, since strong cell immunity is predictive of less serious CMV disease [60, 61]. This assay was also used to show that higher values of cell-mediated immunity in the blood of pregnant women are associated with risk of fetal CMV transmission [44, 62]. This surprising observation might be due to the fact that the number of specific T cells increases with viremia. However, experimental data do not agree with these findings and demonstrate that the number of $CD4^+$ T cells correlates with protection [28].

The assay linearity has been shown to be comparable to ICS [55] but ELISPOT for CMV-specific T cells was shown to be more sensitive than ICS [63].

ELISpot is, so far, the most sensitive method to determine T cell frequencies, but it is costly and laborious and, above all, it is hard to standardize for clinical purposes because ranges of frequencies vary greatly in the general population.

QuantiFERON-CMV (QT, Qiagen) is an assay that measures IFNγ release after stimulation of $CD8^+$ T cells in whole blood with a cocktail of peptides binding a range of different HLA-I haplotypes, designed on the basis of a variety of CMV proteins. Again, controls are stimulation with phytohemagglutinin or no stimuli [64]. One mL of heparinized whole blood is incubated for 15–24 h, and then IFNγ content is measured in plasma by ELISA. It is CE marked (i.e., approved by the European Community and legally placed on the market in Europe) for clinical diagnostic use in Europe but not yet FDA approved in the United States. The assay yields positive and negative results when >0.2 or <0.2 International Units (IU) of IFNγ/mL, respectively, is recovered from CMV-stimulated supernatants. The result is obtained after subtraction of the value of the unstimulated sample and only if the value in PHA-stimulated blood is >0.5 U/mL. In addition, an indeterminate result can be obtained when no IFNγ can be found in the CMV or in the PHA-stimulated blood, where patients have low numbers of PBLs. The advantage of QT is its ease in clinical application, requiring minimal sample processing and technical expertise. However, the determination of IFNγ release by $CD8^+$ T cells alone may be a limitation, since $CD4^+$ T cells are as relevant. In addition, the use of peptides may lead to false negative results for rare HLA haplotypes.

Positive QT results in the week of the onset of CMV reactivation or, alternatively, 2 months after allogeneic hematopoietic stem cell or kidney transplantation were shown to correlate with a lower risk of complicated reactivation [60, 65]. Among QT-positive patients, roughly 6% develop CMV disease, versus 22% of QT-negative ones. The group with the highest risk of CMV reactivation was the indeterminate group, where 58% of patients developed CMV disease at 12 months after transplantation [66].

Although QT was shown to be as good as ELISPOT in determining the risk of CMV infection in kidney transplant recipients [60], results from QT analysis were not found to correlate to the risk of transmission of congenital CMV, as opposed to ELISPOT [44].

Transplant patients with positive results have been shown to have a reduced risk of CMV reactivation or of severe disease upon discontinuation of antiviral prophylaxis [65, 67].

QT-CMV was compared to ELISPOT performed with a CMV pp65 peptide mix (10 μg/mL; AID) in monitoring CMV cell immunity in kidney transplant patients. The assays were shown to display good correlation and similar sensitivities and specificities [60]. However, both assays in their present format failed to detect all CMV-reactive individuals and would profit from improvement of the selected antigen. Whole CMV virions were proposed to overcome failure of certain HLA types to bind the mixture of pp65 peptides [59]. In contrast, another study compared the usefulness of ELISPOT and that of CMV-QT in predicting transmission of congenital CMV infection by pregnant women. In this study, mother CMV-specific T cell frequencies in mothers determined by ELISPOT, but not by QT, correlated with congenital CMV, together with maternal viremia and viruria ($p < 0.05$) and correlated negatively with CMV IgG-avidity ($p < 0.01$) [44].

In comparison to ICS, QT is as specific but less sensitive [68]. However, ICS is definitely more difficult to standardize and not suitable for automation.

6. Concluding Remarks

CMV infection may represent a risk for transplanted, immunocompromized, or immunologically immature individuals. In transplanted patients, measuring viremia levels for CMV is useful to determine whether the virus has reactivated due to immune suppression. However, patients may or may not clear CMV spontaneously, and therefore more detailed clinical diagnostic tools are warranted to set personalized cutoffs for antiviral therapy, given that laboratory assays, such as the determination of antibody titer, isotype, and avidity, are of limited utility to assess risk of transmission of infection from mother to fetus. For these reasons, many clinical studies

have attempted to measure CMV-specific T cell reactivity as an indicator to predict the outcome of infection in different clinical situations. Indeed, most studies agree that quantifying T cell response to CMV can be useful and at least one assay, QT, has been standardized for clinical diagnostic use. Future milestones may be as follows: (1) first future milestone is to extend observations concerning the benefits of determining T cell reactivity in different clinical situations; (2) second one is to improve the currently available methods, for example, by allowing QT to determine $CD4^+$ T cell reactivity besides CD8. This may be achieved by including whole recombinant CMV antigens, virions, or defined MHC class II-binding peptides as stimulators of IFNγ release and may lead to more reliable and generalized cutoffs for antiviral therapy. (3) The third future milestone is to adapt methods, that is, CMV ELISPOT, which may be more sensitive than others, to the clinical practice.

Competing Interests

The authors have no competing interests regarding the publication of this paper.

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Review Article

State of the Art, Unresolved Issues, and Future Research Directions in the Fight against Hepatitis C Virus: Perspectives for Screening, Diagnostics of Resistances, and Immunization

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Hepatitis C virus (HCV) still represents a major public health threat, with a dramatic burden from both epidemiological and clinical points of view. New generation of direct-acting antiviral agents (DAAs) has been recently introduced in clinical practice promising to cure HCV and to overcome the issues related to the interferon-based therapies. However, the emergence of drug resistance and the suboptimal activity of DAAs therapies against diverse HCV genotypes have been observed, determining treatment failure and hampering an effective control of HCV spread worldwide. Moreover, these treatments remain poorly accessible, particularly in low-income countries. Finally, effective screening strategy is crucial to early identifying and treating all HCV chronically infected patients. For all these reasons, even though new drugs may contribute to impacting HCV spread worldwide a preventive HCV vaccine remains a cornerstone in the road to significantly reduce the HCV spread globally, with the ultimate goal of its eradication. Advances in molecular vaccinology, together with a strong financial, political, and societal support, will enable reaching this fundamental success in the coming years. In this comprehensive review, the state of the art about these major topics in the fight against HCV and the future of research in these fields are discussed.

1. Introduction

Among infectious diseases, hepatitis C virus (HCV) still represents a major public health threat, with a dramatic burden from both epidemiological and clinical points of view. Chronically infected individuals are estimated to reach 150–170 million worldwide and estimates of incidence, performed in the United States by the Center for disease control and prevention (CDC), reported nearly 30,000 new HCV infections in 2013 [1, 2].

Although HCV infection is characterized by a global diffusion, its prevalence greatly differs according to geographic area [3, 4]. Central Asia, Eastern Europe, the Midwest of North Africa region, and Central and Western Sub-Saharan Africa present high HCV prevalence rates, with figures ranging between 3.1% and 5.4%; regions with intermediate prevalence rates are Southern Sub-Saharan Africa, Central Europe, Australia, and Latin America, with values between 1% and 1.4%; low prevalence is found in Oceania (0.1%), Caribbean (0.8%), and Western Europe (0.9%) [3].

After acute infection, 75% of infected subjects become chronically infected and approximately 20% of this population develops liver cirrhosis during the two decades after infection if left untreated [5, 6]. However, since in most cases acute infection was asymptomatic, most HCV infections are clinically silent until the disease reaches a late stage: HCV was estimated to cause 25% of all cases of liver cirrhosis and cancer worldwide and to account for more than 500,000 deaths per year [7].

In recent years, substantial advances have been made to understand HCV biology and to develop a new generation of effective direct-acting antiviral agents (DAAs) able to

cure HCV. However, several challenges hamper an effective control of HCV spread worldwide. In fact, the emergence of drug resistance and the suboptimal activity of these therapies against diverse HCV genotypes have been observed and have been associated with treatment failure. Moreover, the high costs of these drugs and the high prevalence of HCV-infected individuals, especially in low-income countries, jeopardized the affordability for the healthcare system to treat all infected patients in developed countries and, even more, in developing countries [8, 9]. Finally, effective screening strategy is required to early identify and treat all HCV chronically infected patients thus limiting the infection transmission risk as well as the progression to cirrhosis or hepatocellular carcinoma and reducing the healthcare costs [10, 11]. For all these reasons, a preventive HCV vaccine remains a cornerstone in the road to significantly reduce the HCV spread globally.

This comprehensive review summarized the state of the art about three major unresolved issues in the fight against HCV: which are the perspectives for the universal screening of HCV? Do we need DAAs resistance testing in the future? How close is an effective preventive HCV vaccine?

2. Which Are the Perspectives for the Universal Screening of HCV?

The rate of underdetection of HCV infection is still relevant because of clinical, educational, technical, organizational, and economic issues. In fact, recent estimates suggest that most of people with HCV remain undiagnosed or unaware of their HCV infection [12, 13].

Another criticism is represented by the difficulty in early diagnosing HCV infection. Indeed, few people are diagnosed during the acute phase because it is usually asymptomatic [14]. Furthermore, the 55–85% of persons who do not spontaneously clear the virus within 6 months develop chronic infection and remain asymptomatic for decades after infection, during which infection may be transmitted to other persons. Chronically infected patients usually become symptomatic when the HCV-induced liver damage is advanced and the therapy may be contraindicated [14]. The risk of late diagnosis is associated also with the limited access to HCV testing in many countries where HCV prevalence is high, such as African and Central-East Asian countries [15].

Therefore, it is crucial to implement the most sensitive and specific approaches to diagnose chronic HCV infection before the development of liver damage and to assure the linkage to care of infected patients [16].

2.1. Screening Tests for HCV Infection. A testing strategy for HCV infections characterized by high sensitivity and specificity should be established.

The WHO recommends offering the HCV serology test to individuals belonging to population with high HCV prevalence or who have a history of HCV risk exposure/behavior [14]. Given that HCV antibodies can be detected two months following the infection and are also detectable in patients who have cleared the virus, a positive result for antibodies against viral proteins (anti-HCV) should be followed by a nucleic acid testing using a reverse transcription polymerase chain reaction (RT-PCR) for the detection of HCV RNA and to confirm chronic HCV infection [14, 17]. Molecular testing is more technically demanding than the serology but it guarantees positive results already after few weeks following the exposure.

Among serologic screening test, the enzyme immunoassays (EIAs) are generally used [18]. EIAs are characterized by high sensitivity and specificity, fast processing, high reliability, relatively low costs, and the possibility of automation, useful for large volume testing [19–21]. In particular, the third generation test of EIAs is available [22] and has an estimated sensitivity and specificity of 98.9% and 100%, respectively, in patients with chronic liver disease [23]. However, the serological window period of this assay is generally more than 40 days [24] and it should not be used in infants younger than 18 months because of the possible reaction with maternal antibody [25].

Recently, the possibility of a single serologic assay for the detection of HCV active infection has become available. This test is based on the identification of the HCV core antigen (HCVAg) that is detectable in acute infection almost at the same time as HCV RNA [26] and persists during all phases of infection [27], indicating its potential use as a less expensive technique than molecular assays.

In consideration of improving the testing access, some countries such as US [28] and France [29] validated the use of Point of Care (POC) not only for HIV but also for HCV infection screening. Although rapid tests have lower sensitivity than standard serological tests and require a specific organization to allow traceability of results, they are characterized by relevant advantages such as the minimal equipment required, the ease of obtaining the samples and of performing the test, and the short time to obtain the results [30, 31]. However a recent meta-analysis that compared seven POC demonstrated that OraQuick had the highest test sensitivity and specificity and showed better performance than a third generation enzyme immunoassay in seroconversion panels [32]. The availability of POC rapid tests represents an opportunity to broaden the screening strategies to people outside the healthcare structures [11], thus helping the achievement of individuals at highest risk, such as people who inject drugs, the homeless, and the incarcerated.

2.2. Identifying Patients with HCV. The variability of the risk factors and history of behaviors linked to HCV infection, depending on the geographical setting and population studied, make the identification of the target population for screening policies challenging worldwide.

In many high-income countries HCV screening recommendations are targeted to subjects with a history of HCV risk exposure or behavior and certain medical conditions and who belong to a population of known high HCV prevalence [33, 34]. In particular, at risk populations include those who have received medical procedures such as hemodialysis or dental interventions in healthcare facilities with inadequate infection control practices, persons who have received blood transfusions, an organ transplant, or tissue graft prior to the time when HCV serological testing of blood

donors was introduced or in countries where it is not routinely performed, subjects who inject drugs or who have used intranasal drugs, persons who have received tattoos, body piercing, or scarification procedures in settings where infection control practices are substandard, children born to mothers infected with HCV, healthcare workers who sustained a needle stick or mucosal splash exposure from a patient with HCV, and patients with HIV infection who have unprotected sex with men, prisoners, and previously incarcerated persons (Class I, Level B recommendation) [10, 16, 35].

In 2012, the Center of Disease Control and Prevention (CDC) and the US Preventive Services Task Force (USPSTF) extended the HCV screening recommendation to all persons born from 1945 through 1965, without prior ascertainment of HCV risk factors (Class I, Level B recommendation) [35–37]. The rationale of this recommendation is based on the evidence that the risk-based strategy alone failed to identify more than 50% of HCV infections because of healthcare provider limitations in ascertaining risk factor information [38, 39] and due to patients' underreporting of their own risk behaviors (e.g., injecting drug use) or exposure (e.g., iatrogenic infection). Furthermore, persons in the 1945 to 1965 birth cohort accounted for about 75% of all HCV infections, with a five times higher prevalence (3.25%) than other persons [10, 37]. A recent retrospective review demonstrated that 68% of persons with HCV infection would have been screened through a birth cohort approach testing, while just 27% would have been identified with the risk-based strategy [40]. The cost-effectiveness of one-time birth cohort testing is comparable to that of current risk-based screening strategies [36]. Nevertheless, the implementation birth cohort screening is challenging [10]. The inclusion of queries and reminder flags among the electronic medical record (EMR) could sensitize physicians [41]. However, various types of EMR exist in USA and many reminders requiring time to be addressed are triggered during each visit [10]. Furthermore, the identification of the best setting to implement the screening of birth cohort represents a further issue. Available evidences suggest that the hospital setting allows reaching better results than the outpatients' visits such as routine colon cancer screening colonoscopy [42–45].

In Japan, where the overall prevalence of HCV infection is comparable with the USA, but different transmission patterns determined different age-specific prevalence, the national screening for HCV in both the high-risk group and the general population from ages 40 to 70 started at 2002 and has demonstrated to be cost-effective in containing the epidemic [46, 47].

In Europe, accurate estimates of HCV incidence and prevalence are not available; however prevalence estimates vary from 0.4% (Austria, Cyprus, Germany, Denmark, France, and United Kingdom) to 1.5% (Israel and Italy) in Western Europe and from 0.7% (Czech Republic) to 4.5% (Moldova) in Central and Eastern Europe [46]. HCV screening programs are implemented for organ and blood donors as well as for patients undergoing hemodialysis, but not for risk groups such as injecting drug users, who currently represent the leading cause of transmission across Europe [48, 49]. Furthermore, the rising issue of fluid immigration patterns from countries with high prevalence such as Egypt has not been addressed yet [50].

Developing nations, where the population-based prevalence of anti-HCV may reach as high as 11% (Mongolia) to 15% (Egypt), are less likely to be able to afford screening [46]. Many of these countries are afflicted by political and social unrest that may influence risk factors of HCV spread, such as trauma, use of intravenous drugs, poor water and electric supply, overcrowding, and lack of financial resources and infrastructure [46].

Considering the existing barriers that limit the implementation of screening strategies such as low provider knowledge about HCV and its related risk factors [13, 51–54], the WHO Guidelines Development Group conducted a systematic review to find the evidences about the most effective strategies to promote HCV testing [16]. Sensitizing the practitioners about the importance of early HCV diagnosis through in-service training sessions or mailed information, provision of additional clinic staff, routine offer of testing to all patients, or placing reminders in medical records resulted more effectively in increasing uptake of testing, detecting HCV antibody-positive cases, and the number of attendances and referral to specialist care than media-/information-based targeted approaches (e.g., invitations to information sessions for care providers, leaflets, and posters).

2.3. Recommendations for Frequency of HCV Screening Testing. CDC and the US Preventive Services Task Force (USPSTF) recommend a one-time HCV test in asymptomatic persons belonging to the 1945 to 1965 birth cohort and subjects with exposures, behaviors, and conditions that increase risk for HCV infection.

As regards the correct periodicity of testing persons at risk for ongoing exposure to HCV, evidence is lacking. Thus, physicians should determine the frequency of testing on the basis of the risk of reinfection. Owing to the high incidence of HCV infection among HIV-infected men who have unprotected sex with men and subjects who inject drugs [55–60], the HCV Guidance by the Infectious Diseases Society of America (IDSA) and the American Association for the study of liver diseases (AASLD) recommended that at least annual HCV testing is in these subgroups [35].

2.4. Linkage to Care. In order to enter the patients in the cascade of care and thus provide them with the so-called "continuum of care," the detection of HCV-infected patients should be followed by linkage to care via a physician referral with expertise in evaluation and treatment HCV infection [10, 61]. However, available evidences show that many gaps between the HCV diagnosis and care cascade still exist [62, 63].

These gaps may be both patient- and practitioner-related. The most common patient-related barriers to treatment initiation include contraindications because of the presence comorbidities, lack of acceptance, and access of treatment, due to often asymptomatic course of the infection, long treatment duration and possible adverse reactions of the treatment, high cost, and distance to specialist [64–66]. In

Table 1: Direct-acting antivirals (DDAs) approved for HCV treatment or investigated in clinical trials (updated in September 2016).

Class	Generation	Approved substances (developing company)	Substances currently tested in clinical trials (developing company) [phase of development]
NS3/4A protease inhibitors	First generation	Telaprevir (Janssen, Mitsubishi) Boceprevir (Merck) Simeprevir (Janssen) Paritaprevir (AbbVie) Asunaprevir (Bristol-Myers Squibb) Vaniprevir (Merck)	
	Second generation	Grazoprevir (Merck)	ABT-493 (AbbVie) [Phase 3] GS-9857 (Gilead Sciences) [Phase 3]
NS5A inhibitors	First generation	Daclatasvir (Bristol-Myers Squibb) Ledipasvir (Gilead Sciences) Ombitasvir (AbbVie) Elbasvir (Merck) Velpatasvir (Gilead Sciences)	Odalasvir (Janssen) [Phase 2] Ravidasvir (Presidio) [Phase 2/3]
	Second generation		ABT-530 (AbbVie) [Phase 3] MK-8408 (Merck) [Phase 2]
Nucleotide analogue inhibitors of NS5B RNA-dependent RNA polymerase	First generation	Sofosbuvir (Gilead Sciences)	MK-3682 (Merck) [Phase 2] AL-335 (Janssen) [Phase 2]
Nonnucleoside inhibitors of NS5B RNA-dependent RNA polymerase	Palm-1 inhibitors	Dasabuvir (AbbVie)	

particular, the frequency and the severity of side effects related to pegylated interferon and ribavirin (PEG-INF/RBV) combination represent primary factors affecting both the initiation of therapy and its continuation. The tolerability of HCV therapy remained low also with the addition of first generation DAAs (telaprevir and boceprevir) to the above-mentioned therapeutic regimen [66]. The availability of new generation DAAs has improved the tolerability of the antiviral therapy [67].

Moreover, the linkage to care is critical among patients coinfected with HIV [68, 69], those with underlying psychiatric and substance use disorders, and those with social instabilities such as homelessness and incarceration [70].

General practitioner-related barriers include lack of expertise in HCV treatment, lack of specialty referral resources, resistance to treating persons currently using illicit drugs or alcohol, and concern about cost of HCV treatment [71–73].

Evidence-based interventions to address linkage to care have been summarized by Meyer JP and colleagues [61] and by the HCV Guidance by the Infectious Diseases Society of America (IDSA) and the American Association for the study of liver diseases (AASLD) recommended that at least annual HCV testing is in these subgroups [35].

These interventions include the integration of HCV screening, evaluation, and treatment center with other medical or social services, such as correctional facilities and programs providing needle exchange, substance abuse treatment, and methadone maintenance [74–76]. Multidisciplinary case management and social support have been demonstrated to facilitate the efficacious treatment of HCV-infected patients with psychiatric illness or substance use [77].

In order to address lack of access to specialists, models involving close collaboration between primary care practitioners and subspecialists also through telemedicine and multidisciplinary networks of specialists have been implemented [65, 78, 79]. Furthermore, the decreased duration and better safety profile of current HCV therapy may increase the number of mid-level practitioners and primary care physicians able to appropriately manage and treat HCV infection [35].

Additional strategies for improving linkage to and retention in care could be the use of patient navigators or care coordinators [80, 81]; however, the efficacy and effectiveness assessment of these interventions is still ongoing.

The extension of best and new strategies for linkage to HCV care is essential to optimize the impact of HCV treatment.

3. Do We Need DAAs Resistance Testing in the Future?

Treatment of chronic hepatitis C has rapidly evolved from PEG-INF/RBV to more potent highly effective DAAs combination therapies. In particular, DAAs act on key stages of the HCV lifecycle and are classified on the basis of their molecular target and mechanism of action [82]. Four categories of DAAs are currently available: NS3/4A protease inhibitors, NS5A inhibitors, nucleotide analogue inhibitors of NS5B RNA-dependent RNA polymerase (RdRp), and nonnucleoside inhibitors of RdRp. Table 1 summarizes DAAs approved for HCV treatment. DAAs combination with other

Table 2: Recommended regimen for the treatment of HCV with direct-acting antivirals-based combination therapies.

Genotype	Presence of cirrhosis	Recommended regimen	Alternative regimen	Strength of recommendation	Quality of evidence
1a	Without cirrhosis	DCV + SOF (12 weeks) LDV + SOF (12 weeks)[a]	SMV + SOF (12 weeks)[c] OBV + PTV/r + DSV + R (12 weeks)	Strong	Moderate
	With cirrhosis	DCV + SOF (24 weeks) DCV + SOF + R (12 weeks) LDV + SOF (24 weeks) LDV + SOF + R (12 weeks)[b]	SMV + SOF (24 weeks)[c] SMV + SOF + R (12 weeks)[c] OBV + PTV/r + DSV + R (24 weeks)		
1b	Without cirrhosis	DCV + SOF (12 weeks) LDV + SOF (12 weeks)[a]	SMV + SOF (12 weeks) OBV + PTV/r + DSV (12 weeks)	Strong	Moderate
	With cirrhosis	DCV + SOF (12 weeks) LDV + SOF (12 weeks)	SMV + SOF (24 weeks) SMV + SOF + R (12 weeks) OBV + PTV/r + DSV + R (12 weeks)		
2	Without cirrhosis	SOF + R (12 weeks)	DCV + SOF (12 weeks)	Strong	Low
	With cirrhosis	SOF + R (16 weeks)	DCV + SOF (12 weeks)		
3	Without cirrhosis	DCV + SOF (12 weeks) SOF + R (24 weeks)		Strong	Low
	With cirrhosis	DCV + SOF + R (24 weeks)	SOF + PegIFN + R (12 weeks)		
4	Without cirrhosis	DCV + SOF (12 weeks) LDV + SOF (12 weeks)	SMV + SOF (12 weeks) OBV + PTV/r + R (12 weeks)	Strong	Moderate
	With cirrhosis	DCV + SOF (24 weeks) DCV + SOF + R (12 weeks) LDV + SOF (24 weeks) LDV + SOF + R (12 weeks)[b]	SMV + SOF (24 weeks) SMV + SOF + R (12 weeks)[c] OBV + PTV/r + R (24 weeks)		
5	Without cirrhosis	LDV + SOF (12 weeks)	SOF + PegIFN + R (12 weeks)	Conditional	Very Low
	With cirrhosis	LDV + SOF (24 weeks) LDV + SOF + R (12 weeks)[b]	SOF + PegIFN + R (12 weeks)		
6	Without cirrhosis	LDV + SOF (12 weeks)	SOF + PegIFN + R (12 weeks)	Conditional	Very Low
	With cirrhosis	LDV + SOF (24 weeks) LDV + SOF + R (12 weeks)[b]	SOF + PegIFN + R (12 weeks)		

DCV: daclatasvir; LDV: ledipasvir; SMV: simeprevir; SOF: sofosbuvir; OBV: ombitasvir; PTV: paritaprevir; DSV: dasabuvir; R: ribavirin; r: ritonavir; PegIFN: pegylated interferon.

[a]Treatment may be shortened to 8 weeks in treatment-naïve persons without cirrhosis if their baseline HCV RNA level is below 6 million (6.8 log) IU/mL. The duration of treatment should be shortened with caution.

[b]If platelet count $<75 \times 103/\mu$L, then 24 weeks' treatment with ribavirin should be given.

[c]If positive for the Q80K variant, a simeprevir/sofosbuvir regimen should not be chosen.

DAAs and/or ribavirin has been widely investigated in clinical trials and current recommendation by the World Health Organization (WHO) for the treatment of HCV with DAAs-based combination therapies is outlined in Table 2 [16]. During 2016, two novel DAAs combinations, grazoprevir/elbasvir and sofosbuvir/velpatasvir, have been licensed in US and Europe, and others are currently under evaluation in phases II and III clinical trials and may be available in the coming months [83].

The efficacy of treatment containing DAAs is very high in terms of sustained virological response (SVR); nonetheless 10–15% of therapeutic failure is observed in clinical practice, mainly associated with the selection of DAAs-resistant viral variants, resulting from mutations produced by amino acid

substitutions in the target virus protein that reduce viral sensitivity to DAAs [84]. Resistance-associated variants (RAVs) may be present, despite being usually at low levels even before the beginning of DAAs treatment due to the great genetic variability of HCV [84]. Moreover, genotype 3 virus consistently demonstrates lower SVR rates to DAAs, despite higher SVR rates in other forms of the virus [85].

Since DAAs are widely used for treatment of HCV, the role of resistance-associated variants (RAVs) is becoming clearer.

Natural polymorphisms, before treatment, associated with resistance to NS3/4A, NS5A, and NS5B inhibitors have a considerable prevalence in DAAs naïve patients that is variable and depends on HCV genotype and subtype [86]. These variants may be selected rapidly during treatment with DAAs with the possible consequences of a viral breakthrough and treatment failure [86–88]. For example, the preexisting NS3/4A Q80K, mainly found in patients with HCV genotype 1a (5%–48%), reduces SVR rates in genotype 1a infected patients treated with the protease inhibitor simeprevir in combination with PEG-INF/RBV in comparison to those without Q80K [89, 90]. In phase III clinical trials of simeprevir and PEG-INF/RBV HCV genotype 1 and genotype 4 infected treatment-naïve patients and prior relapsers achieved SVR at week 12 rates of approximately 80% [91–95]. Response rates to therapy were lower in HCV genotype 1 patients with Q80K compared with HCV genotype 1 patients without this polymorphism [7, 9, 11]. The Q80K polymorphism is frequently observed in HCV genotype 1a [96]; meanwhile the occurrence is close to zero in HCV genotype 1b with the only exception of France where 11% of genotype 1b has Q80K [97, 98]. On the basis of these data, the European Medical Agency (EMA) and the Food and Drug Administration (FDA) strongly recommend testing for the presence of Q80K in HCV genotype 1a patients who are candidates to therapy with simeprevir and PEG-INF/RBV and discourage the use of this drug when Q80K is detected.

In the IFN-free regimen the presence of Q80K was associated with treatment failure only in a small sample size of patients with genotype 1a and cirrhosis: a phase 3 study (OPTIMIST-2) evaluated the efficacy and safety of 12 weeks of simeprevir plus sofosbuvir in HCV genotype 1 infected treatment-naïve or treatment-experienced patients with cirrhosis. Of the 72 patients with chronic HCV genotype 1a, SVR rate was lower for the 34 with Q80K at baseline than for the 38 without Q80K (74% versus 92%) [99]. For this reason, screening for the presence of the Q80K polymorphism should be considered before initiating simeprevir in combination with sofosbuvir in cirrhotic patients infected with HCV genotype 1a.

Reduced sensitivity to the first generation protease inhibitors (PIs) (boceprevir and telaprevir) and to the more recent PIs was associated with other NS3/4A mutations, such as those involving the amino acid positions V36, T54, R155, A156, and D168. The list of the major NS3/4A RAVs is reported in Table 3.

The RAVs conferring resistance to NS5A inhibitors more frequently occurred as natural variants in HCV G1 infected patients naïve to DAAs [100] and seemed to be more problematic in the setting of retreatment. Y93H is the most frequent baseline NS5A RAV in G1b, followed by L31M/V, while NS5A RAVs are less frequent in G1a [86, 87, 101–103]. L31M confers low-medium level resistance to daclatasvir and ledipasvir, while Y93H/N confers medium-high level resistance to all three approved NS5A inhibitors [100, 104] in G1a but only for ledipasvir in G1b.

Recently, the FDA recommended testing for the presence of virus with NS5A resistance-associated polymorphisms (substitutions at amino acid positions 28, 30, 31, or 93) in genotype 1a, regardless previous treatment history and cirrhosis status, before the combination therapy with grazoprevir and elbasvir to determine dosage regimen and duration (for 12 or 16 weeks and if ribavirin should be added) [101].

It is noteworthy that the persistence of NS5A RAVs has been observed in about 85% of patients with treatment failure over 1-2 years after the start of therapy [100, 105–107]. Therefore, testing polymorphism could be useful prior to retreatment decision because persistent NS5A RAVs could impact the second line therapy success [90]. The list of the major NS5A RAVs is reported in Table 4.

Regarding the NS5B RAVs, no cross resistance is observed across currently approved nucleotide and nonnucleoside polymerase inhibitors. Nucleos(t)ide inhibitors demonstrate activity against different HCV genotypes and have a high resistance barrier [97]. The principal mutation that confers decreased susceptibility to sofosbuvir is S282T and more recently the variants L159F (with/without L320F and C316N) and V321A were detected in patients with failed treatment [108]. M414T and S556G variants were observed in G1a and S556G in G1b patients who did not achieve an SVR after a nonnucleoside analog inhibitor dasabuvir-based regimen [104]. The most relevant NS5B RAVs are reported in Table 5.

Resistance testing at baseline and following treatment failure is not yet indicated, as more data are needed to demonstrate its role in clinical practice, but the unresolved issue is if it may be useful to individualize the best treatment option for each patient in the future. With the exception of Q80K and NS5A RAVs detection in G1a patients before starting simeprevir plus PEG-INF/RBV and grazoprevir plus elbasvir, respectively, whether resistance mutation testing should be done in treatment-naïve patients remains controversial.

Some authors highlight the importance of resistance testing in patients who fail multiple DAAs for deciding retreatment and selecting salvage therapy [90, 108, 109]. More recently, Sarrazin encourages also the baseline testing in patients treated with a combination of a first generation NS3/4A protease and NS5A inhibitor with a low barrier of resistance. Instead, in case of treatment with high antiviral activities and high genetic barrier to resistance, the evaluation of additional predictors of response is to be assessed. Moreover, baseline resistance testing may be used to choose the best DAAs regimen in patients with shortened treatment duration or with liver cirrhosis. Sarrazin sustained that in the future it is to evaluate if baseline testing could be cost-effective

Table 3: Natural prevalence of NS3/4A inhibitor resistance-associated variants (RAVs) detected by population sequencing and mean fold change in resistance associated with RAVs.

Position	Variant	Resistant to	Natural prevalence in HCV genotype							Mean fold change in resistance compared to wild-type replicon						References
			1a	1b	2	3	4	5	6	Boceprevir	Telaprevir	Simeprevir	Asunaprevir	Paritaprevir	Vaniprevir	
V36	A/C/G	BOC/TVR/PTV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 142, 143]
V36	M	BOC/TVR	0.2–0.6%	0.1%	n.d.	n.d.	n.d.	n.d.	n.d.	3 (GT 1a)			2 (GT 1a)	2 (GT 1a)		[87, 142–147]
F43	I/L/S/V	SMV/ASV/PTV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[86, 87, 148, 149]
T54	A	BOC/TVR	0.1–1.9%	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.	2 (GT 1a) 3 (GT 1b)						[87, 144, 147, 150–153]
T54	S	BOC/TVR	0.4–3.1%	1.2–2.0%	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 142, 143, 149]
V55	A	BOC/TVR	2.8%	0.4%	n.d.	n.d.	n.d.	n.d.	n.d.	3 (GT 1b)			1 (GT 1b)			[87, 145–147, 153]
Y56	H	PTV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[86, 87, 144]
Q80	K	SMV/ASV/PTV	4.8–75.0%	0.5–1.2%	n.d.	n.d.	n.d.	n.d.	n.d.			11 (GT 1a) 8 (GT 1b)	3 (GT 1a) 1 (GT 1b)			[144, 146, 147, 154]
Q80	R	SMV/ASV	0.8%	0.6–0.7%	n.d.	n.d.	n.d.	n.d.	n.d.			6	1			[86, 87, 89, 144]
S122	R	SMV/ASV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			21				[86, 144, 150]
R155	K/I/G/L/M/T/Q/S	BOC/TVR/SMV/ASV/PTV	0.2–0.9%	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 142]
A156	F/N/S/T/V	BOC/TVR/SMV/ASV/PTV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[86, 87, 89, 142, 148, 149]
V158	I	BOC	n.o.	0.1%	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 143]
D168	E	SMV/ASV/PTV	0.2–0.3%	0.1–1.4%	n.d.	n.d.	n.d.	n.d.	n.d.			38 (GT 1b)	58 (GT 1a) 78 (GT 1b)	14 (GT 1a)		[86, 87, 89, 100, 142, 148, 149, 153, 155]
D168	G/H/V/TY	SMV/ASV/PTV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.							[86, 87, 148]
V170	A	BOC/TVR	n.a.	0.1%	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 142]
M175	L	BOC	n.a.	0.8–1.1%	n.d.	n.d.	n.d.	n.d.	n.d.							[87, 143]

BOC: boceprevir; TVR: telaprevir; SMV: simeprevir; ASV: asunaprevir; PTV: paritaprevir.
n.a.: not applicable because of different natural amino acid sequence in the respective HCV geno-/subtype (V170 and M175 are the dominant amino acids in GT1b).
n.o.: not observed.
n.d.: no data available.
GT: genotype.

Table 4: Natural prevalence of NS5A inhibitor resistance-associated variants (RAVs) detected by population sequencing and mean fold change in resistance associated with RAVs.

Position	Variant	Resistant to	Natural prevalence in HCV genotype							Mean fold change in resistance compared to wild-type replicon			References
			1a	1b	2	3	4	5	6	Daclatasvir	Ledipasvir	Ombitasvir	
K24	G/N	LDV	n.o.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.				[156]
K24	R	LDV	<1–1.5%	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.				[156, 157]
M28	A	DCV/LDV	0.5%	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.				[156]
M28	G	LDV	n.o.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.				[86, 156]
M28	T	DCV/LDV/OMV	0.4–1.8%	n.a.	n.d.	n.o.	82.0% (M28L)	n.d.	n.d.	205	61	8,965	[87, 100, 156, 158–160]
M28	V	OMV	3.5%	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.			58 (GT 1a)	[86, 87, 100, 160–163]
F28	S	DCV	n.a.	n.a.	n.o.	n.d.	n.d.	n.d.	n.d.				[164]
L28	F/T	DCV/OMV	n.a.	n.o.	8.0%	n.d.	n.d.	n.d.	n.d.				[86, 87, 164]
Q30	H/R/E/L/T	DCV/LDV/OMV	0.3–1.3%	n.a.	n.d.	90.4–100%	50.0–100%	n.d.	n.d.				[86, 87, 156, 158, 165]
R30	H	DCV	n.a.	0.4%	n.d.	n.d.	n.o.	n.d.	n.d.				[87, 166]
R30	S	DCV	n.a.	n.a.	n.d.	n.d.	10%	n.d.	n.d.				[166]
R30	G/H	DCV	n.a.	n.a.	n.d.	n.d.	n.o.	n.d.	n.d.				[166]
A30	K	DCV	n.a.	n.a.	n.d.	2.3–6.3%	n.d.	n.d.	n.d.				[167]
L31	M	DCV/LDV	0.9–1.8%	2.1–6.3%	74.0–85.0%	1%	92.5–100%	n.d.	n.d.	105 (GT 1a) 3 (GT 1b)	554 (GT 1a)		[86, 87, 100, 145, 146, 151, 158, 160, 163, 164, 166–169]
L31	I/F/V	DCV/LDV/OMV	n.o.	0.7–1%	n.d.	n.o.	n.d.	n.d.	n.d.	15 (L31V)			[86, 87, 100, 156, 158, 159, 164, 166–168, 170]
P32	L	DCV/LDV	n.o.	<0.5%	n.d.	n.o.	n.o.	n.d.	n.d.				[86, 87, 100, 158]
S38	F	LDV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.				[86, 100]
H58	D	DCV/LDV/OMV	<1%	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.		1,127	243	[86, 87, 100, 144, 157]
P58	D	LDV	n.a.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.				[86, 100]
A92	K	LDV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.				[86, 100]
A92	T	LDV	n.o.	2.8%	n.d.	n.d.	n.d.	n.d.	n.d.				[86, 100]
C92	R	DCV	n.a.	n.a.	n.o.	n.d.	n.d.	n.d.	n.d.				[164]
Y93	C/F/N	DCV/LDV/OMV	n.o.–0.6%	n.o.–0.7%	n.d.	n.o.	n.d.	n.d.	n.d.				[86, 87, 100, 158]
Y93	H	DCV/LDV/OMV	<1.5%	3.8%–14.1%	n.o.	1.3–8.3%	5–13%	n.d.	n.d.	12		77	[86, 87, 100, 156, 158, 159, 164–167, 169–175]
Y93	S	LDV	<0.5%	<0.5%	n.d.	n.d.	n.d.	n.d.	n.d.				[156]

DCV: daclatasvir; LDV: ledipasvir; OBV: ombitasvir.
n.a.: not applicable because of different natural amino acid sequence in the respective HCV geno-/subtype (K24, M28, Q30, and H58 are the dominant amino acids in GT1a; F28 is the dominant amino acid in subtype 2a and L28 in subtype 2b; A30 is the dominant amino acid in GT3).
n.o.: not observed.
n.d.: no data available.
GT: genotype.

Table 5: Natural prevalence of NS5B nucleotide and nonnucleoside inhibitor resistance-associated variants (RAVs) detected by population sequencing and mean fold change in resistance associated with RAVs.

Position	Variant	Resistant to	Natural prevalence in HCV genotype							Mean fold change in resistance compared to wild-type replicon		References
			1a	1b	2	3	4	5	6	Sofosbuvir	Dasabuvir	
S28	2T	SOF	n.o.	n.o.	n.o.	n.o.	n.d.	n.d.	n.d.	16		[87, 142, 176–180]
M289	I/L	SOF	n.o.	1.8%	3.5%	n.d.	n.d.	n.d.	n.d.			[176]
C316	Y	DSV	0.2–1.2%	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.		1472 (GT 1a) 1569 (GT 1b)	[86, 87, 100, 165, 175]
C316	N	DSV/SOF	n.o.	10.9–35.6%	n.d.	n.d.	7.9%	n.d.	n.d.			[86, 87, 165, 181]
C316	H	DSV/SOF	n.o.	1.9–2.1%	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 165]
L320	F	SOF	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[176]
S368	T	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86]
N411	S	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 142]
M414	T	DSV	0.5%	0.4%	n.d.	n.d.	n.d.	n.d.	n.d.		32 (GT 1b)	[87, 100, 175, 182]
M414	I	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[87, 142]
E446	K/Q	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[165]
Y448	C	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 87, 142]
Y448	H	DSV	0.2%	1.3%	n.d.	n.d.	n.d.	n.d.	n.d.		975 (GT 1a)	[87, 144]
A553	I/T/V	DSV	6%	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[165]
G554	S/D	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 87, 142]
S556	G	DSV	0.6–3.1%	7.0–16%	100%	100%	97%	n.d.	n.d.		30 (GT 1a) 11 (GT 1b)	[86, 163, 181, 182]
S556	N/R	DSV	0.6–1.2%	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 87, 182]
G558	R	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[165]
D559	G	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[86, 87, 142]
Y561	H	DSV	n.o.	n.o.	n.d.	n.d.	n.d.	n.d.	n.d.			[165]

SOF: sofosbuvir; DSV: dasabuvir.
n.o.: not observed.
n.d.: no data available.
GT: genotype.

in order to prevent nonresponse to very expensive treatment, particularly in regions with economical restriction [100].

4. How Close Is an Effective Preventive HCV Vaccine?

The development of an effective HCV vaccine is hampered by several factors. First, HCV is characterized by an extraordinary genetic variability resulting from the lack of proofreading activity of the NS5B RNA-dependent polymerase [110]. This determines an impressive error rate per replication cycle that, in combination with the short viral half-life and the rapid turnover, leads to the generation of multiple distinct but closely related HCV variants, known as "quasispecies," in one infected subject [111, 112]. Mutated viruses have the ability to persist in infected people by escaping immune control of cytotoxic T lymphocytes (CTL) and antibodies against different regions of the viral envelope [113, 114].

In addition to evading antibody and CTL recognition by passively mutating its genome in response to immune pressure, HCV exploits several further strategies to escape adaptive immune response and achieve a high rate of chronic infection. In fact, HCV, through a yet-unknown mechanism, fails to properly initiate the CD4+ T cell response at the beginning of infection and determines a rapid immune exhaustion and depletion of CD8+ T cells [115–117]. Moreover, HCV is able to hide from humoral immunity and pass from cell to cell without being exposed to the circulating antibodies by (i) binding low density lipoproteins, thus limiting the production of neutralizing antibodies during acute infection, (ii) decreasing viral immunogenicity through the presence of three glycans at the CD81 binding site of E2 glycoprotein, (iii) infecting surrounding cells through cell-to-cell contact mediated by CD81 and Claudin-1, and (iv) inducing interfering antibodies by constant mutation [118].

The weaker and ineffective adaptive immune response to HCV mainly contributes to the infection progressing into a chronic state. On the other hand, these immune evasion mechanisms have relevant implications in the development of the HCV vaccine. In fact, an effective vaccine should be able to induce strong neutralizing antibodies as well as powerful cellular immune responses during the very first stages of the HCV infection, before the virus has the chance to activate its many immune escape mechanisms [119].

A further barrier that has challenged the research on HCV vaccine is the lack of convenient experimental model systems for the study of HCV pathogenesis and vaccine design. To date, the only suitable infectious animal model is the chimpanzee. However, some ethical issues, the high cost of acquiring and maintaining the chimpanzees, and their limited supply restricted the use of these animals and the statistical power of studies [8, 120]. Furthermore, immune response of chimpanzees to HCV greatly differs from humans. Therefore, immunological results obtained with this model should be prudently interpreted [121, 122].

Several strategies have been adopted to develop an effective preventive HCV vaccine. They ranged from the traditional strategy of producing recombinant envelope proteins combining them with adjuvant substances to complex manufacturing of viral vectors directing the expression of multiple viral antigens. All strategies have been targeted to enhance the T cell response and, in particular, to determine a long-lasting cellular immune responses involving helper CD4+ and CD8+ T cells rather than only improving humoral responses.

Despite two decades of research efforts, few HCV candidate vaccines have reached the clinical trials phase and the evidences on both the efficacy and safety of these vaccines in humans remain limited (Table 6).

The first prophylactic HCV vaccine tested in human beings was a C-terminally shortened recombinant E1 protein adjuvant with aluminum hydroxide, called T2S-918/InnoVac-C, demonstrating good tolerability and good antibody response against E1. Moreover, a robust specific cellular immune response towards E1 was stimulated in all vaccinees [123]. However, the studies on this candidate vaccine were stopped in 2007.

Further HCV prophylactic vaccines based on recombinant proteins combined with adjuvant substances have been developed and evaluated in clinical trials.

A recombinant E1/E2 heterodimer vaccine (derived from HCV 1a) adjuvant with MF59C was tested in phase I clinical trial involving 60 healthy subjects. Vaccine demonstrated inducing neutralizing antibodies and T cell responses to E1/E2 in all subjects. Although the vaccine was safe and well-tolerated, its usage was prevented by manufacturing difficulties [124].

Another approach, based on recombinant protein, consisted of combining recombinant HCV core protein with an adjuvant substance, called ISCOMATRIX™. In a phase I clinical trial, conducted on 30 healthy volunteers, all subjects except one demonstrated antibodies against HCV core protein, without indication of a dose response. However, T cell responses were detected in only two subjects who received the highest dose of vaccine. The candidate vaccine was generally well-tolerated [125].

Also the development strategies based on HCV peptides vaccine that were able to induce HCV specific T cell responses by presenting vaccine peptide to the T cell receptor via HLA molecules have obtained some encouraging findings.

In particular, a peptide vaccine, known as IC41 and consisting of five synthetic peptides derived from conserved regions of core, NS3 and NS4 proteins of HCV genotypes 1 and 2 with a poly-L-arginine adjuvant, was evaluated in 128 healthy volunteers in a phase I clinical trial where it resulted in being safe and well-tolerated [126]. On the other hand, IC41 elicited few interferon-producing cells and dose-dependent T cell immune responses, even though a correlation between higher responder rates with dose and number of vaccinations was demonstrated [127]. In a successive randomized clinical trial, 54 healthy subjects received either subcutaneous or intradermal IC41 vaccine weekly (16 injections) or every other week (8 injections). One group additionally received imiquimod, an activator of the toll-like receptor (TLR) 7. Results showed that IC41 induced significant immunological responses in all groups with responder rates of up to 100%, even though imiquimod was not able to increase immunogenicity but was associated with a lower immune

Table 6: Preventive hepatitis C virus vaccine tested in clinical trials.

Type of vaccine	Viral component	Adjuvant	Phase of clinical trial	Study population	Year	Reference
Recombinant protein	Recombinant E1 protein	Aluminum hydroxide	I	20 healthy subjects	2004	[123]
	Recombinant E1 and E2 proteins	MF59	I	60 healthy subjects	2010	[124]
	Recombinant core protein	ISCOMATRIX	I	60 healthy subjects	2009	[125]
Peptide	Five synthetic peptides derived from conserved regions of core, NS3, and NS4 proteins of HCV genotypes 1 and 2 (IC41)	Poly-L-arginine	I	128 healthy subjects	2006	[126]
	Five synthetic peptides derived from conserved regions of core, NS3, and NS4 proteins of HCV genotypes 1 and 2 (IC41)	Poly-L-arginine	I	54 healthy subjects	2010	[127]
Virally vectored	Human adenovirus rare serotype 6 (HADV6) and chimpanzee Ad 3 (ChAd3) expressing the HCV nonstructural proteins	—	I	30 healthy subjects	2012	[128]

response. Intradermal injections caused more pronounced reactions, especially erythema and edema, but immunization with IC41 resulted generally in being safe and well-tolerated [127].

More innovative strategies to develop an effective HCV preventive vaccine include the manufacturing of DNA vaccine and viral vectors expressing HCV genes. DNA vaccines showed the capability of inducing cytotoxic lymphocyte responses in animal models; however, the induced immunity is often brief, weak, and unlikely to be effective in infection prevention. Viral vectors able to express foreign antigens represent an effective tool to induce a broader CD4+ and CD8+ T cell responses, compared to peptide vaccines.

Two preventive vaccines based on human adenovirus rare serotype 6 (HADV6) and chimpanzee Ad 3 (ChAd3) expressing the HCV nonstructural proteins were tested in a phase I clinical trial conducted on 36 healthy volunteers. The study demonstrated that both vaccines induced by specific T cell responses against multiple HCV proteins and T cell were capable of recognizing heterologous HCV strains (genotypes 1a and 3a). These data suggested that an adenoviral vector strategy may induce sustained T cell responses of a magnitude and quality associated with protective immunity, thus encouraging studies of novel prophylactic vaccines for HCV [128].

On the basis of some promising data obtained in phase I clinical trials, different types of prophylactic HCV vaccine are approaching phase II and phase III clinical trials [129]. In the next future, research efforts should be focused on improving the selection of the viral component and the proper administration regimens as well as on the safety and the tolerability of candidate preventive HCV vaccines.

5. Further Therapeutic Options

Research efforts for the development of alternative therapeutic options have been made contextually to the development of DAAs and the research on preventive vaccines. To date, several approaches have been adopted in the development and production of HCV therapeutic vaccines including peptide vaccines, recombinant protein vaccines, DNA vaccines with different carriers, and virally vectored vaccines.

Some studies were conducted among chronic hepatitis C patients, obtaining promising results in terms of T cell proliferation and IFN-γ responses and SVR rates within patients treated with interferon [130, 131].

Clinical trials, conducted by administering candidate HCV therapeutic vaccines in HCV chronic infected patients, have demonstrated HCV specific immune responses and transiently reduction of viral RNA, but the vaccines were not able to completely clear HCV infection or consistently reduce viral titers [130, 132, 133].

Some authors discussed the possibility to improve the efficacy of these vaccines by prior treatment with DAAs to first suppress HCV viremia [9], but the promising results obtained with the new generation of DAAs and their combination in curing HCV patients may overcome the use of therapeutic vaccination as an effective strategy for HCV treatment.

A further therapeutic option to cure HCV is represented by neutralizing monoclonal antibodies (mAbs) directed against HCV envelope glycoproteins E1 and E2, which are involved in the HCV entry into host cells or against phospholipids expressed on infected host cells [134, 135]. With respect to mAbs directed against viral antigens, due to the low immunogenicity of E1 glycoprotein, the research mainly focused on mAbs targeted conserved regions of E2 glycoprotein. To date, two mAbs directed against this target have been investigated in phase I and phase II clinical trials in the prevention of HCV reinfection during and after liver transplantation.

Two studies involved anti-HCV/2 human mAb HCV-AB68 demonstrating only a modest and short-lasting reduction in viremia [136, 137]. More recently, MBL-HCV1 showed good tolerability, reduced viral load, and delayed median time to viral rebound compared to placebo treatment in a phase II clinical trial involving 11 patients infected with HCV genotype 1a [138].

As regards mAbs against host receptors, a phase II unpublished clinical trial involving bavituximab was conducted to study the safety profile and early virological response in HCV genotype 1 infected patients treated with this mAb in combination with ribavirin in comparison with patients treated with PEG-INF/RBV [135].

In recent years, novel mAbs with immunomodulatory effects have been explored. Among these, mAbs that target programmed death 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and CD3 receptors were studied in phase II clinical trials with encouraging results in terms of safety, tolerability, and viral response [139–141].

The most likely future for clinical use of mAbs is represented by regimen for HCV treatment consisting of mAb cocktails or combination of mAbs with other available drugs with the main goal of avoiding viral escape and the development of resistance.

6. Conclusions

Substantial advances have been made in HCV research and treatment in recent years. Although highly effective anti-HCV drugs are now available, these treatments remain poorly accessible because of their high costs; moreover, their efficacy is challenged by the high frequency of resistance-associated mutations. A further major obstacle for the control of the disease is represented by the lack of availability of an effective screening strategy to identify all people in need of treatment. Therefore, even though new drugs may contribute to impacting HCV spread worldwide, substantially modifying the natural history of the disease, eradication will be reached only through the development of an effective prophylactic vaccine. Future research directions should bridge this gap and progress in the comprehension of biological and immunological mechanisms of the disease and advances in molecular vaccinology, together with a strong financial, political, and societal support, will enable reaching this fundamental success in the coming years.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All the authors contributed equally to this paper.

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Review Article

The CXCL10/CXCR3 Axis and Cardiac Inflammation: Implications for Immunotherapy to Treat Infectious and Noninfectious Diseases of the Heart

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Accumulating evidence reveals involvement of T lymphocytes and adaptive immunity in the chronic inflammation associated with infectious and noninfectious diseases of the heart, including coronary artery disease, Kawasaki disease, myocarditis, dilated cardiomyopathies, Chagas, hypertensive left ventricular (LV) hypertrophy, and nonischemic heart failure. Chemokine CXCL10 is elevated in cardiovascular diseases, along with increased cardiac infiltration of proinflammatory Th1 and cytotoxic T cells. CXCL10 is a chemoattractant for these T cells and polarizing factor for the proinflammatory phenotype. Thus, targeting the CXCL10 receptor CXCR3 is a promising therapeutic approach to treating cardiac inflammation. Due to biased signaling CXCR3 also couples to anti-inflammatory signaling and immunosuppressive regulatory T cell formation when activated by CXCL11. Numbers and functionality of regulatory T cells are reduced in patients with cardiac inflammation, supporting the utility of biased agonists or biologicals to simultaneously block the pro-inflammatory and activate the anti-inflammatory actions of CXCR3. Other immunotherapy strategies to boost regulatory T cell actions include intravenous immunoglobulin (IVIG) therapy, adoptive transfer, immunoadsorption, and low-dose interleukin-2/interleukin-2 antibody complexes. Pharmacological approaches include sphingosine 1-phosphate receptor 1 agonists and vitamin D supplementation. A combined strategy of switching CXCR3 signaling from pro- to anti-inflammatory and improving Treg functionality is predicted to synergistically lessen adverse cardiac remodeling.

1. Introduction

The chemokine receptor CXCR3 is a Class A seven-transmembrane-domain or G protein-coupled receptor (GPCR) that is involved primarily in chemotaxis of certain immune cells, inhibition of angiogenesis, and Th1 cell polarization [1–3]. CXCR3 is expressed by various effector T lymphocytes, including $CD4^+$ T helper 1 (Th1) cells, $CD8^+$ cytotoxic T lymphocytes (CTL), and $CD4^+$ and $CD8^+$ memory T cells, as well as monocytes, M1 macrophages, natural killer (NK) cells, subsets of B-cells, mast cells, endothelial cells, and vascular smooth muscle cells [1–4]. CXCR3 couples to $G\alpha_i$ protein [5, 6] and although not extensively studied, it has been shown to activate a number of signaling pathways that are generally associated with GPCRs such as increases in intracellular calcium and activation of MAP kinases and PI3K/Akt signaling [4, 7, 8]. The principal agonists of CXCR3 are CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-TAC). The human equivalent of the murine form of CXCR3 is CXCR3A and unless noted otherwise CXCR3 is used in this review to include both murine and human isoforms. Two additional splice variants of CXCR3 are expressed in humans, CXCR3B and CXCR3-alt. CXCR3B, which couples to G_s, is the receptor isoform expressed in microvascular endothelial cells and is linked to inhibition of angiogenesis and induction of apoptosis [2, 3]. Besides CXCL9, CXCL10,

and CXCL11, CXCR3B and CXCR3 are activated by CXCL4 and CXCL4L1, chemokines that are released by platelets and have been implicated in atherogenesis and acute coronary syndrome [3, 9, 10]. CXCR3-alt is a truncated form of CXCR3 that is selectively activated by CXCL11 [2–4].

CXCR3 is associated with the pathophysiology of Th1-type diseases, including infections of various etiologies and autoimmune disorders [1, 3]. Although CXCR3 is activated by CXCL9, CXCL10, and CXCL11, the outcome is different with growing evidence that CXCL9 and CXCL10 are essentially proinflammatory, while CXCL11 has anti-inflammatory actions [11, 12]. Over the last decade, numerous studies have documented elevated circulating levels of CXCL10 in wide-ranging infectious and autoimmune diseases, autoimmune encephalomyelitis, Crohn's disease, tuberculosis, thyroid autoimmune diseases, and type 1 diabetes, as well as several cancers [13–18]. Recent evidence from us and others [1] has revealed the importance of the CXCL10/CXCR3 axis in cardiovascular diseases. As discussed elsewhere [11], CXCL9 and CXCL10 are two of only 8–10 chemokines that are sufficient to sustain an inflammatory response. In addition, the homing signature for memory T cells to the heart from mediastinal lymph nodes is c-Met$^+$CCR4$^+$CXCR3$^+$. While c-Met triggering supports cardiotropic T cell recirculation, CXCR3 and CCR4 engagement *via* tissue-released CXCL10 and CCL4, respectively, sustains recruitment in heart inflammation [19, 20]. In this review, we present an overview of the role of CXCL9 and CXCL10 in infectious and noninfectious diseases of the heart and its implications for immunotherapy.

2. CXCR3 Biased Signaling

Recently, Zohar et al. [21] showed that CXCL9 and CXCL10 drive effector Th1/Th17 cell polarization via STAT1, STAT4, and STAT5 activation, thereby promoting inflammation. In contrast, CXCL11, which exhibits relatively higher binding affinity for CXCR3, drives development of FOXP3 (forkhead box P3)-negative IL-10^{high} T regulatory 1 (Tr1) cells and IL-4^{hi} Th2 cells *via* STAT3 and STAT6 activation and was demonstrated to dampen inflammation [21]. The opposite actions of the CXCR3 agonists are likely the consequence of the biased signaling that is a fixture of GPCRs, which can activate both G protein-dependent and protein-independent signaling cascades, the latter occurring *via* β-arrestin 2 recruitment [1, 11, 12]. Biased allosteric agonists of CXCR3 that selectively activate β-arrestin or G protein-dependent signaling are in development and may have utility in immunotherapy [22].

3. Coronary Artery Disease (Ischemic Heart Disease)

Coronary artery disease (CAD), which progresses to coronary heart disease, is a leading cause of death in the USA and globally [23, 24]. CAD is caused by atherosclerosis within the arteries of the heart, a chronic inflammatory condition associated with waxy plaque buildup [25]. CXCR3 expressing monocytes/macrophages, Th1 cells, NK cells, and CTL cells play a critical role in atheromatous plaque progression and eventual disruption [1]. Ruptured or ulcerated plaques cause formation of a thrombus that precipitates an acute coronary syndrome, such as unstable angina or a heart attack.

Endothelial dysfunction, increased vascular permeability, increased expression of adhesion molecules on endothelial cells for leukocytes, and increased plasma levels of low density lipoprotein (LDL) are initiating factors in atherosclerosis [1]. LDL, which accumulates in the intima, undergoes oxidation by macrophages and endothelial cells, as well as by VSMC that migrate into the intima from the media and proliferate. In response to the oxidized LDL and plasma LDL, endothelial cells secrete proinflammatory cytokines and chemokines (MCP-1/CCL2, fractalkine, and CXCR3 ligands) that attract monocytes, which differentiate into dendritic cells or macrophages that accumulate oxidized LDL to become foam cells. T cells are recruited into the intima, and dendritic and NK cells help induce the CD4$^+$ Th1 phenotype, which is the most abundant T cell population in human atherosclerotic plaques [26]. CXCR3 is required for optimal Th1 generation [27]. Th1 cells, as do NK cells, produce IFN-γ, which contributes to Th1 polarization, activates proinflammatory M1 macrophages, and induces apoptosis. The atheromatous plaque that builds up in the artery wall is made up of an accumulation of lipids, fibrous connective tissue, macrophages, and cellular debris that arises from the cytolytic actions of oxidized LDL, NK cells, IFN-γ, and CTL cells on macrophages, foam cells, VSMC, and endothelial cells. A fibrous coat of extracellular matrix proteins produced by VSMC stabilizes the plaque, but proinflammatory M1 macrophages secrete metalloproteinases in response to IFN-γ that degrade the fibrous cap and enhance its vulnerability to rupture.

CXCL10 is reported to be expressed by endothelial cells, smooth muscle cells, and macrophages during the formation of atherosclerotic lesions in both preclinical and clinical studies [28, 29]. Suppression of CXCL10 bioactivity in Apo-E deficient mice resulted in a more stable plaque phenotype with less macrophage activation, along with more smooth muscle cells and collagen abundance [30]. The mechanistic role of CXCL10 in the pathogenesis of atherosclerotic plaque growth and destabilization is not yet resolved. Of note, CXCL10 concentrations increase in patients with a more vulnerable plaque phenotype [30]. Unstable plaques have increased levels of Th1, NK, and CTL cells and decreased levels of anti-inflammatory regulatory T (Treg) cells [31]. Recent studies show that the relative levels of Treg cells are reduced and their functionality is impaired in patients with CAD [32, 33]. Knockout of CXCL10 in the apolipoprotein E-deficient mouse model of atherosclerosis was associated with increased Treg cell numbers and activity, along with a reduction in lesion formation [34].

Circulating levels of CXCL10 are elevated in patients with coronary artery disease [35, 36]. Notably, CXCL10 was also reported to be produced by the endothelium of mouse coronary blood vessels infused with angiotensin II [37], human coronary artery endothelial cells treated with TNF-α [38], and rat cardiac microvascular endothelial cells subjected to hypoxia/ischemia [39]. Patients with acute myocardial infarction (AMI) showed significantly higher serum levels of CXCL10 than control subjects and patients with stable

angina pectoris [40]. Although serum CXCL10 levels were negatively correlated with infarct size, these results in terms of pathogenic implications and determining cause *versus* effect relationships have limitations. First, during AMI there is a massive systemic inflammatory insult in which CXCL10 levels are expected to be high. It would be interesting to test blood concentration of CXCL10 within the first 3 hours after angina onset during AMI when systemic activation is not yet started. Secondly, the pathogenic mechanisms of plaque rupture may involve factors acting locally without necessarily showing a high systemic blood concentration. It would be interesting to analyze CXCL10 in samples of blood obtained by thrombus-aspiration during coronary artery percutaneous intervention (PCI) in patients with unstable coronary artery disease. In patients with first-time ST-segment elevation AMI, high circulating levels of CCL4, CXCL16, CXCL8, and CXCL10 within the first week after PCI were found to be positively correlated with the degree of myocardial damage [41].

4. Kawasaki Disease

Kawasaki disease is an autoimmune disease that manifests as a systemic vasculitis with a predilection for coronary arteries [42]. The disease occurs in children under 5 years of age and a preexisting viral infection may have a role in its development. During the acute phase of Kawasaki disease the immune system is highly activated and includes both Th1 and Th2 subsets [43]. Recently, Ko et al. [44] reported that CXCL10 is a good biomarker/predictor of Kawasaki disease and, furthermore, that CXCR3 is activated in the T cells of patients with acute Kawasaki disease. In addition, several studies report that numbers and functionality of Treg cells are reduced in Kawasaki patients [45–48].

5. Myocarditis and Chagas Heart Disease

Myocarditis or inflammation of the myocardium is a heterogeneous group of disorders initiated by various pathogens, including worms, bacteria, protozoa, rickettsia, and most commonly viruses [49]. Myocarditis may lead to heart failure and sudden death. Autoimmunity after viral myocarditis is thought to cause dilated cardiomyopathy, which is characterized by ventricular dilation and contractile dysfunction [50, 51].

CXCL10 is elevated in the heart following viral and nonviral infection and has the characteristics of a biomarker in rodent models of myocarditis [52, 53]. Yue et al. [54] reported findings showing that CXCL10 contributes to the pathogenesis of viral myocarditis. In their study, myocarditis was induced with Coxsackievirus B3 (CVB3), the primary cause of viral myocarditis, in mice that overexpressed a CXCL10 mutant protein without functional activity in order to antagonize endogenous CXCL10. These mice exhibited ameliorated disease progression, including reduced cardiac thickening (due to inflammatory edema), inflammation, and cell death, as well as improved survival when compared to wild-type mice [54]. The authors concluded that CXCL10 plays a crucial role in recruitment of Th1 cells to the heart, leading to the increase in detrimental proinflammatory Th1 cytokines. These findings have implications for interferon treatment, which is beneficial for some forms of viral myocarditis [55]. Following CVB3 infection, the rise in IFN-γ stimulates CXCL10 expression in cardiac myocytes and other cardiac cells. Yuan et al. [56] reported that CXCL10 inhibits CVB3 replication at early stage of infection, consequently protecting cardiac myocytes from damage and improving heart function. This antiviral activity of CXCL10 entails the regulation of natural killer (NK) cell infiltration into the myocardium and associated IFN-γ expression. However, the transient antiviral effect of CXCL10 was shown to be insufficient for viral clearance and in preventing death during acute inflammation stages in their mouse model. Other chemokines or cytokines were proposed to play an important role in clearance of viruses. No simple explanation seems to explain the disparate findings of Yuan et al. [56] and Yue et al. [54] on whether myocardial CXCL10 is harmful or beneficial in the context of acute CVB3 myocarditis, although timing and dosage levels of CXCL10 and effective viral clearance *versus* Th1 recruitment are likely contributing factors.

Evidence indicates the major contribution of autoimmunity to the etiology of myocarditis and thus adoptive transfer of Tregs and/or stimulating their differentiation are promising therapeutic approaches [49, 50]. Another possible immunotherapy approach is humanized monoclonal antibodies targeting IL-17 derived from Th17 cells [49]. Both Th1 and Th17 cells drive myocarditis, with Th17 cells playing an important part in the development of dilated cardiomyopathy [57].

Chagas disease is a tropical disease that results from infection with the protozoan parasite *Trypanosoma cruzi* and affects ~10 million individuals worldwide but is most prevalent in Latin America [58, 59]. In some, 20–30%, of infected individuals, chronic infection leads to a potentially fatal cardiomyopathy known as Chagas heart disease, generally 10–20 years after the initial infection [60, 61]. Chagas heart disease is characterized by marked inflammation and fibrosis of the heart, along with cardiac edema, myofibrillar destruction, chamber dilation, and loss of contractile function. The etiology of Chagas heart disease is not fully understood and likely multifactorial, with a contribution of an autoimmune response due in part to molecular mimicry between antigenic determinants of *T. cruzi* and human (cardiac) antigens [62, 63]. With heart failure in Chagas disease, myocardial levels of $CD8^+$ and $CD4^+$ T cells are increased, with a predominance of $CD8^+$ T cells [61, 64, 65]. In addition, the myocardium exhibits a strong Th1 cytokine profile with increased expression of IFN-γ and IL-18 genes that correlate with ventricular dilation [64, 65]. Circulating levels of IFN-γ are elevated during chronic Chagas disease [61] and were reported to be inversely correlated to left ventricular ejection fraction (LVEF) [66]. In contrast, Chagas-related heart failure is associated with reduced myocardial levels of Treg cells [61, 64], and circulating Treg activity was reported to be reduced in moderate or severe cardiomyopathy with activity directly correlated to LVEF [66].

Several studies have implicated CXCL10 in Chagas cardiomyopathy. Increased plasma levels of CXCL10 were

detected in patients with chronic Chagas disease [67], and LV mRNA expression levels of *CXCL10* were found to be elevated in patients with Chagas cardiomyopathy [68]. Recently, evidence was provided that polymorphisms in the *CXCL9* and *CXCL10* genes controlled the expression of chemokines in the myocardium and the degree of myocarditis in Chagas cardiomyopathy [69].

Behçet's disease is an autoimmune or autoinflammatory disorder common in the Middle East, Asia, and Japan. The basis for the pathogenesis of Behçet's disease is not known, although a number of factors have been proposed to have a role, including viral, bacterial, environmental, genetic, and immune factors. Behçet's is caused by small-vessel systemic vasculitis that very often affects the heart in diverse ways, including endomyocardial fibrosis, intracardiac thrombus, endocarditis, pericarditis, myocarditis, coronary arteritis, myocardial infarction, and valvular disease [70]. The cardiomyopathy may be ischemic, nonischemic, or inflammatory in nature and may manifest as asymptomatic systolic or diastolic dysfunction or overt systolic or diastolic heart failure [70]. Recently, monocytes of Behçet's patients were found to have dysfunctional posttranscriptional regulation of CXCL10 mRNA that resulted in overexpression of CXCL10 protein with IFN-γ stimulation [70]. Thus, overexpression of CXCL10 may contribute to the pathogenesis of Behçet's disease. In general, the role of CXCL10 in immune-mediated and autoimmune myocarditis is little studied; however, based on studies of infective myocarditis, a critical role for CXCL10 is likely. The role of CXCL10 in cardiac allograft transplantation rejection is discussed elsewhere [1].

6. LV Hypertrophy and Nonischemic Heart Failure

After CAD, hypertension is the most common risk factor for heart failure and accounts for ~25% of heart failure cases [71]. In the elderly, as many as 68% of heart failure cases are linked to hypertension and community-based studies indicate that hypertension contributes to heart failure in 60% of patients [72]. Hypertension causes a number of adverse remodeling events at the cellular and tissue level of the heart, including cardiac myocyte hypertrophy and gene reprograming, activation of cardiac fibroblasts, interstitial and perivascular fibrosis, and capillary refraction [73–75]. These alterations ultimately cause marked changes in the overall geometry of the heart that may progress to heart failure and the inability of the heart to adequately meet the oxygen and energy demands of the body. Heart failure (HF) may manifest clinically with either preserved or reduced LVEF, which are designated HFpEF (so-called diastolic heart failure) and HFrEF (systolic heart failure), respectively [75].

Hypertension results in concentric LV hypertrophy, which may progress to ventricular dilation and eventual HFrEF because of poorly understood means that may include ischemic injury [76, 77]. Related to this, volume overload due to fluid retention and impaired kidney function may cause a dilated pattern of eccentric LV hypertrophy with hypertension that leads to HFrEF [77]. Generally, cardiac remodeling with hypertension reflects a combination of both concentric and eccentric patterns of remodeling [77]. Concentric hypertrophy is also a characteristic of HFpEF, which typically has hypertension as the major comorbidity [78]. In addition, microvascular dysfunction concomitant to hypertension is thought to be a contributing factor for HFpEF [79]. The relative importance of CXCL10 in concentric *versus* eccentric LV hypertrophy, as well as their progression to heart failure, is not known.

Numerous preclinical and clinical studies have implicated marked activation of neurohormonal drive to the heart in the pathoetiology of LV hypertrophy and its progression to heart failure [80]. Neurohormonal drive, namely, activation of the sympathetic and renin-angiotensin-aldosterone systems, is generally thought to directly cause adverse remodeling of the heart. At the same time, there is evidence for indirect actions of neurohormonal stimulation on cardiac remodeling *via* activation of innate immunity and inflammation, especially the induction of heart-derived proinflammatory cytokines [81]. In chronic heart failure, an increased Th1/Th2 ratio is seen [82], but whether increased Th1 cell levels contribute to heart failure progression or simply are a consequence of heart failure is unresolved.

Exciting new findings have now implicated adaptive immunity and T cells more directly as causal agents in hypertensive LV hypertrophy and resultant heart failure by poorly understood means. These preclinical studies employed the mouse model of transverse aortic constriction- (TAC-) induced heart failure to mimic the impact of high blood pressure on the heart. We observed that circulating levels of CXCL9 and CXCL10 are elevated in TAC mice [83]. Laroumanie et al. [84] reported increased recruitment of activated $CD4^+$ and $CD8^+$ T cells and elevated levels of several chemokines for T cells and monocytes, including CXCL10, in ventricular tissues from mice with TAC-induced heart failure. TAC-induced ventricular dilation and fibrosis was prevented and contractile dysfunction was attenuated in mice deficient in mature B and T lymphocytes due to knockout of RAG2, although cardiac hypertrophy was still observed. T cell replenishment in RAG2 knockout mice restored the TAC-induced heart failure phenotype. In addition, elimination of $CD4^+$ T cells (MHCII knockout) but not $CD8^+$ T cells ($CD8^+$ knockout) prevented TAC-induced cardiac fibrosis and failure, suggesting a critical involvement of T helper cells. This conclusion was further supported by the observation that mice with transgenic T cell receptor specific for ovalbumin did not develop heart failure and fibrosis with TAC. Altogether these findings suggest that activation of $CD4^+$ T cells in hypertension causes interstitial and perivascular fibrosis that leads to functional and morphological changes in the heart conducive to the development of heart failure. However, it should be noted that an earlier study reported that coronary vessels of RAG1 knockout mice exhibited more intimal hyperplasia and perivascular fibrosis compared to wild-type mice following TAC [85]. The basis for the discrepant findings of the two studies is not clear. More recently, Nevers et al. [86] also investigated the role of T cells in cardiac remodeling in response to TAC-induced pressure overload. They observed that the development of

systolic dysfunction was associated with the kinetics of T cell infiltration into the left ventricle and evidence was provided that most of the infiltrating T cells were IFN-γ secreting Th1 cells. LV systolic and diastolic function were preserved with TAC in T cell deficient mice (T cell receptor (TCR) knockout), and LV hypertrophy, fibrosis, and inflammation were markedly attenuated. In addition, T cell depletion with an anti-CD3 antibody prevented heart failure in wild-type mice. Unresolved at present is the identity of the antigen(s) responsible for T cell activation in LV hypertrophy and heart failure, and the potential contribution played by the loss of regulatory mechanisms that normally protect the heart from T cells [87].

In contrast to the involvement of adaptive immunity in TAC, Ma et al. [88] provided evidence that $CD8^+$ T cells play a critical role in perivascular and interstitial fibrosis in the angiotensin II infusion model of hypertensive cardiac remodeling through the recruitment and activation of macrophages. They found that $CD8^+$ T cells are recruited to the heart and activated by IFN-γ secreting myocardial cells; recruited macrophages in turn are activated by $CD8^+$ T cells in contact-dependent, but TCR-independent means. A possible contribution of $CD4^+$ T cells to the actions of $CD8^+$ T cells will need to be explored.

Circulating levels of CXCL10 are elevated in patients with untreated essential hypertension [89]. In a small cohort, we observed that the CXCR3 chemokines, including CXCL10, were present in elevated concentrations in the plasma of patients with symptomatic diastolic LV dysfunction indicative of HFpEF or early stage HFrEF [90]. The magnitude of their increase was independent of the extent of hypertension and the CXCR3 agonists enhanced diagnostic accuracy over and beyond NT-pro BNP. More recently, we reported that circulating CXCL10, MIP-1α, and CD40 ligand were the best indicators for differentiating healthy and heart failure subjects [91]. We found that serum CXCL10 levels were increased in patients with symptomatic heart failure as indexed by NYHA classification II through IV and were positively correlated with serum levels of Th1 proinflammatory cytokines. The findings of these two studies are consistent with the idea that inflammation is involved in the pathogenesis of heart failure with CXCL10 playing a central role.

Numerous preclinical studies and recent genome-wide association studies (GWAS) support a role for both cytotoxic ($CD8^+$) T cells and Th ($CD4^+$) lymphocytes in human hypertension [92, 93]. However, accumulating evidence from experimental studies indicates that increasing Treg cell levels in hypertension is an effective strategy to preserve cardiac function, attenuate cardiac hypertrophy and fibrosis, and prevent heart failure progression, independent of any blood pressure lowering effects [94–96]. Reduced circulating levels of Treg cells in heart failure patients have been reported in several studies [97–99].

The role of CXCL10 in other forms of nonischemic heart failure with reduced ejection fraction, such as restrictive cardiomyopathy, ion channelopathies, and diabetic cardiomyopathy, awaits investigation. Recently, Di Luigi et al. [100] reported that the phosphodiesterase type 5 inhibitor sildenafil decreased elevated circulating CXCL10 levels in subjects with diabetic cardiomyopathy, suggesting that sildenafil could be used pharmacologically to mitigate CXCL10-associated inflammation in diabetic cardiomyopathy. Recent findings support a role for CXCL10 in right ventricular (RV) remodeling as well. Waehre et al. [101] found that several chemokines, most notably CXCL10, are upregulated in the pressure-overloaded right ventricle and play a role in myocardial extracellular matrix remodeling in an animal model of pulmonary stenosis. CXCL10 is implicated also in RV dysfunction and inflammation following experimental pulmonary embolism in rats [102].

7. Implications for Immunotherapy

The chemokine receptor CXCR3 and its agonist CXCL10 are potential drug targets to treat various cardiovascular diseases.

Potential immunotherapies for cardiac inflammation are as follows:

Treg Stimulation

(i) *IL-2/anti-IL-2* complex treatment to enhance Treg number and activity

(ii) *Targeted cytokine-infused nanoparticles* to stabilize and expand Tregs *in vivo*

(iii) Intravenous immunoglobulin (IVIG) therapy to boost Treg activity

(iv) *Vitamin D* to modulate formation and activity of Tregs

(v) *Atorvastatin* to enhance Treg number and activity

(vi) *FTY720* to increase Treg levels and activity

(vii) Adoptive Treg cell transfer

Immunosuppression

(i) *Immunoadsorption* to remove circulating antibodies and boost Treg activity

(ii) Phosphodiesterase type 5 inhibitor to decrease CXCL10 formation

(iii) *CXCL11* to stimulate biased GPCR anti-inflammatory signaling

(iv) *PPAR-γ agonists* to block CXCL9, CXCL10, and CXCL11 formation

Levels of CXCL10 are generally elevated with chronic cardiac inflammation, which is associated with enhanced Th1 polarization and infiltration into the myocardium. CXCR3 plays a key role in recruiting various leukocytes to the heart, including monocytes, effector lymphocytes, and CTL cells [1]. Peroxisome proliferator-activated receptor- (PPAR-) γ agonists may be a potential pharmacological treatment to block CXCL9, CXCL10, and CXCL11 formation in patients, as PPAR-γ agonists show a strong inhibitory effect on their expression and production *in vitro* [103]. Pioglitazone, which lacks the adverse cardiovascular effects of older thiazolidinediones and may be cardiovascular protective, looks promising in this regard, although pioglitazone is contraindicated in

Table 1: Immunological Mediators in Chronic Inflammation of the Heart.

Disease	Elevated Th1	Depressed Tregs	Elevated CXCL10
CAD	1; 27; 31; 32	32–34	35–37
Kawasaki	44	45	46–49
Myocarditis/Chagas	58; 62; 65; 66	62; 65; 67	68; 69; 71
Hypertrophy	83; 84; 86; 92; 93	97–99	84; 89–91

heart failure patients likely due to fluid retention [104–107]. Another potential therapeutic approach is the phosphodiesterase type 5 inhibitor sildenafil, which was recently reported to decrease CXCL10 gene expression and protein secretion in human cardiac myocytes and decrease circulating CXCL10 in subjects with diabetic cardiomyopathy [100]. There is substantial evidence that sildenafil has protective effects against adverse remodeling of the heart [108].

Although CXCL9, CXCL10, and CXCL11 all bind to CXCR3, there is evidence that these agonists activate opposing responses due to biased signaling that is a fixture of G protein-coupled receptors [1, 11, 12]. Whereas CXCL9/CXCL10/CXCR3 interactions drive effector Th1 polarization, CXCL11/CXCR3 binding seems to induce an immunotolerant state characterized by T lymphocyte polarization into regulatory Tr1 lymphocytes that produce anti-inflammatory IL-10 [11, 12]. Therefore, inhibiting CXCR3 may not yield definitive findings. Biased agonists have been developed that exert CXCL11-like actions at CXCR3 [22] but have not as yet been assessed in experimental models of cardiovascular diseases. An alternative approach might involve a biological compound. CXCL11 has a short half-life *in vivo*. To address this shortcoming, Zohar et al. [21] generated a stabilized form of CXCL11 by creating a fusion protein in which CXCL11 was linked to IgG1. When administered during ongoing autoimmune encephalomyelitis, the fusion protein suppressed the disease by increasing the number of IL-10-secreting Tr1-like cells (direct effect) and reducing Th1 polarization.

Targeting CXCR3 might be more effective in treating chronic heart inflammation in combination with approaches to enhance Treg numbers or activity, which are generally reduced in cardiovascular diseases (Table 1). Regulatory T cells are immunosuppressive and anti-inflammatory, and a growing number of experimental studies have shown their beneficial effects on the heart in various experimental models of coronary artery disease [109, 110], Kawasaki disease [111], myocarditis and dilated cardiomyopathies [112–117], Chagas heart disease [118, 119], hypertensive LV hypertrophy [95, 96], and nonischemic heart failure [94, 109, 120]. Although not discussed here, boosting Treg numbers or activity in the heart is reported to be beneficial as well in experimental models of infarction-driven remodeling [121–124]. Increasing levels of IL-10-secreting Treg/Tr1 cells may be particularly advantageous, as IL-10 has anti-inflammatory and protective actions on the vasculature and heart [125–132]. Intravenous immunoglobulin (IVIG) therapy has been shown to be effective in treating acute Kawasaki disease in 80–90% of Kawasaki patients with rapid resolution of clinical symptoms and reduced risk of coronary disease [133]. Although the exact basis for the effectiveness of IVIG therapy is not clear, IVIG therapy is thought to modulate the inflammatory process and recent evidence indicates that IVIG therapy acts in part by stimulating an immature myeloid population of dendritic cells that secretes IL-10 and favors expansion of Fc-specific natural Treg cells [134]. IVIG may have promise for treating viral myocarditis [55] and might be effective in treating Chagas [135], as well as chronic heart failure, including both ischemic and idiopathic dilated cardiomyopathies [136].

Adoptive transfer of Tregs and/or stimulating their differentiation are promising therapeutic approaches to target cardiac inflammation [49, 50], although the concerns that must be overcome to make adaptive transfer routine therapy in humans are considerable [137]. However, the safety and efficacy of Treg immunotherapy in humans is supported by preliminary clinical trials for treating graft versus host disease [137, 138]. Immunoadsorption, which is a promising approach for treating myocarditis and dilated cardiomyopathy, may have beneficial effects by not merely removing circulating antibodies, but increasing Treg activity [49, 139–141].

It may be feasible to selectively enhance Treg numbers using a pharmacological approach. The prodrug FTY720 (Fingolimod) is phosphorylated *in vivo* and has been shown to trap naïve and memory T cells in the thymus and secondary lymphoid organs by downregulating sphingosine 1-phosphate receptor 1 (S1P1) [142]. Activation of S1P1 is linked to inhibition of Treg cell differentiation [142]; however, FTY720 and phosphorylated FTY720 increase Treg levels and activity *in vivo* and *in vitro* [143–146]. The exact mechanism is unclear but may require higher doses [144]. The inhibitory effects of atorvastatin on inflammation in acute coronary syndrome (ACS) may be due to its beneficial effects on natural Tregs [147]. In patients with ACS, atorvastatin treatment increased the percentage and inhibitory ability of natural Tregs.

IL-2 plays a critical role in Treg cell activity, growth, and survival, but there may be insufficient IL-2 to sustain their *in vivo* potentiation [12]. Emerging evidence indicates that enhancing Treg cell numbers and activity with low-dose IL-2 treatment is effective in treating autoimmune and inflammatory diseases [12, 148–151], although there may be intrinsic risk as IL-2 is also a key growth factor for effector $CD4^+$ T cells and NK cells [12]. An IL-2/anti-IL-2 immune complex is reported to preferentially expand Treg cells with little or no effect on other cells [121]. Delivery of the complex to mice before TAC was recently reported to attenuate LV hypertrophy, inflammation, and contractile dysfunction, while increasing LV levels of Treg cells [94]. Inert biodegradable nanoparticles represent another promising platform for stabilizing and expanding Tregs *in vivo*. McHugh et al. [152] recently reported success in using nanoparticles loaded with the Treg inducers IL-2 and TGF-β and targeted to $CD4^+$ T cells with conjugated antibodies. These nanoparticles were demonstrated to induce $CD4^+$ Tregs *in vitro*, even in the

presence of proinflammatory cytokines, and expand their number in mice *in vivo*.

Vitamin D status has been linked to chronic heart failure and vitamin D supplementation improves LV structure and function in heart failure patients [153]. Recent evidence indicates a modulatory role of the vitamin D system in the formation and activity of regulatory T cells [154]. Vitamin D deficiency was associated with reduced numbers and impaired function of naïve $CD45RA^+$ regulatory T cell in chronic heart failure patients [120]. In addition, the vitamin D receptor agonist BXL-01-0029 was shown to inhibit IFN-γ and TNF-α-induced CXCL10 secretion by fetal human cardiac myocytes and reduce CXCL10 protein secretion and gene expression by $CD4^+$ T cells [155]. Whether a vitamin D receptor agonist or supplementation increases Treg levels and reduces CXCL10 levels in heart failure patients will need to be assessed.

8. Conclusions and Future Perspectives

The last decade has witnessed an impressive advance in our understanding of the role of the immune system in the pathophysiology of cardiovascular diseases. Beyond an expected role in allograft disease and the development and progression of atherosclerosis, an abundant body of evidence supports a significant contribution of the immune system to many other cardiovascular settings, ranging from the development and maintenance of hypertension to cardiac and vessel remodeling (whether constrictive or expansive) in response to hemodynamic stress [156], in both health and disease. The challenge now is to translate this knowledge for the benefit of patients suffering from cardiovascular diseases. Targeting the CXCL10/CXCR3 axis and cardiac inflammation may open new pharmacological venues for treating heart failure or coronary artery disease to supplement current drugs that target the sympathetic or renin-angiotensin systems, or platelets. This will require selectively targeting the most critical immune pathways in order to optimize interference with pathological processes, while preserving protective and homeostatic immune functions. Antigen-specific modulation of the immune system, for example, through systemic delivery of nanoparticles coated with disease-relevant peptides bound to major histocompatibility complex class II (pMHCII) to expand endogenous antigen-specific Tregs [157], is an optimal strategy in settings where an antigen-specific adaptive immune response has been involved in disease development or progression. However, several cardiovascular diseases will probably resist the identification of a specific pathogenic antigen and will require a broader, although targeted, regulation of the immune response, for example, through administration of low-dose IL-2 to promote endogenous Tregs [149]. With regard to CXCR3 pathway, besides the therapeutic possibilities listed above, we believe that the identification and development of selective Evasins [158] or Evasin-like peptides that differentially bind and neutralize CXCL9 or CXCL10 versus CXCL11 may provide an interesting therapeutic strategy to limit pathogenic while preserving regulatory CXCR3 functions.

Competing Interests

The authors have no competing interests to declare.

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Research Article

Delta Procalcitonin Is a Better Indicator of Infection Than Absolute Procalcitonin Values in Critically Ill Patients: A Prospective Observational Study

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Purpose. To investigate whether absolute value of procalcitonin (PCT) or the change (delta-PCT) is better indicator of infection in intensive care patients. *Materials and Methods*. *Post hoc* analysis of a prospective observational study. Patients with suspected new-onset infection were included in whom PCT, C-reactive protein (CRP), temperature, and leukocyte (WBC) values were measured on inclusion (t_0) and data were also available from the previous day (t_{-1}). Based on clinical and microbiological data, patients were grouped *post hoc* into infection- (I-) and noninfection- (NI-) groups. *Results*. Of the 114 patients, 85 (75%) had proven infection. PCT levels were similar at t_{-1}: I-group (median [interquartile range]): 1.04 [0.40–3.57] versus NI-group: 0.53 [0.16–1.68], $p = 0.444$. By t_0 PCT levels were significantly higher in the I-group: 4.62 [1.91–12.62] versus 1.12 [0.30–1.66], $p = 0.018$. The area under the curve to predict infection for absolute values of PCT was 0.64 [95% CI = 0.52–0.76], $p = 0.022$; for percentage change: 0.77 [0.66–0.87], $p < 0.001$; and for delta-PCT: 0.85 [0.78–0.92], $p < 0.001$. The optimal cut-off value for delta-PCT to indicate infection was 0.76 ng/mL (sensitivity 80 [70–88]%, specificity 86 [68-96]%). Neither absolute values nor changes in CRP, temperature, or WBC could predict infection. *Conclusions*. Our results suggest that delta-PCT values are superior to absolute values in indicating infection in intensive care patients. This trial is registered with ClinicalTrials.gov identifier: NCT02311816.

1. Introduction

Treatment of severe sepsis and septic shock remains a major challenge in the critically ill, and it is still one of the leading causes of death worldwide [1]. Despite increased awareness of the importance of early resuscitation, mortality in North America and Europe ranges between 28 and 41% [2]. Based on a consensus agreement sepsis is defined as infection in the presence of systemic inflammatory response syndrome (SIRS) [3]. However, the signs of SIRS are nonspecific and can often be seen in several (none septic) critically ill conditions. Fever, tachycardia, or leukocytosis on their own has low sensitivity and specificity [4, 5]. Detailed microbiological results are often only available after 24 hours or later, and negative results do not necessarily rule out infection. Nevertheless, early diagnosis of infection in critically ill patients is of utmost importance, and delay in starting appropriate antibiotic therapy may lead to lethal events [6]. However, giving antibiotics unnecessarily to every acutely ill patient is an unacceptable practice for several reasons [7]. Therefore, fast reacting biomarkers of infection have been used for almost 50 years to help the clinician, of which C-reactive protein (CRP) and procalcitonin (PCT) are the most often used and studied [8].

Procalcitonin is a fast reacting biomarker with a half-life of around 24 hours [9]. Its sensitivity and specificity for bacterial infection seem to be superior compared to CRP [10, 11]. However, it must be considered that the same absolute values of PCT cannot be used in all circumstances. It has been reported that PCT levels are higher in surgical compared to medical patients [12], and elevated PCT can also be present without infection, in conditions such as trauma [13] and surgery [14] or after cardiac arrest [15]. There is some evidence that evaluating PCT kinetics may be superior to absolute values [12, 16].

In this study, our aim was to investigate whether the absolute value of PCT measured in critically ill patients on the day when infection was suspected, or the change in PCT (delta-PCT) from the day before to the day when infection was suspected, was a better indicator of infection.

2. Methods

2.1. Patient Selection. This prospective observational study was part of the Early Procalcitonin Kinetics (EProK) study, which was undertaken between October 2012 and October 2013 and approved by the Regional and Institutional Human Medical Biological Research Ethics Committee, University of Szeged, Hungary (WHO-3005; 19.04.2012, Chairperson Professor T. Wittmann). A detailed description of the EProK study and the final results are published elsewhere [17]. The investigation was performed at the University of Szeged (Szeged, Hungary), Albert Szent-Györgyi Health Center in four tertiary intensive care units. The study was registered at ClinicalTrials.gov with the registration number: NCT02311816. Written informed consent was obtained from all subjects or from their relatives.

2.1.1. Inclusion Criteria. In the EProK study all patients over 18 years with suspected infection on admission or during their stay on the intensive care unit were screened for eligibility. Patients were enrolled, when the attending intensive care specialist suspected infection, based on (1) suspected source which could be identified, (2) new onset organ dysfunction, and (3) body temperature, PCT, CRP, and the decision to start empirical antibiotic therapy. Once the original EProK study was completed, in a *post hoc* analysis those patients in whom PCT and CRP values were available from the previous day (t_{-1}) were included in the current analysis.

2.1.2. Exclusion Criteria. Exclusion criteria included patients younger than 18 years, who had received antibiotic therapy in the previous 48 hours, and those who received acute renal replacement therapy 24 hours before enrollment. Patients were also excluded following cardiopulmonary resuscitation and with end stage diseases with a "do not resuscitate" order. Immunocompromised patients (human immunodeficiency virus infection, bone marrow transplantation, malignant haematological disorders, and chemotherapy) were also excluded.

2.2. Subgroups and Definitions. Diagnosis of infection was based on a *post hoc* analysis of mainly microbiological results but also clinical parameters and biochemical results which were evaluated by two experts (infectologist, EH, and an intensivist, FJ) blinded for the PCT data apart from the first PCT measurement (t_0, see below). The experts also took into consideration the recommendations of international guidelines [18, 19]. Based on these results, patients were grouped into "infection-" (I-) and "noninfection-" (NI-) groups.

For subgroup analysis, patients were divided into "medical" and "surgical" groups. The medical-group represented patients who had had no surgical intervention before and during the study period and for source control did not require surgery. In the surgical-group infection either was related to an operation or required surgery for source control [12].

2.3. Protocol and Data Collection. Whenever infection was suspected by the attending physician, the signs of infection and the suspected source were recorded, which included high/low body temperature (<36°C; >38°C), high/low white blood cell count (<4,000; >12,000 million/mL), acute worsening of the clinical picture (hemodynamic instability, worsening PaO_2/FiO_2 ratio, and deterioration in mental status or any other clinical sign indicating infection). Microbiological specimens were collected from all suspected sources immediately before the administration of the first dose of antibiotics (t_0).

2.3.1. Data Collection. After enrollment, demographic data, signs of infection, the suspected source of infection, and corresponding microbiological samples were registered. The length of intensive care unit and hospital stay, 28 days, and the overall mortality were also documented.

2.3.2. Procalcitonin Measurement. It is common practice in our ICU to measure PCT daily in critically ill patients. Procalcitonin levels were documented from the previous day of enrollment (t_{-1}) and immediately before the initiation of ABs (t_0). Core temperature, C-reactive protein (CRP), and white blood cell count (WBC) were also recorded with every PCT measure. The flow chart of the data collection is summarised in Figure 1.

Serum PCT levels were measured with Cobas 6000 analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Analyzer reagents (Elecsys® B·R·A·H·M·S PCT assay) were developed in collaboration with B·R·A·H·M·S corporation (Hennigsdorf, Germany) and Roche Diagnostics (Mannheim, Germany). Procalcitonin was determined by electrochemiluminescence immunoassay (ECLIA) serum on the automated Roche Elecsys and Cobas immunoassay analyzers.

2.3.3. Microbiological Staining and Antibiograms. Microbiological tests were performed and sent at t_0, before the first antibiotic dose was administered and if needed they were repeated on the following days, to identify infection.

2.4. Statistical Analysis. Data were analyzed using IBM SPSS Statistics Version 20 (Armonk, NY, USA) and Systat Software Inc. SigmaPlot 12.5 (London, UK) software. For continuous

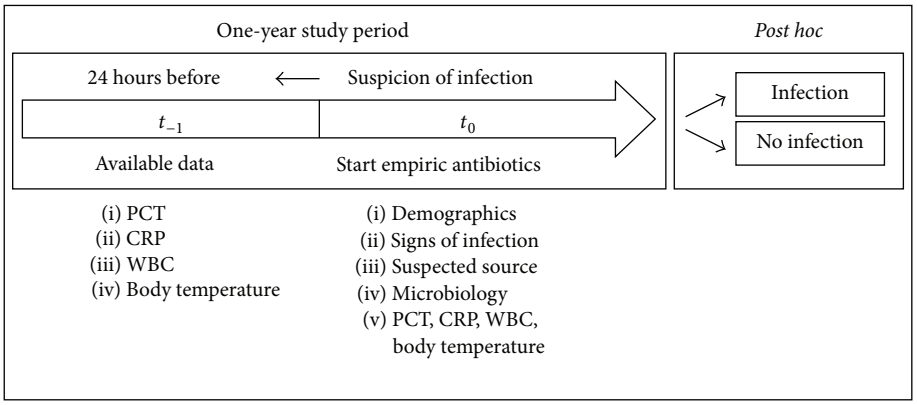

Figure 1: Flow chart.

data, the Shapiro-Wilk tests were performed to assess normal distribution. Demographic data were analyzed between groups with Student's t-test or nonparametric data with the Mann-Whitney U test as appropriate. Categorical data were compared using χ^2 tests. Biomarkers were analyzed by using Two-Way Repeated Measures Analysis of Variances (All Pairwise Multiple Comparison Procedures: Holm-Sidak method). Logistic regression, receiver operating characteristic (ROC) curve, and the respective areas under the curves (AUC) were calculated for PCT, CRP, body temperature, and white blood cell count levels. The best cut-off values were determined using the Youden index ($J = \max[\text{Sens} + \text{Spec} - 1]$). The test parameters (sensitivity, specificity, positive, and negative predictive values) were compared by their 95% confidence intervals. Logistic regression analysis was used to determine the best combination of parameters and cut-offs for predicting infection. The level of $p < 0.05$ was defined as statistically significant. Data are given as mean ± standard deviation or median (interquartile range) as appropriate.

The "delta" was considered as the changes in the absolute values (subtracting t_{-1} from t_0); the percentage values were calculated as $[(t_0/t_{-1}) \times 100 - 100]$.

3. Results

Over the one-year study period all ICU patients were screened for eligibility and 209 patients were recruited into the EProK study. Out of the 209 patients in the current *post hoc* analysis we include 114 cases where PCT values were available from the previous day. Demography and outcomes characteristics for the entire cohort are summarised in Table 1. Out of the 114 patients, 85 (75%) patients were identified as having proven infection and in 29 (25%) patients the presence of infection was highly unlikely. Disease severity scores and outcomes were similar in the two groups, but the NI-group required less organ support.

The clinical and laboratory signs of infection on which the clinicians suspected infection at the time of inclusion (t_0) are summarised in Table 2. Although all indices were higher in the I-group, but only the altered level of consciousness, hemodynamic instability, and the PCT was significantly different between the two groups.

Regarding the suspected source of infection, generally there was nonsignificant difference between the groups, but significantly more patients were suspected of having abdominal related infection in the NI-group. Detailed data on the isolated pathogens and their sources are summarised in the Supplemental Digital Content Tables S5–7 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/3530752).

3.1. PCT, CRP, WBC, and Temperature Values at t_{-1} and t_0

3.1.1. Total Sample. Measurement results at t_{-1} and t_0 in the I- and NI-groups are shown in Figure 2. PCT absolute values were similar at t_{-1}, but by t_0 in the I-group levels were significantly higher compared to the NI-group and there was also a significant increase from t_{-1}, while there was no such change in the NI-group. There was no significant difference in CRP and WBC count between the two groups nor could we find significant changes from t_{-1} to t_0. There was no difference between the groups for body temperature but there was a statistically significant increase in the NI-group by t_0. It is of note that body temperature remained <38°C in almost all patients.

3.1.2. Medical and Surgical Patients. Measurement results in medical ($n = 80$) and surgical ($n = 34$) patients are summarised in Table 3. In the surgical subgroup PCT absolute values were significantly higher than in the medical cohort, but the pattern of change was similar. In the NI-group there was a slight, but statistically significant increase in medical patients from t_{-1} to t_0, while there was no significant change in surgical patients, where levels actually decreased slightly. However, in the I-group there was an almost 3-fold increase in the PCT levels.

Regarding the CRP, body temperature, and WBC count, there was no significant changes over time and no differences between medical and surgical patients.

3.2. Predictive Value for Indicating Infection. The predictive value for infection for the absolute values of PCT, CRP, temperature, and WBC count can be seen in Figure 3 and is summarised in Table 4. Only PCT had a significant predictive value, but with a poor AUC (Figure 3). However, regarding

Table 1: Demographics, organ support, and outcome in the entire cohort.

	Total	NI-group	I-group	p value
Age (years)	65 (22.5)	67 (25.5)	65 (22)	0.772
Gender (M/F)	69/45	15/14	54/31	0.261
Body height (cm)	170 (12)	167 (19)	170 (11)	0.766
Body weight (kg)	73 (25)	80 (25)	70 (20)	0.345
SAPS II points	62.2 ± 20.5	62.7 ± 25.5	66.1 ± 18.6	0.513
SAPS II PM (%)	77.2 (52.1)	64.0 (75.9)	78.5 (42.1)	0.437
ICU days before enrollment	1 (3)	1 (3)	1 (3)	0.669
Mechanical ventilation	80 (70.2%)	12 (41.4%)	68 (80.0%)	<0.001
Vasopressor therapy	69 (60.5%)	13 (44.8%)	56 (65.9%)	0.045
ICU LOS (day)	9 (12)	8 (8)	9 (12)	0.089
ICU survival	84 (73.7%)	24 (82.8%)	60 (70.6%)	0.199
Hospital LOS (day)	17 (20)	14 (17)	19 (22)	0.050
Hospital survival	67 (58.8%)	20 (68.9%)	47 (55.3%)	0.197
28-day survival	64 (56.1%)	19 (65.5%)	45 (52.9%)	0.239

Data are given as mean ± standard deviation or median (interquartile range) as appropriate. M: male; F: female; SAPS: simplified acute physiology score; PM: predicted mortality; ICU: intensive care unit; LOS: length of stay; mechanical ventilation and vasopressor therapy represent data at the day of enrollment.

Table 2: Clinical signs and suspected source of infection at enrollment (t_0).

	Total $n = 114$	NI-group $n = 29$	I-group $n = 85$	p value
Fever (<36°C; >38°C)	55 (48.2%)	13 (44.8%)	42 (49.4%)	0.670
WBC (>12 or $<4 \times 10^9$/L)	82 (71.9%)	22 (75.9%)	60 (70.6%)	0.585
Impaired gas exchange	82 (71.9%)	18 (62.1%)	64 (75.3%)	0.171
Impaired consciousness	59 (51.8%)	9 (31.0%)	50 (58.8%)	0.010
Hemodynamic instability	74 (64.9%)	13 (44.8%)	61 (71.8%)	0.009
PCT (ng/mL)	3.37 (9.22)	1.12 (1.36)	4.62 (10.72)	0.018
CRP (mg/L)	182.75 (158.5)	147.60 (156.50)	208.80 (140.60)	0.301
Respiratory	72 (63.2%)	17 (58.6%)	55 (64.7%)	0.557
Soft tissue	13 (11.4%)	2 (6.9%)	11 (12.9%)	0.377
Abdominal	14 (12.3%)	7 (24.1%)	7 (8.2%)	0.024
Urinary tract	5 (4.4%)	0	5 (5.9%)	0.182
Bloodstream	6 (5.3%)	2 (6.9%)	4 (4.7%)	0.648
Central nervous system	4 (3.5%)	1 (3.4%)	3 (3.5%)	0.984

WBC: white blood cell count, PCT: procalcitonin, and CRP: C-reactive protein. The PCT and CRP values are presented as median (interquartile range).

Table 3: PCT, CRP, body temperature, and white blood cell count in medical and surgical patients with and without infection.

		NI-group		I-group	
		t_{-1}	t_0	t_{-1}	t_0
Medical	PCT (ng/mL)	0.26 (0.57)	0.54 (1.16)*	0.89 (1.52)	3.17 (5.9)*#
	CRP (mg/L)	136.7 (159.1)	141 (125.9)	150 (184.3)	164.2 (145.3)
	BT (°C)	36 (1.02)	37 (0.82)*	36.9 (1.23)	37 (1.6)
	WBC ($\times 10^9$/L)	14.32 (8.9)	15.4 (8.64)	12.06 (6.36)	13.76 (10.16)
Surgical	PCT (ng/mL)	3.5 (9.91)	2.89 (9.33)	3.83 (22.55)	14.9 (58.06)*#
	CRP (mg/L)	95 (342.5)	163 (327.4)	199.5 (130.1)	243.2 (112.7)
	BT (°C)	36.5 (2)	36.5 (2.4)	36 (1)	36.9 (1.1)
	WBC ($\times 10^9$/L)	8.99 (7.37)	14.56 (9.65)	11.9 (10.06)	10.91 (9.9)

Data are presented as median (interquartile range). PCT: procalcitonin, CRP: C-reactive protein, BT: body temperature, and WBC: white blood cell count; * $p < 0.05$ within groups and # $p < 0.05$ between groups.

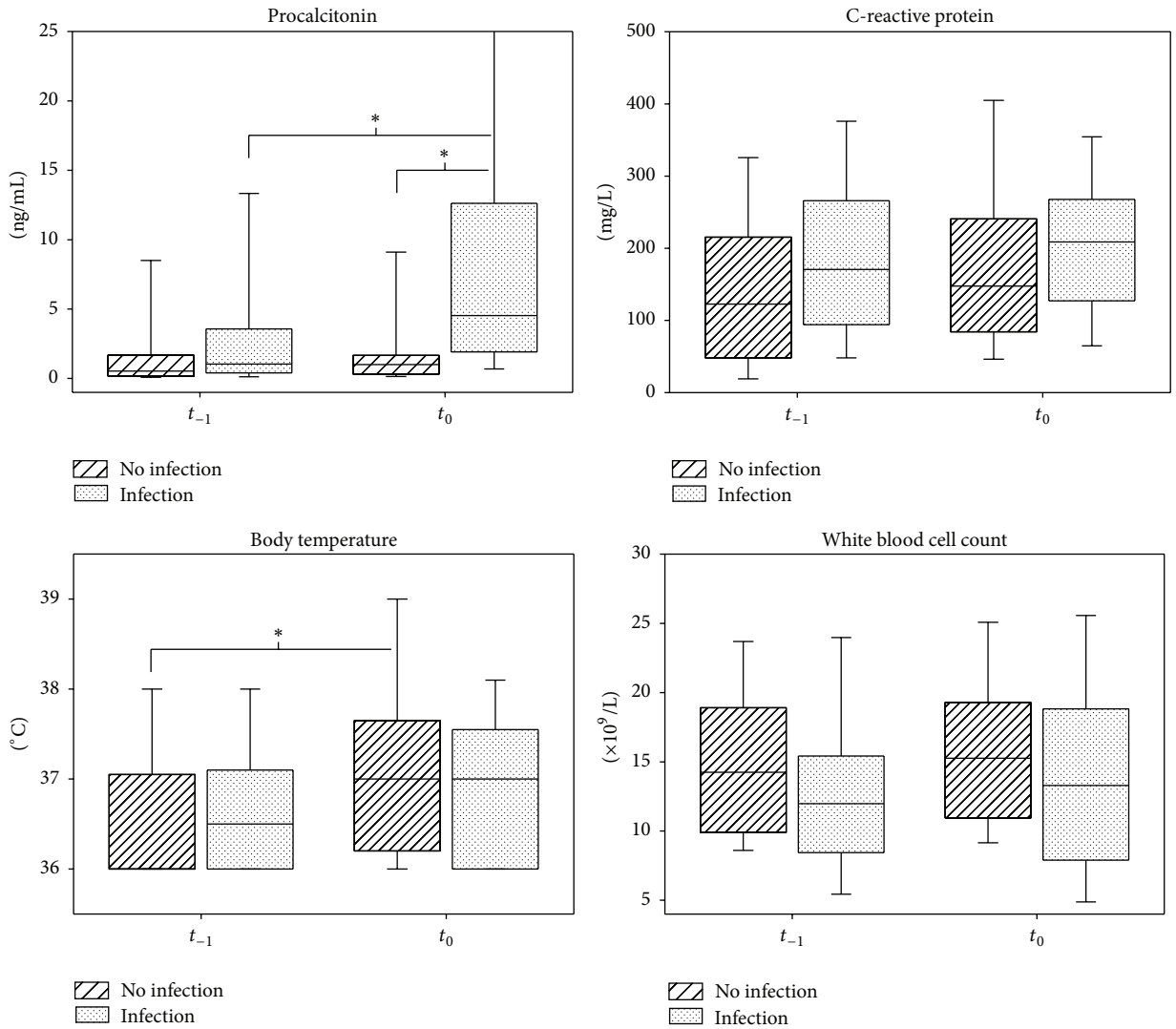

Figure 2: PCT, CRP, body temperature, and WBC count absolute values in the total cohort. Boxplots present median (interquartile range) 10th and 90th percentile. $*$ indicates $p < 0.05$.

Table 4: The predictive value of the absolute values, percentage, and delta changes of PCT, CRP, temperature, and WBC count for infection in the total cohort.

		Absolute value			Percentage changes			Absolute value changes		
		AUC	95% CI	p value	AUC	95% CI	p value	AUC	95% CI	p value
Total	PCT	0.64	0.52–0.76	0.022	0.77	0.66–0.87	<0.001	0.85	0.78–0.92	<0.001
	CRP	0.60	0.47–0.72	0.103	0.54	0.41–0.66	0.530	0.54	0.42–0.65	0.536
	BT	0.52	0.39–0.63	0.804	0.56	0.44–0.68	0.300	0.56	0.44–0.68	0.322
	WBC	0.60	0.48–0.70	0.125	0.51	0.40–0.61	0.852	0.51	0.39–0.61	0.924
Medical	PCT	0.67	0.54–0.80	0.016	0.76	0.63–0.88	<0.001	0.83	0.73–0.92	<0.001
	CRP	0.58	0.44–0.72	0.248	0.57	0.42–0.70	0.359	0.57	0.44–0.70	0.306
	BT	0.51	0.37–0.64	0.858	0.64	0.50–0.77	0.055	0.64	0.49–0.77	0.060
	WBC	0.57	0.44–0.70	0.329	0.56	0.43–0.68	0.441	0.57	0.43–0.69	0.365
Surgical	PCT	0.78	0.58–0.97	0.025	0.80	0.59–1.00	0.014	0.94	0.85–1.00	<0.001
	CRP	0.56	0.23–0.87	0.654	0.56	0.29–0.81	0.654	0.54	0.31–0.76	0.749
	BT	0.52	0.22–0.80	0.898	0.63	0.39–0.85	0.306	0.63	0.39–0.86	0.296
	WBC	0.63	0.44–0.82	0.277	0.67	0.47–0.86	0.166	0.71	0.49–0.90	0.108

AUC: area under the ROC curve, CI: confidence interval, PCT: procalcitonin, CRP: C-reactive protein, BT: body temperature, and WBC: white blood cell count.

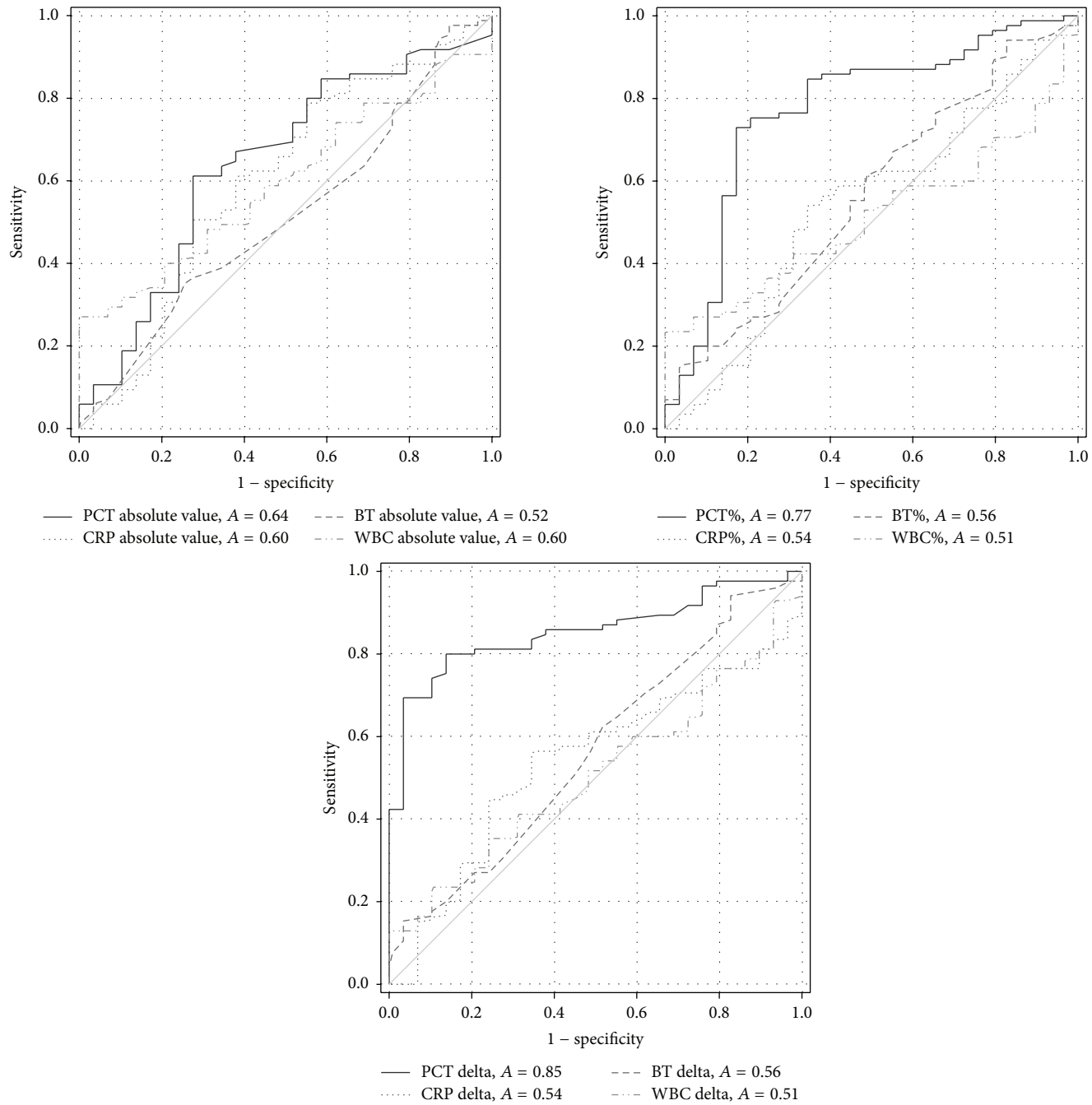

Figure 3: The predictive value of the absolute values, percentage, and delta changes of PCT, CRP, temperature, and WBC count for infection in the total cohort.

the percentage and delta changes CRP, temperature and WBC counts diagnostic value did not change, while PCT's AUC for both percentage and delta changes had a significantly better performance for predicting infection. Similar patterns were observed in the medical and surgical subgroups (Table 4).

3.3. Best Cut-Off Value. The best cut-off values were defined for PCT only as there was no significant predictive value for the other parameters, as determined by the Youden index. For the PCT absolute value it was 0.84 ng/mL with a sensitivity of 61% (95% CI: 50–72) and specificity 72% (53–87) to indicate infection in the ICU. Regarding the percentage change a PCT increase of >88% from t_{-1} to t_0 had a sensitivity of 75% (65–84) and specificity of 79% (60–92) and a PCT delta change of >0.76 ng/mL had a sensitivity of 80% (70–88) and specificity of 86% (68–96) to indicate infection.

Data were also analyzed using the logistic regression model for finding the best combination of these four parameters together to predict infection in the ICU. However, none of the combinations tested improved the performance for predicting infection (data not shown).

4. Discussion

The main finding of this observational study was an increase in PCT levels from the day before (t_{-1}) to the day when infection was suspected (t_0) predicted infection, while in patients with no proven infection PCT remained unchanged. Furthermore, regarding the conventional indicators of infection such as WBC, body temperature, and CRP, neither the absolute values nor their change from t_{-1} to t_0 could predict infection.

Diagnosing infection in the critically ill is challenging. Appropriate decision making has paramount importance as any delay in adequate antibiotic treatment of sepsis and septic shock evokes worsening morbidity and mortality results [6, 20]. On the other hand unnecessary antibiotic administration in patients without infection has led to the emergence of multidrug-resistant bacteria [21, 22], complications related to the side effects of the antibiotics themselves and an increased burden of healthcare expenses [23]. Despite its importance, there is no gold standard for diagnosing/proving infection in the critical care setting.

In our study 75% of patients had proven infection. This complex *post hoc* analysis of all results is fundamentally different from "labelling" patients as septic, based solely on the Surviving Sepsis Guideline criteria at the time of initial assessment as seen in several studies [24, 25]. Although our method also has some uncertainties, it provides a more robust approach utilising all data, clinical, biochemical, and microbiology alike, to aid in the diagnosis of patients with bacterial infection. However, it is also important to acknowledge that there is no gold standard to diagnose infection; therefore despite all our efforts, some patients in the NI-group may have had culture negative infection.

In our investigation it was found that conventional indicators of infection such as body temperature and white cell count had less value in diagnosing infection. Levels of WBC count remained elevated on both days and there was no significant change over time. This phenomenon can be explained by the nonspecific activation of the immune cascade as often seen in ICU patients [26]. Although there was a statistically significant increase in body temperature in the NI-group, levels largely remained below 38°C in almost all patients. These results are in accordance with recent findings that increased temperature alone does not predict infection [27].

Although microbiology remains the gold standard for confirming pathogens, results only come back at least 24–48 hours after sampling. Furthermore, in several cases results remained negative, despite obvious signs of infection. In order to help the diagnostic process several novel biomarkers of infection have been developed [8]. However, all biomarkers share the same limitations that "one size will not fit all," due to the complex pathomechanism and the heterogeneity of patients.

The two most commonly used markers in infection/sepsis diagnostics are PCT and CRP [8]. Procalcitonin is detectable in the serum a few (2–4) hours after the onset of bacterial infection. It reaches its peak within 24 hours and then starts to decline with adequate treatment by around a 50% daily decrease according to its half-life [9]. In contrast, CRP has a delayed response. It reaches its maximum value usually after 48 hours of an insult and in general it lags behind the actual events of the inflammatory and clinical process. Furthermore, CRP levels are generally elevated in most ICU patients regardless of the aetiology. In our study neither the absolute values of CRP nor its delta changes were able to indicate new onset infection. Patients had elevated CRP values with a median of almost 200 mmol/L for the whole cohort, which makes interpretation very difficult. Furthermore the kinetics did not show any significant change over time. Therefore, our results question the place of CRP measurements for diagnosing infection on the ICU.

The most important finding of the current study was to show the superiority of PCT kinetics over the absolute values to indicate new onset infection in the ICU. However, this requires at least daily measurements of PCT, which has been common practice in our ICU in critically ill patients in whom infection cannot be excluded. Our current findings are in accordance with those reported by Tsangaris et al. [16]. They also measured PCT daily and observed a twofold increase of PCT levels from the day before to the day when there was a sudden onset of fever in patients with proven infection, but no change in PCT was found in patients without infection. They concluded that, in patients treated chronically in the ICU, PCT values on the day of fever onset must be compared to values measured the previous day in order to define whether this rise in temperature was due to infection or not. An important difference between their and our study is that in our patients body temperature merely reached 38°C; in fact most of these patients were apyrexial, despite 75% having proven infection. Therefore, we recommend to evaluate PCT kinetics not only in the onset of fever, but whenever infection is suspected on the ICU. Based on the current results, the best cut-off values were also determined for change in PCT, which were >88% and >0.76 ng/mL delta change from t_{-1} to t_0. The reasons why a given absolute value of any biomarker, not just PCT, may be of limited value as compared to its changes can be explained by the pathomechanism of systemic inflammation. It was a very important discovery that after trauma, burns, ischemia-reperfusion, pancreatitis, major surgery, and so forth, the same or similar molecules are released predominantly from the mitochondria, as after an infectious insult. Based on aetiology these are called "damage-associated molecular patterns" (DAMP), or "pathogen associated molecular patterns" (PAMP). Once similar mediators/proteins are released they act on the same receptors of monocytes inflicting a similar inflammatory response, including PCT release and subsequent organ dysfunction [28, 29].

Indeed, PCT levels were found to be severalfold higher in surgical compared to medical patients in septic shock despite the similar clinical manifestation and severity of

the clinical picture [12]. This explains why PCT levels were elevated in our surgical patient population without proven infection, with median values of around 3.5 (NI-group) and 3.8 (I-group) ng/mL at t_{-1}. The corresponding PCT values in medical patients were substantially lower (0.26 and 0.89 ng/mL, resp.). Although levels were higher in the I-group at t_{-1}, this difference did not reach statistical significance while there was a severalfold increase in the I-group in both medical and surgical patients with no change in kinetics in the NI-groups.

In two large recent multicenter trials the authors could not show any benefit from a PCT-based approach in antibiotic management in the ICU [30, 31]. However, in both studies the threshold for intervention was a PCT of >1 ng/mL. As 40% of the patients in both trials were surgical, in whom this threshold for intervention may be too low, one cannot exclude that these patients may have had received antibiotics unnecessarily. This overuse of antibiotics may be one of the reasons for the worse outcome in the PCT-guided group in both studies. Our study provides further evidence that changes or kinetics of PCT may be superior to absolute values.

The current study has several limitations. Firstly, one may argue that there was a selection bias; in other words, physicians suspected infection more often when they observed a PCT increase in a patient. Although this cannot be excluded completely, at the time when the study was performed, PCT collection was not the routine practice within the department, and delta-PCT was not included among the criteria of inclusion either. The whole idea of retrieving PCT data from the day before came after we analyzed the original EProK database. Secondly, despite all our efforts of allocating patients into the I- and NI-groups, this took place in a *post hoc* fashion. The available clinical results were analyzed in a blinded manner for delta-PCT (apart from PCT values at t_0) and thoroughly by our experts; however, one cannot exclude the possibility of inappropriate judgment during the decision making. The lack of gold standard for diagnosing infection is aggravated by this obscurity when configuring groups. Furthermore, the sample size was generally small, especially to be able to draw firm conclusions regarding the medical, surgical subgroups, although the trend in our results was certainly promising. Finally, it remains uncertain why PCT values were measured on the previous day before starting empiric antibiotic therapy in more than 50% of the 209 patients of the EProK study. Therefore, some selection bias cannot be excluded. The median day of inclusion into the study from ICU admission was 1 day, indicating that 50% of patients had PCT measurements on the ward/Accident and Emergency Unit, before admission. However, this may also reinforce the importance of measuring PCT values consecutively.

5. Conclusion

The main finding of this observational study was that an increase in PCT levels from the day before (t_{-1}) to the day when infection was suspected (t_0) predicted infection, while in patients with no proven infection PCT remained unchanged. Based on the data presented a single PCT measurement may not be adequate to differentiate between an infectious and noninfectious inflammatory response. This means that the kinetics of procalcitonin values based on daily measurements are superior to absolute values in diagnosing infection on the ICU and absolute values of procalcitonin may be of limited use. Both absolute values and kinetics of C-reactive protein are poor indicators of infection; furthermore, conventional indicators of infection such as white cell count and body temperature have limited use for predicting infection in the ICU. The clinical implication of these results is that daily PCT measurements in patients at high risk of infection allow the opportunity to evaluate PCT kinetics, which may improve diagnostic accuracy and rationalise antibiotic therapy on the ICU and improve outcome.

Competing Interests

János Fazakas and Zsolt Molnár receive lecture fees from Thermo Fisher Scientific, BRAHMS Gmbh. The other authors declare no competing interests regarding the publication of this paper.

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Research Article

In Vitro Effects of Some Botanicals with Anti-Inflammatory and Antitoxic Activity

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Several extrinsic factors, like drugs and chemicals, can foster autoimmunity. Tetracyclines, in particular oxytetracycline (OTC), appear to correlate with the emergence of immune-mediated diseases. Accumulation of OTC, the elective drug for gastrointestinal and respiratory infectious disease treatment in broiler chickens, was reported in chicken edible tissues and could represent a potential risk for pets and humans that could assume this antibiotic as residue in meat or in meat-derived byproducts. We investigated the *in vitro* anti-inflammatory properties of a pool of thirteen botanicals as a part of a nutraceutical diet, with proven immunomodulatory activity. In addition, we evaluated the effect of such botanicals in contrasting the *in vitro* proinflammatory toxicity of OTC. Our results showed a significant reduction in interferon- (INF-) γ production by human and canine lymphocytes in presence of botanicals ($^{*}p < 0.05$). Increased INF-γ production, dependent on 24-hour OTC-incubation of T lymphocytes, was significantly reduced by the coincubation with *Haematococcus pluvialis*, with *Glycine max*, and with the mix of all botanicals ($^{*}p < 0.05$). In conclusion, the use of these botanicals was shown to be able to contrast OTC-toxicity and could represent a new approach for the development of functional foods useful to enhance the standard pharmacological treatment in infections as well as in preventing or reducing the emergence of inflammatory diseases.

1. Introduction

The immune system has the fundamental role of not only protecting and defending the organism against infections but also controlling homeostasis and health maintenance against infections, autoimmune diseases, and tumor onset [1]. Depending on the pathogen or on antigen, two different immune responses can occur: the humoral and the cellular responses [2]. Moreover, the immune system can be classified into two fundamental phases: the innate and acquired (or adaptive) responses [3]. Innate immunity is present in vertebrates and in nonvertebrates, represents the first-line defence in the species and is based on cells (i.e., macrophages, polymorphonuclear cells, and natural killer lymphocytes) and on some mechanisms, mediated by soluble substances (i.e., complement proteins, antibodies, natural compounds, etc.) that defend the plants and animals from infections [4]. Conversely, adaptive immunity is present only in vertebrates and is a host defence related to several specific cellular mechanisms that specifically recognize the

antigens and are fundamentally expressed by B and T lymphocytes, plasma cells, and antibodies [5]. The $CD4^+$ T helper (T_H) lymphocytes represent key cells in the polarization of inflammatory/noninflammatory immune response: T_H1 and T_H2 are the most common [6]. The T_H1 response is characterized by the a secretion of INF-γ, which optimizes the bactericidal macrophages capability, induces the production of opsonizing and complement-fixing antibodies, and fosters the establishment of an optimal CTL response. The T_H2 response is characterized by interleukin- (IL-) 4, IL-5, IL-10, and IL-13 release, which results in the activation of B cells to make neutralizing noncytolytic antibodies, leading to humoral immunity [6].

Exacerbation and endurance of T_H1 response have been associated with the emergence of inflammatory diseases [6] and autoimmune disorders [7]. In particular, INF-γ appears to play a pivotal role in inducing autoimmune responses [8–16].

Several extrinsic factors, like drugs and chemicals, can foster the development of autoimmunity [17–22]. In this regard, the use of tetracyclines appears to correlate with the emergence of autoimmune diseases [23–29]. Concerning this, OTC represents the main drug used to control gastrointestinal and respiratory diseases in broiler chickens. Its accumulation was demonstrated in chicken edible tissues [30] and could represent a potential risk also for pets and humans that could assume this antibiotic as a residue in meat or in meat-derived byproducts. Recently, we published two papers evidencing the *in vitro* toxicity of bone meal-derived OTC from intensive poultry farming, in terms of apoptosis induction [31], as well as the proinflammatory cytokines, that is, INF-γ, release from peripheral blood mononuclear cells (PBMCs) cultures [32]. Moreover, we evidenced that the presence of significant concentrations of OTC in gym trained human subjects was linked to the presence of food intolerances [33]. Therefore, we hypothesized a possible modulatory activity exerted by a pool of botanicals derived from medical plants, which are successfully used in several commercially available nutraceutical diets. Intriguingly, many botanicals could have the capability to modulate the immune system [34]. In this regard, it is well known that the immunomodulatory activity of acemannan, a mucopolysaccharide extracted from *Aloe vera*, related to modulation of nitric oxide release that modulate classes I and II MHC cell surface antigens involved in antigen presentation [35, 36]. The same immunomodulating activity was observed for fermented *Carica papaya* able to increase T_{reg} cells, reduce INF-γ^+CD4^+ T cells, and possibly alter the growth of several cancer cell lines [37–39]. As to Maitake mushroom (*Grifola frondosa*), many reports have shown its ability to downregulate cytokine secretion, such as Tumor Necrosis Factor- (TNF-) α and INF-γ, as well as to inhibit adhesion molecule expression and cell-mediated immunity enhancement [40–44]. Downregulation of overexpressed cytokines in different inflammatory and immune-related inflammatory conditions was also reported for curcumin extracted from turmeric (*Curcuma longa*) [45–47]. Antiproliferative and chemopreventive effects are known to be also exerted by other curcuminoids, for example, demethoxycurcumin, bisdemethoxycurcumin, and alpha-turmerone [48, 49]. Cytokine downregulation is also performed by *Glycine max* (soybean) isoflavones that interfere with leukocyte endothelial adhesion ability [50–54]. In more detail, isoflavones, that is, genistein, can suppress dendritic cell function and cell-mediated immunity.

It is noteworthy that some botanical principles, which have been investigated in this study such as astaxanthin (from *Haematococcus pluvialis*), resveratrol (from *Polygonum cuspidatum*), and *Cucumis melo*, are characterized by antioxidant and anti-inflammatory properties as well as modulation properties towards $CD8^+$ T-cell proliferation [55, 56]. Anti-inflammatory but also oxidative stress preventing activity has been also ascribed to *Cucumis melo* extract due to its high activity on superoxide dismutase [57, 58].

Recently, we published a paper evidencing the role for a nutraceutical diet in regulating the immune response in canine *Leishmaniosis* along with standard pharmacological treatment [59]. In particular, the presence of *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* in the diet correlated with a significant decrease in T_H1 response, in terms of INF-γ production. Such evidence highlighted the anti-inflammatory effects of these specific botanicals. In addition, we suggested the anti-inflammatory effects of several botanicals added to specific diets in relieving inflammatory conditions in chronic pathologies affecting dogs [59–63].

Based on these premises, the aim of our study was to investigate the potential anti-inflammatory properties of those 13 botanicals having immune-modulatory effect as supplemented diet regulating the immune response in *Leishmaniosis* [59]. In particular, we tested the *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* and their ability to counteract the proinflammatory toxicity of OTC *in vitro*.

2. Materials and Methods

2.1. Culture Medium and Botanicals. To evaluate the cellular production of cytokines, human and canine PBMCs were incubated overnight with an *ad hoc* culture medium. Briefly, the first step was the solubilization of 1 gr of powder of each plant-derived substance in an appropriate chemical vehicle depending on solubility degree. In particular, *Ascophyllum nodosum* (pure powder of *Ascophyllum nodosum* seaweed, laminarin content min. 2.3%, and fucoidans content min. 11.4% [64]), *Aloe vera* (*Aloe vera* gel 200 : 1 powder, aloin content min. 1% [65]), *Cucumis melo* (lyophilized extract of melon, superoxide dismutase min. 1 UI/mg [57]), *Polygonum cuspidatum* (powder obtained from dried *Polygonum cuspidatum* roots, resveratrol content min. 8% [66]), *Camellia sinensis* (standardized decaffeinated green tea leaves powder, catechins content min. 75% [67]), *Carica papaya* (Papaya fermented granular, rich in papain [68]), *Glycine max* (Soy powder, 40% isoflavones [69]), and *Grifola frondosa* (maitake carpophore dry extract, polysaccharides content min. 20.0%

[70]) were solubilized in 10 mL of PBS 1x, with the exception of *Glycine max* that was added to 30 mL of PBS 1x to gain the full solubilization. *Haematococcus pluvialis* (standardized beadlets of *Haematococcus pluvialis* extract, astaxanthin content min. 2.5% [71]) was solubilized in 5 mL of dimethyl sulfoxide and 5 mL of PBS 1x. *Echinacea purpurea* (*Echinacea purpurea* dried extract, polyphenols content min 4% [72]), *Piper nigrum* (black pepper powder, piperine content min. 95% [73]), *Curcuma longa* (turmeric dried extract, curcuminoids content min. 95% [74]), and *Punica granatum* (standardized powdered extract from pomegranate, ellagic acid content min. 20% [75]) were solubilized in 4 mL of ethanol and 6 mL of water.

The solubilized botanicals were added to RPMI 1640 culture medium (Sigma-Aldrich, Milan, Italy) to obtain the *ad hoc* medium in the proportion of 1 : 10 (vehicle-solubilized substance : RPMI 1640) to preserve the good quality of cellular condition in the culture.

The cytokine cell production was evaluated in presence of the *ad hoc* medium containing the solubilized individual substance or a mixture containing all the solubilized botanicals. The vehicles employed for the solubilization were used as specific controls in the same proportion of *ad hoc* medium (1 : 10, vehicle : RPMI 1640). The mixture was composed by all *ad hoc* medium from the botanicals in a variable percentage according to that contained in the commercial canine food, previously used as immunomodulating diet able to reduce INF-γ production [59]. Briefly, the mixture contained 66.3% of *Ascophyllum nodosum*, 3.1% of *Aloe vera*, 6.1% of *Cucumis melo*, 1.5% of *Polygonum cuspidatum*, 1.5% of *Camellia sinensis*, 3.1% of *Carica papaya*, 4.6% of *Glycine max*, 6.3% of *Grifola frondosa*, 1.1% of *Haematococcus pluvialis*, 3.1% of *Echinacea purpurea*, 0.6% of *Piper nigrum*, 2.3% of *Curcuma longa*, and 1.5% of *Punica granatum*. The obtained mixture was added to RPMI 1640 culture medium to obtain the *ad hoc* medium in the proportion of 1 : 10 (vehicle/mixture : RPMI 1640) to preserve the good quality of cellular condition in the culture.

Ascophyllum nodosum, *Aloe vera*, *Cucumis melo*, *Polygonum cuspidatum*, *Camellia sinensis*, and *Haematococcus pluvialis* were purchased from Italfeed S.r.l, Milano (Italy).

Carica papaya, *Glycine max*, *Echinacea purpurea*, *Punica granatum*, *Piper nigrum*, and *Curcuma longa* were purchased from Nutraceutica S.r.l, Monterenzio, Bologna (Italy) while *Grifola frondosa* was purchased from A.C.E.F. S.p.a., Fiorenzuola D'Arda, Piacenza (Italy).

All the botanicals are in form of powder and are free from genetically modified organisms (Reg. 1829/2003-1830/2003 EC), gluten, bovine transmissible spongiform encephalopathy, and food allergens (DIR 2003/89/EC and 2006/142/EC).

2.2. Human and Canine Donors and Cell Preparation. The human blood collection from 10 healthy donor volunteers (5 males and 5 females, 20–30 years old) was performed at the Haemotrasfusional Center of University of Naples "Federico II," according to standard procedures and used within the 3 hours from the collection.

Peripheral blood was collected from ten healthy dogs (5 males and 5 females, 5–9 years old and ranging between 15 and 35 kg in weight). All dogs were enrolled with the owner consent in the Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II." Human or canine PBMCs were isolated by centrifugation on Lymphoprep (Nycomed Pharma) gradients, as previously described [59, 76]. Obtained PBMCs were considered as mixed population of T and non-T lymphocytes.

2.3. Monoclonal Antibodies, Detection of Intracellular Cytokine Production, and Flow Cytometry. For the immunefluorescent staining a panel of fluorescent-labelled monoclonal antibodies (mAbs) was used to evaluate the human CD3, CD8, INF-γ, and IL-4, as well as a panel of isotype-matched mAb controls (Becton Dickinson Pharmingen, San Jose, California). In addition, we used several fluorescent-labelled mAbs against canine CD3, CD4, CD8, CD45, INF-γ, and IL-4 molecules and isotype-matched controls (Serotec Ltd., London, UK).

To analyze the production of INF-γ and IL-4 cytokines, 2×10^6/mL purified PBMCs were incubated overnight (10–12 hours) in the *ad hoc* medium of each botanical or of mixture (see Section 2.1). In particular, to obtain the cytokine production, PBMCs were always cultured in presence of 500 ng/mL of phorbol-12-myristate-13-acetate (PMA) and 1 μg/mL of Ionomycin (Sigma-Aldrich), as described in [77]. To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/mL of Brefeldin-A (Sigma-Aldrich), as described in [77].

To test the ability of botanicals in contrasting the toxic role of OTC, we used the commercial preparation of the drug (Oxytetracycline 20%®, TreI, Reggio Emilia, Italy). 1 μg of OTC [31] was added to cell culture and incubated for overnight (10–12 hours) as previously described [32]. In addition, *Haematococcus pluvialis* or *Glycine max* or the mixture *ad hoc* medium was used in the coincubation of cells with OTC and all along the overnight (10–12 hours) culture.

At the end of overnight (10–12 hours) incubation, the above incubated cells were fixed and permeabilized by using a commercial cytokine staining kit following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA, USA). Briefly, the cell fixing and permeabilization procedure were of 20 min at 4°C each. At the end of procedure, PBMCs were washed twice by centrifugation (800 ×g) in RMPI 1640 culture medium.

PBMCs were stimulated overnight with PMA and Ionomycin, cultured in a medium containing the botanicals solubilization buffer (vehicle), and used as control points. The proportion of vehicle and RPMI 1640 was the same of *ad hoc* medium (1 : 10 ratio).

The intracellular cytokine production was evaluated by using the triple staining technique and analyzed by flow cytometry (FACSCalibur platform) and CellQuest Software (Becton Dickinson Pharmingen, San Jose, California). The analyzed cells were always gated (R1 in dot plot of Figures 1(a) and 2(a)) on forward scatter (FSC) and side scatter (SSC) FACS parameters (cell size and cell complexity, resp.) to reasonably select the region of viable lymphocytes in order to avoid any interference due to the possible presence of death cells.

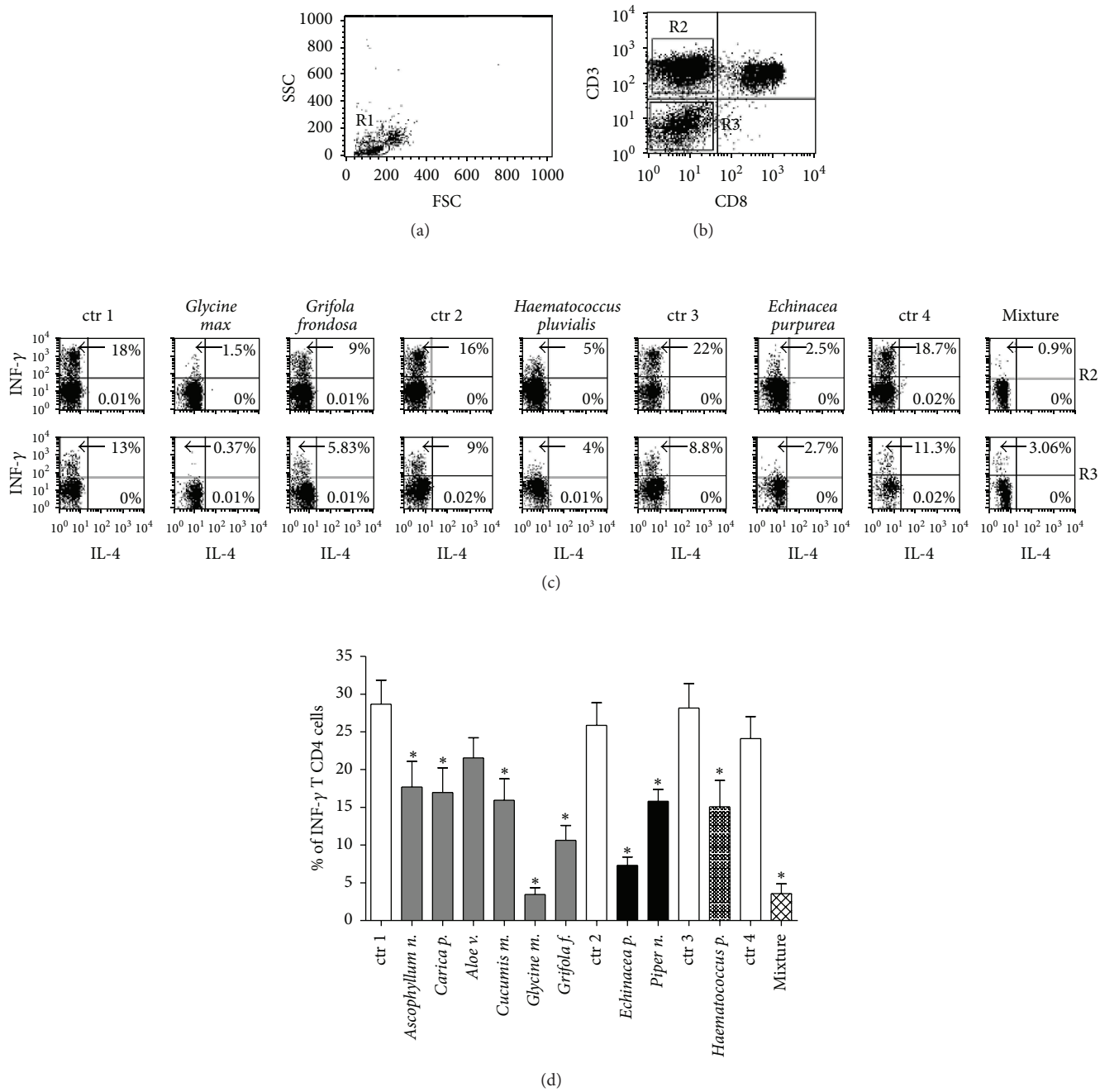

Figure 1: The effects of botanicals on cytokine production by human PBMCs. (a) shows the gating on viable lymphocytes (R1 in dot plot graph) based on FSC and SSC parameters (see Section 2); (b) represents the gating on T_H lymphocytes ($CD3^+$ $CD8^-$ as R2 in the dot plot graph) and on non-T cells ($CD3^-$ $CD8^-$ cells as R3 in the dot plot graph); and (c) shows the INF-γ and IL-4 production in human T_H lymphocytes and non-T cells incubated with *ad hoc* medium derived from botanicals or from mixture (see Section 2). Cytokine production was evaluated as percentage of INF-γ (y-axis) and IL-4 (x-axis) producing cells. The percentage of INF-γ (upper left quadrant inside the dot plots) and IL-4 (low right quadrant inside the dot plots) producing CD4 T (R2) and non-T (R3) cells are reported. The different cell incubations with *ad hoc* medium derived from botanicals or from mixture (see Section 2) are indicated on the top of each graph. (d) reports the statistic representation of 10 experiments on human $CD4^+$ T Lymphocytes evaluated as percentage of INF-γ producing cells, $^*p < 0.05$. The different cell-incubations with *ad hoc* medium derived from botanicals or from mixture (see Section 2) are indicated on the top of each column. The abbreviation "ctr" in (c) and (d) indicates the basal cytokine production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2); specifically, ctr 1 (*Ascophyllum n.*, *Carica p.*, *Aloe v.*, *Cucumis m.*, *Glycine m.*, and *Grifola f.*), ctr 2 (*Echinacea p.*, *Piper n.*), ctr 3 (*Haematococcus p.*), and ctr 4 (the mixture of all the botanicals).

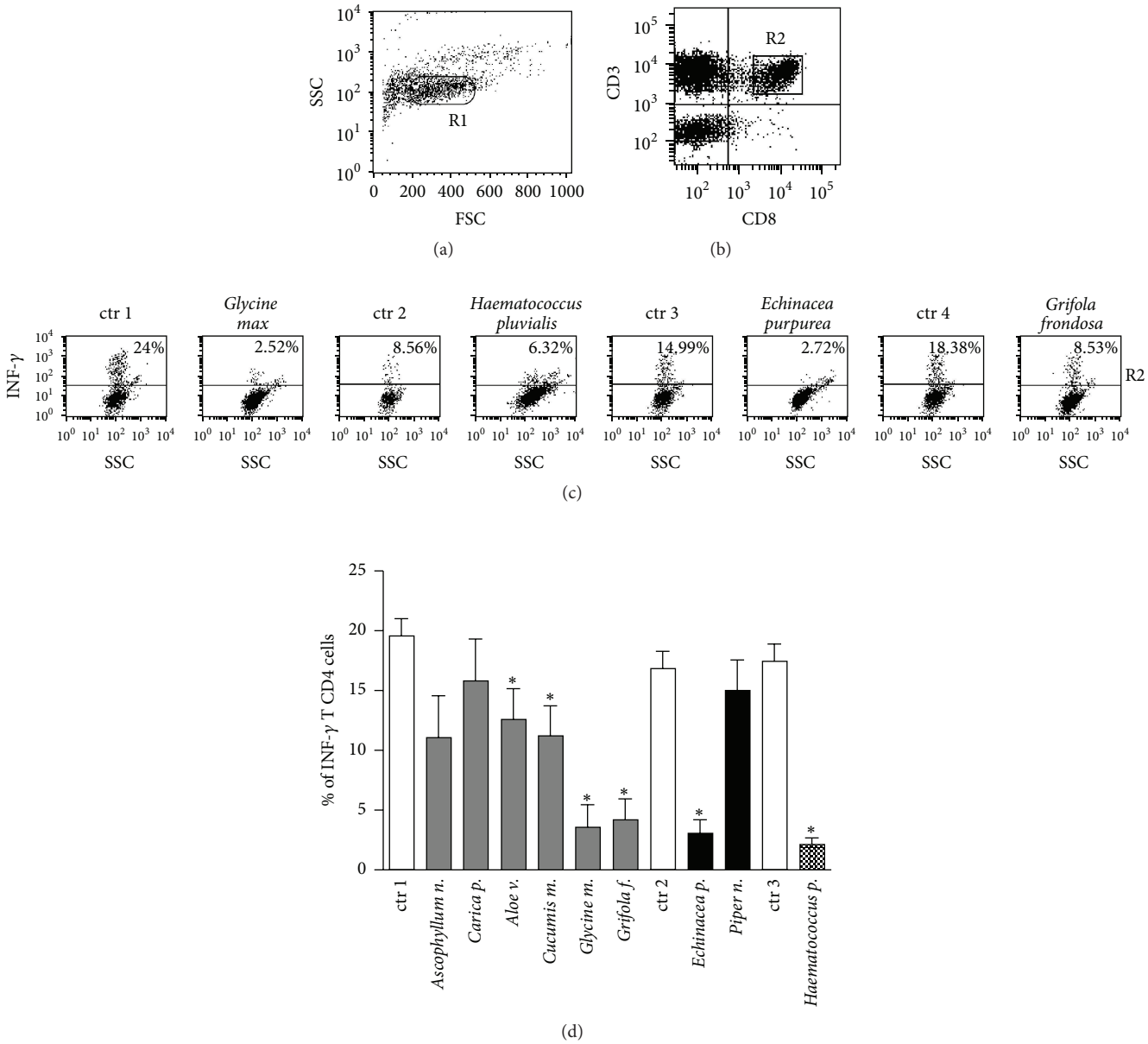

Figure 2: The effects of botanicals on INF-γ production by canine PBMCs. (a) shows the gating on viable lymphocytes (R1 in dot plot graph) based on FSC and SSC parameters (see Section 2). (b) represents the gating on $CD4^+$ T lymphocytes ($CD3^+$ $CD8^-$ as R2 in the dot plot graph). (c) reports the results from one representative experiment showing the percentage (the number in upper quadrant) of INF-γ producing canine $CD4^+$ T lymphocytes gated on R2 (y-axis); x-axis indicates the SSC parameter (see Section 2). The different coincubations of cells with *ad hoc* medium or mixture (see Section 2) are indicated on the top. (d) shows the statistic representation the INF-γ production by canine $CD4^+$ T Lymphocytes evaluated as percentage of INF-γ producing cells in 10 representative experiments, $^*p < 0.05$. The abbreviation "ctr" in (c) and (d) indicates the basal INF-γ production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2): specifically, ctr 1 (*Ascophyllum n.*, *Carica p.*, *Aloe v.*, *Cucumis v.*, *Glycine m.*, and *Grifola f.*), ctr 2 (*Echinacea p.*, *Piper n.*), and ctr 3 (*Haematococcus p.*).

2.4. Statistical Analysis. Data are presented as the means ± standard error of the mean (SEM) and were firstly checked for normality using the D'Agostino-Pearson normality test. The Kruskal-Wallis followed by Dunn's multiple comparisons analysis was performed. A $^*p < 0.05$ was considered significant. Statistics was performed by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1. The Anti-Inflammatory Effect of Botanicals as Significant Reduction of INF-γ Production in Human T and Non-T Lymphocytes. We focused on INF-γ production, as the main proinflammatory cytokine able to foster the T_H1 and non-T cell immune responses involved in several etiopathogenic

mechanisms at the basis of inflammatory-mediated disease [6].

As shown in Figure 1, the overnight incubation (10–12 hours) with each botanical as well as with a mix of all botanicals induced a significant decrease in INF-γ production in the T_H lymphocytes ($CD3^+$ $CD8^-$ cells gated as R1 in the dot plot graphs of Figures 1(b) and 1(c) and reported as mean ± SEM of 10 experiments in Figure 1(d)) and in non-T cells, mainly represented by NK lymphocytes ($CD3^-$ $CD8^-$ cells gated as R2 in the dot plot graphs of Figure 1(c)). In particular, the individual incubation with *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to reduce INF-γ production. Intriguingly, despite the obtained slightly decrease in cytokine production, the *Aloe vera* incubation did not induce a significant reduction from the statistical point of view (Figure 1(d)). In this regard, we cannot rule out that a larger number of experiments (more than the 10 performed in this study and summarized in Figure 1(d)) or a higher concentration of substance could confirm the reduction in lymphocyte INF-γ production by the incubation with this botanical.

The basal IL-4 production was undetectable or only slightly detectable in T and non-T lymphocytes, as expected in PBMCs from healthy human donors after exposure to PMA and Ionomycin [77] and was not modulated after the overnight incubation with the botanicals (Figure 1(c)). Each specific vehicle, used to solubilize the botanicals, was used as control and the obtained value was substracted from each experimental point to obtain the correction following the formula "the value obtained from cell culture in presence of botanicals – the value obtained from cell culture in presence of the vehicle alone = corrected experimental point value." It is of note that even if the used vehicles appeared to not induce significant cell death in the culture, the flow cytometry analysis was always performed by gating on viable cells to avoid any possible interference dependent on death cells (see Figure 1(a) and Section 2.3). Moreover, the *ad hoc* medium from botanicals did not exert effect in absence of PMA and Ionomycin stimulation (data not shown).

3.2. The Anti-Inflammatory Effect of Botanicals as Significant Reduction of INF-γ Production in Canine $CD4^+$ T Lymphocytes. The individual incubation with *Ascophyllum nodosum*, *Cucumis melo*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to significantly decrease the INF-γ production in the $CD4^+$ lymphocytes (dot plot graphs in Figure 2(c), summarized in Figure 2(d)). In contrast, the incubation with *Carica papaya* or with *Piper nigrum* seemed not to induce a statistically significant reduction (Figure 2(c)). Also, in this case, as referred to in human experiments, we cannot rule out that a larger number of experiments (more than the 10 performed in this study, summarized in Figure 2(d)) or a higher concentration of the substances could confirm the reduction in lymphocyte INF-γ production by the incubation with these two botanicals.

IL-4 production was undetectable in T lymphocytes, as expected in PBMCs from healthy dogs after exposure to PMA and Ionomycin [59], and was not modulated after the overnight incubation with the botanicals (data not shown).

The specific vehicles, employed to solubilize the substances, were used as controls and the resulting values were substracted from experimental points, as described (see Section 3.1). Flow cytometry analysis was always performed by gating on viable cells to avoid any possible interference dependent on death cells (see Figure 2(a) and Section 2.3).

3.3. The Anti-Inflammatory Effect of Botanicals as Significantly Contrasting Effect on INF-γ Production Dependent on OTC Exposure of Human T Lymphocytes. Notably, the individual incubation with *Haematococcus pluvialis* or with *Glycine max* was able to contrast the previously demonstrated proinflammatory effect of OTC in human T lymphocytes [32]. Indeed, the increased INF-γ production, dependent on 24-hour OTC-incubation of T lymphocytes, was strongly reduced by the coincubation with *Haematococcus pluvialis* or *Glycine max* (Figures 3(a) and 2(b), resp.). Note that the individual incubation with the botanicals, other than *Haematococcus pluvialis* and *Glycine max*, was unable to contrast OTC-toxicity (data not shown), while the mixture of all substances exerted a significant effect. Nevertheless, as referred to in previous sections, we cannot rule out that a larger number of experiments or a higher concentration of each substance could confirm the anti-OTC effect also for the other tested botanicals.

The specific vehicles, used to solubilize the substances, were considered as controls and the resulting values were substracted from experimental points, as described (see Section 3.1).

4. Conclusions

This study was inspired by two recently published *in vivo* observations in which we suggested a potential anti-inflammatory effect of some nutraceutical diets, containing the studied botanicals, in infectious and inflammatory diseases [59–61].

In particular, we observed that a diet enriched by *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to reduce proinflammatory T cell responses in canine *Leishmaniosis* [59] and the clinical feature of ear inflammation in chronic otitis in dogs [60].

Here, we observed the *in vitro* effect of *Ascophyllum nodosum*, *Cucumis melo*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* in reducing *in vitro* proinflammatory cytokine production by human and canine PBMCs. These botanicals appeared to exert a potential anti-inflammatory effect that was evident in the reduction of INF-γ production in human T and non-T cells and in canine T lymphocytes. Conversely, *Aloe vera*,

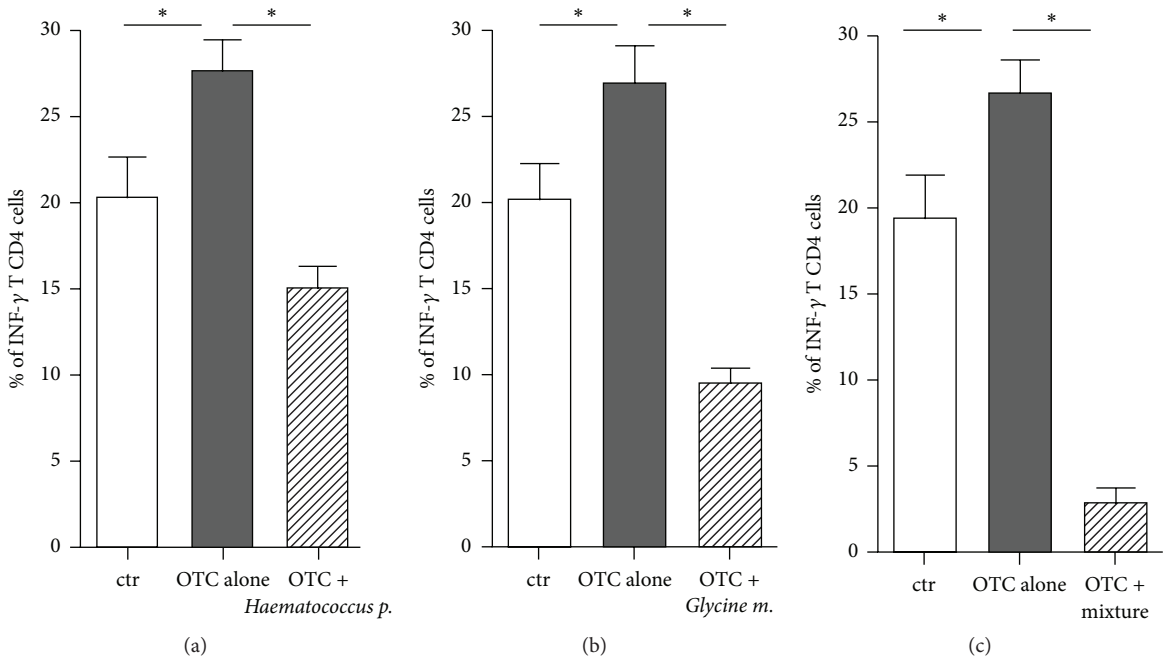

Figure 3: Statistic representation of the INF-γ production in human $CD4^+$ T Lymphocytes after the OTC exposure and the contrasting effects after botanicals challenge in 10 representative experiments. (a) *Haematococcus p.*; (b) *Glycine m.*; and (c) the mixture of all the botanicals. Cytokine production was evaluated as percentage of INF-γ producing T $CD4^+$ cells. All the incubations (basal, OTC alone, and OTC + botanical) were performed in the *ad hoc* medium based on the vehicle used to solubilize the botanical, so that the abbreviations "ctr" indicate the basal INF-γ production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2). $^*p < 0.05$.

Carica papaya, and *Piper nigrum* appeared to be ineffective in reducing this cytokine production. These results seem to be contradictory with the data observed in dogs [59], where the diet containing all these botanicals, including *Aloe vera*, *Carica papaya*, and *Piper nigrum*, exerted a therapeutic effect by reducing the inflammatory aspects of *Leishmaniosis*. Such apparent contradiction may be explained by the different sensitivity between *in vitro* and *in vivo*, as well as by the fact that *in vivo* botanicals are probably synergized in the combined administration as in the diet. In this regard, this latter consideration fits with *in vitro* effect obtained by the mixed incubation with all substances that induced the INF-γ decrease.

Moreover, as stated in Section 3, we cannot rule out that a larger number of experiments or a higher concentration of substances could confirm the reduction in lymphocyte INF-γ production also by *Aloe vera*, *Carica papaya*, and *Piper nigrum*.

Taken together, our observation highlighted the relevance for the use of botanicals to modulate the inflammatory responses in both dogs and humans. Indeed, exacerbation and the persistence of T_H1 response frequently result in the emergence of inflammatory diseases [6] and autoimmunity disorders [7] and the increase of INF-γ production is associated with autoimmunity in humans [8–16]. In addition, some of the botanicals used in this study were previously suggested as antioxidants and immune-modulating substances to reach the physiological status in several models of disease in human [55, 78] and animals [59, 60, 79–83].

Moreover, this study was also inspired by our recent paper, which evidenced the *in vitro* toxicity of OTC in terms of inflammatory response increase by human lymphocytes [32]. In this regard, here we evaluated the potential ability of *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* to contrast the OTC-toxicity exerted *in vitro* in human lymphocytes.

Our data suggested that the incubation with the mixture of these botanicals clearly reduced the OTC-induced INF-γ production in T cells. It is of relevance that the individual incubation with *Haematococcus pluvialis* or with *Glycine max* significantly reduced this cytokine production.

Such evidence may shed new light on the misunderstood scenario resulting from the increasing emergence of inflammatory diseases in humans, dogs, and cats [84–89]. Moreover, it has been suggested that tetracycline, in particular OTC, could take part in this scenario and could represent harmful compounds for human health and animals fed meat derived from intensive livestock [25–30, 33, 90].

In conclusion, this study could open an interesting approach regarding the use of anti-inflammatory and antioxidant botanicals in immune-mediated pathologies and in infectious diseases as well as to counteract the effect of several putative toxic substances present in food, such as the OTC, which can cause inflammatory disorders and diseases.

Ethical Approval

This study has been reviewed by Ethical Animal Care and Use Committee of the University of Naples Federico II and received formal Institutional approval (Protocol no. 2015/0071388) in accordance with local and national law, regulations, and guidelines (Circular no. 14 of September 25, 1996, and Italian civil code article 1175).

Consent

Informed consent from human donors was obtained in accordance with the Declaration of Helsinki, as approved within the study protocol by the Institutional Review Board at the Federico II University of Naples.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. This research was performed in collaboration with some scientists from the Division of Research and Development of SANYpet S.p.A. (as indicated in the authors' affiliations) according to scientific and ethical principles of the scientific community.

Authors' Contributions

Gianandrea Guidetti, Alessandro Di Cerbo, Angela Giovazzino, and Valentina Rubino contributed equally to this work.

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