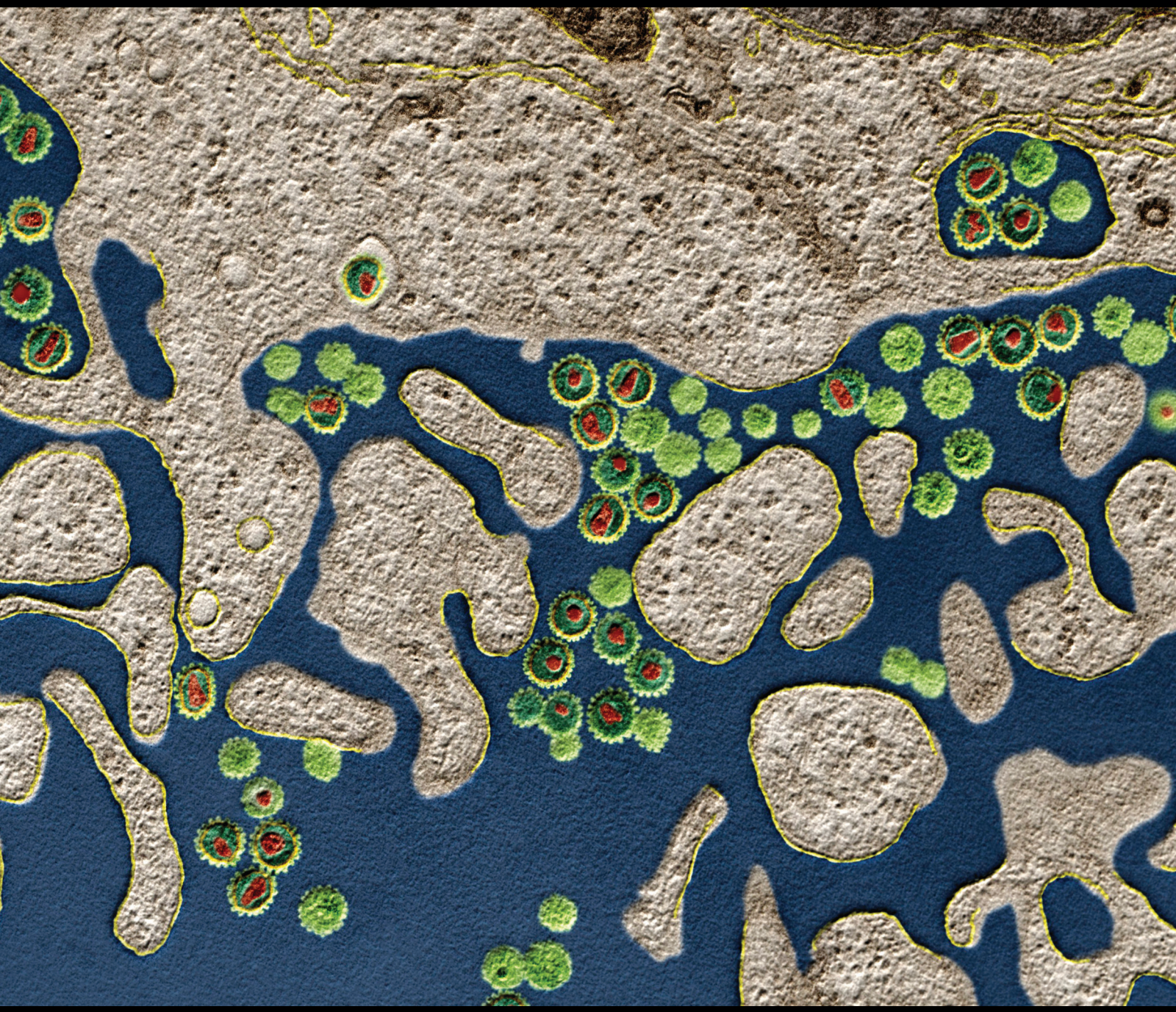
Journal of Immunology Research

Vaccines of the Future: The Role of Inflammation and Adjuvanticity

Guest Editors: Cheol-Heui Yun, Luciana C.C. Leite, Aldo Tagliabue, and Diana Boraschi



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Editorial Board

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Editorial

Vaccines of the Future: The Role of Inflammation and Adjuvanticity

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With the increase of life expectancy worldwide, people expect a better quality of life, and vaccination is possibly one of the best tools for improving it. In addition to developing or improving vaccines for the elimination of infectious diseases such as malaria, tuberculosis and AIDS, the challenge of future vaccination strategies is both to improve efficacy of currently available vaccines for groups of people with frail immunity, such as the elderly or patients with chronic diseases, and to develop new vaccines for non-communicable diseases such as autoimmunity and cancer. More personalized or precise vaccination is one of the aims of future vaccines, taking into account age, gender, health and nutritional status, together with geographical/environmental conditions.

Vaccine efficacy is greatly enhanced by adjuvants. Adjuvants are agents or strategies that cause the initiation and generation of protective memory by inducing a mild innate/inflammatory reaction followed by the amplification of vaccine-specific adaptive immunity. Safety has therefore been a major issue with adjuvants, since adverse effects are hard to avoid and inevitable especially after a strong inflammatory reaction. For a long time, the only adjuvant approved for human use has been Alum (particulate aluminium salts), until the recent development and approval of new adjuvants such as monophosphoryl lipid A (MPL) and oil-in-water emulsions (such as MF59). It is clear that development of new safer adjuvants coincident with the improved design of their use will greatly influence the efficacy of modern vaccines. Novel technologies will allow us to achieve better and safer vaccination and protection through the induction of broadly neutralizing antibodies. Novel adjuvants/delivery systems for multiple antigens will be developed by structural vaccinology, reverse vaccinology, and nanotechnology. With biodegradable nanosized materials, we expect to maximize vaccine efficacy through better targeted delivery and concomitant adjuvanticity.

In this exciting period of reborn interest for vaccines, it is important that we investigate and revisit the mechanism of action of old and new adjuvants and provide insights for their practical use in vaccine formulations. Thanks to the impressive advancements in the knowledge of innate immune mechanisms, including activation of Pattern Recognition receptors, in particular Toll-like receptors (TLR), and the new concept of innate memory, novel adjuvants will be able to selectively activate one or more of these pathways in a controlled fashion, thereby achieving optimal efficacy and reducing adverse effects.

In this special issue, a number of papers will illustrate the most recent advances in the concept of adjuvanticity and controlled inflammation in achieving optimal protection and concomitant safety. E. Töpfer et al. will introduce the concept of innate memory and how this could be exploited for improving vaccine efficacy. A group of papers will then illustrate the most recent advances in exploiting TLR agonism for optimizing adjuvanticity and modulating the resulting immune responses (J. Bortolatto et al., M. Herbáth et al.,

T. Aoshi et al., and N. S. Daifalla et al.). Other contributions will describe new technologies for designing vaccine antigens with endowed adjuvanticity (L. D'Apice et al.), for exploiting extracellular vesicles to concomitantly obtain antigen delivery and adjuvanticity (J. H. Campos et al.), and for precise localization and delivery of plasmid DNA in veterinarian DNA vaccines (D. Dory et al.). A last group of papers deal with the key issue of efficacy and safety. S. Di Mario et al. discuss significant differences in the efficacy of two anti-HPV vaccines in naive versus infected women. A. T. Gunes et al. address the issue of possible effects of vaccination on triggering immune-related affections. Last but not least, D. Lewis and M. Lythgoe close the special issue by presenting a comprehensive platform of systems vaccinology for evaluating the inflammatory and reactogenic effects of adjuvanted vaccines, with the final goal of ensuring the optimal safety of future vaccination strategies.

It is our hope that the knowledge-based development of new adjuvanted vaccines, relying on the modulation of inflammatory responses suitable for the protection, will not only provide improved protection against new and re-emerging infectious diseases, but also enhance the quality of life of the human population.

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Research Article

Stimulation of Innate and Adaptive Immunity by Using Filamentous Bacteriophage fd Targeted to DEC-205

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The filamentous bacteriophage fd, codisplaying antigenic determinants and a single chain antibody fragment directed against the dendritic cell receptor DEC-205, is a promising vaccine candidate for its safety and its ability to elicit innate and adaptive immune response in absence of adjuvants. By using a system vaccinology approach based on RNA-Sequencing (RNA-Seq) analysis, we describe a relevant gene modulation in dendritic cells pulsed with anti-DEC-205 bacteriophages fd. RNA-Seq data analysis indicates that the bacteriophage fd virions are sensed as a pathogen by dendritic cells; they activate the danger receptors that trigger an innate immune response and thus confer a strong adjuvanticity that is needed to obtain a long-lasting adaptive immune response.

1. Introduction

Vaccines are one of the most successful outcomes of modern medicine in improving the global health. Nevertheless, many diseases are still a challenge for vaccine development and the attempt to make new vaccines using more recent technologies has required the use of adjuvants, which enhance the magnitude and modulate the quality of the immune response.

In this context, recent failure in producing functional vaccines against emerging diseases has shown that formulating a vaccine able to induce a protective immunity should involve the innate immunity. To this purpose, adjuvants should be natural ligands or synthetic agonists for pattern-recognition receptors (PRRs) that are the molecules responsible of sensing microbes. Among the PRRs, toll-like receptors (TLRs), C-type lectin-like receptors, and the cytosolic NOD-like receptors sense a broad range of microbial *stimuli*, and the cytosolic RIG-I-like receptors sense viral nucleic acids [1].

PRR activation stimulates the production of proinflammatory cytokines/chemokines and type I Interferons (IFNs) that increase the host's ability to eliminate the pathogen. Thus, the incorporation of pathogen associated with molecular patterns (PAMPs) in vaccine formulations can improve and accelerate the induction of vaccine-specific responses.

The adjuvants currently licensed for human use are alum, an aluminum salt-based adjuvant, AS04, an adjuvant composed of monophosphoryl lipid A (MPL) adsorbed to alum, the oil-in-water emulsions, such as MF59 and AS03, and virosomes, composed of lipids and hemagglutinin [2]. Each of these approved adjuvant components has drawbacks: aluminium-based adjuvants determine macrophagic myofasciitis and delayed-type hypersensitivity [3], while both AS04 and MF59 have cost limitations due to the expensive process of MPL purification and the use of nonrenewable resource as shark oil (for MF59). These considerations highlight the need to develop new types of adjuvants able to interact with the innate immune system.

Here we propose the filamentous bacteriophage antigen display system as a candidate vaccine able to induce both the innate and the adaptive response. This system is based on a nonpathogenic procaryotic virus, well characterized at both structural and genetic level [4]. The use of bacteriophage as antigen delivery system is based on modification of the phage display technology. In particular, it is designed to express

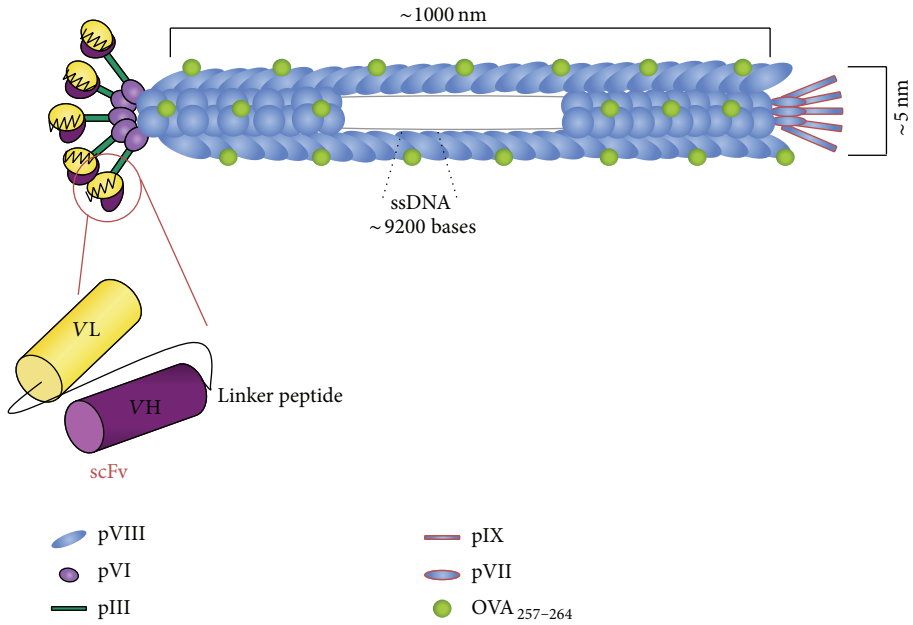

Figure 1: Schematic representation of the engineered filamentous bacteriophage fdOVA/sc-αDEC displaying single chain variable fragment (scFv) anti-DEC-205 molecules as fusion to pIII proteins and the $OVA_{(257–264)}$ peptide as N-terminal fusion to pVIII proteins. The scFv is composed of heavy (VH) and light (VL) chain variable regions of the mouse monoclonal antibody NLDC145, assembled with a (Gly4Ser)3 linker to yield a single-chain fragment binding the mouse dendritic cells DEC-205 receptor. About 9200 bases of engineered genome include about 6400 bases of wild type DNA, the β-lactamase gene conferring the Ampicillin resistance, one additional copy of pVIII gene plus bases coding for the $OVA_{(257–264)}$ peptide, and the sequence encoding for the single chain antibody fragment anti-DEC-205 plus the HA tag.

multiple copies of exogenous peptides (or polypeptides) as fusions to viral capsid proteins. Recombinant virions that carry multiple copies of exogenous sequences can be easily generated cloning a double strand DNA fragment in the phage genome. The protein used to display short antigenic peptides on the phage surface is the pVIII [5]. Such protein, with 2,700 copies per wild type virion, allows the display of a large number of foreign antigenic sequences. Its major limitation relies on the number of amino acids that can be displayed without disrupting the phage assembly [5]. The best strategy to display long exogenous polypeptides is to use the pIII protein, which allows accommodating even a whole protein on the viral surface, although in a maximum of five copies per virion [6].

Thus, the ability of filamentous bacteriophages to tolerate recombinant coat proteins showing short peptides (on pVIII) or bigger polypeptides (on pIII) makes this virus appealing as antigenic carrier. Indeed, it has been already demonstrated to be a powerful delivery system in numerous vaccine development studies [7–11].

We have previously described that the filamentous bacteriophage, when engineered to express antigenic epitopes, elicits T cell help [7] and triggers cytotoxic T cell-mediated response [8]. More recently, we have further improved this delivery system by targeting fd particles to dendritic cells (DCs) *via* DEC-205, an endocytic receptor expressed mainly by dendritic cells [11]. DEC-205 is a C type I lectin-like receptor with ten CRD-like domains and a cytoplasmic tail containing a membrane proximal tyrosine-based region for internalization in clathrin-coated vesicles and a distal region with an EDE amino acid triad for the targeting to late endosome and lysosome and for the recycling to cell surface. DEC-205 is able to internalise and deliver antigens to late endolysosomal compartments allowing the degradation and enhancing efficiency of antigen presentation by dendritic cells [12]. Therefore, it represents a promising receptor for antigen delivery in dendritic cell-targeted vaccines.

As a proof of principle, we have produced a double hybrid bacteriophage expressing the antigenic determinant $OVA_{(257–264)}$ cytotoxic peptide at N-terminus of the pVIII protein and the single chain variable fragment of the NLDC145 antibody directed against the mouse DEC-205 receptor (Figure 1). We have demonstrated that this double-displaying bacteriophage induces stronger antigenic response if compared to nontargeted bacteriophage, enhancing uptake by dendritic cells and inducing DC maturation [11].

The double recombinant bacteriophage represents a powerful delivery system able to target specifically DCs, to promote DC maturation, and to induce specific $CD8^+$ T cell response even if administered in the absence of adjuvants or maturation *stimuli*. The ability of fd bacteriophage targeted to DEC-205 (fdsc-αDEC) to induce this strong immune response in the absence of exogenous adjuvants is due to DEC-205-mediated delivering of fd particles into endolysosomal LAMP-1+ compartments and their subsequent colocalization with the innate immune Toll like receptor TLR9 [13]. TLR9, which detects CpG-rich viral DNA, is thus activated by the single-strand DNA genome rich in CpG motifs, and

this activation leads to an enhanced immunogenicity of the antigenic determinants displayed on the bacteriophage coat [14].

Using a system vaccinology approach based on RNA-Sequencing analysis of bone marrow derived dendritic cells (BMDCs) pulsed with fdsc-αDEC, here we report new insights about the molecular mechanisms by which filamentous bacteriophage induces protective immunity. Our data reveal the ability of this valuable antigen delivery system to induce wide changes in the gene expression pattern of dendritic cells. Such modifications mostly overlap with those induced by different pathogens (bacteria, fungi, and protozoan) in the same cells. Many of the differentially expressed genes are under the control of proinflammatory cytokines and in particular of the interferon molecules. Finally, some of the upregulated genes have been recently described and proposed as biomarkers of vaccine efficacy, strengthening the relevance of our findings.

2. Materials and Methods

2.1. Purification of Bacteriophage Particles and Western Blot. Recombinant fdsc-αDEC (expressing a single chain variable fragment against mouse DEC-205 molecule) and fdOVA/sc-αDEC bacteriophages (expressing $OVA_{(257–264)}$ epitope and the anti-DEC-205 scFv) were in PBS solution and purified as described previously [11]. The hybrid phage preparations carrying the $OVA_{(257–264)}$ peptide displayed 20% copies of the recombinant pVIII protein, as estimated by N-terminal sequence analysis of the purified virions. Elimination of lipopolysaccharide was performed according to Aida and Pabst [15] by extraction with Triton X-114 (Sigma-Aldrich, Milan, Italy) and assessed using the limulus amebocyte lysate (LAL) assay (QCL-1000, Lonza, Basel, Switzerland), according to the manufacturer's instructions. The expression of the scFv anti-DEC-205 in the pIII protein of the purified virions was assessed by Western blot analysis using a mouse anti-HA tag mAb (Roche-Boehringer, Basel, Switzerland). Bands of interest were visualized using enhanced chemiluminescence reagent (Thermo Fisher Scientific, IL, USA) and quantified by densitometry (VersaDoc imaging system and Quantity One Analysis Software, Bio-Rad, Milan, Italy).

2.2. Mice. Six- to eight-week-old female C57BL/6 and ovalbumin ($OVA_{257–264}$) specific TCR transgenic OT-I mice were purchased from Charles River (Lecco, Italy) and were maintained under specific pathogen-free conditions. All experiments with mice were performed in accordance with European union laws and guidelines. All animal studies were approved by our institutional review board and the animal procedures (i.e., immunization and sacrifice) were performed according to rules approved by the ethics committee (permission number 137/2006-a).

2.3. Antibodies and Flow Cytometry. Antibodies used for flow cytometry were all from Biolegend and were as follows: anti-CD8-PE-Cy7 (53-6.7), anti-Vα2-TCR-PE (B20.1), and anti-CD11c-APC (N418). Staining was performed in PBS containing 0.5% BSA for 30 minutes on ice using standard protocols. Data were acquired and analysed by a BD FACSCanto II flow-cytometer and DIVA software (Becton Dickinson, Fullerton, CA).

2.4. BMDCs Differentiation and Culture. BMDCs were produced from precursors isolated from the bone marrow of C57BL/6 mice by culturing them with 200 U/mL of recombinant murine granulocyte/macrophage-colony stimulating factor (GM-CSF) (Peprotech, NJ, USA) in RPMI 1640 (Lonza) medium supplemented with 10% FCS, 100 Units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 55 μM 2-mercaptoethanol (all from GIBCO, Milan, Italy). Cells were collected at day 7 of culture and were assayed for their phenotypes of dendritic cells by staining with the monoclonal antibody anti-CD11c.

2.5. RNA-Seq Library Production, Sequencing, and Data Analysis. BMDCs were plated in presence or absence of 100 μg/mL of the LPS-purified bacteriophage fdsc-αDEC for 20 hours. Total RNA was extracted from cultures using Tri Reagent (Sigma-Aldrich) according to manufacturer's protocol. The integrity and quantity of RNAs were assessed by denaturing agarose gel electrophoresis (presence of sharp 28S and 18S bands) and by spectrophotometry (NanoDrop Technologies). RNA quality was assessed as described in Costa et al. [16]. Paired-end libraries (100×2 bp) were prepared using the TruSeq RNA Sample Preparation Kit (Illumina), following the manufacturer's instructions. Libraries were sequenced on the Illumina HiSeq2000 NGS platform at high coverage. A total of about 220 million paired-end reads were sequenced. Reads quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mapping to the reference mouse genome (mm9) and to RefSeq transcripts was achieved using TopHat version 2.0.10 [17]. Uniquely mapped reads (about 95% of sequenced reads) were used for further analyses. SamTools and BEDTools were used to convert alignment formats and to produce coverage files (bedgraph format). UCSC Genome Browser was used for quality assessment of mapped reads and to inspect gene-specific features. Cufflinks and Cuffdiff were used to quantify gene expression and to identify differentially expressed genes (DEGs) [18]. PANTHER [19] was used to classify DEGs and DAVID [20, 21], to assess gene ontology, and to perform pathway analysis enrichment on the list of DEGs. Interferon-regulated gene analysis was performed using the INTERFEROME 2.0 bioinformatic database [22].

2.6. Adoptive Transfer and T Cell Assays. $CD8^+$ $OVA_{(257–264)}$ specific T cells were purified from spleen of OT-I mice using the $CD8^+$ T cell isolation kit (Miltenyi Biotec). Cells were labelled by incubation with 1 μM CFSE (Biolegend) for 10 minutes at 37°C. The staining was quenched adding ice cold RPMI1640 media containing 10% FCS.

3.5×10^6 CFSE labeled OT-I $CD8^+$ T cells were then injected intravenously into C57BL/6 recipient mice ($n = 5$/group). 24 hours later, mice were immunised subcutaneously with 50 μg of fdOVA/sc-αDEC phage particles in PBS 1X. As control, mice were injected with vehicle alone.

After 3 days, splenocytes were isolated and stained with anti-Vα2-PE and anti-CD8-PE-Cy7 mAbs. The CFSE fluorescence intensity of OT-I cells was then evaluated by flow cytometry as previously described [11].

2.7. Real-Time Quantitative PCR. Total RNA was isolated using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions. For each sample, 500 ng of total RNA was reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer's protocol. cDNAs were then used as template for quantitative real-time polymerase chain reaction assays. Amplification reaction mix contained 1x iTaq Universal SYBR Green Supermix (Bio-Rad), 400 nM of each primer, and 25 ng of cDNA (RNA equivalent) as template. PCR conditions were 95°C 30 sec followed by 40 cycles of 95°C × 5 sec and 60°C × 30 sec. Melting curves were generated after amplification using instrument default setting. Data were collected using the CFX Connect real-time PCR detection system (Bio-Rad); each reaction was performed in duplicate. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and *Actb* was used as housekeeping gene. Primers were designed using Oligo 4.0-s. Sequences of the primers are

Isg15.F: 5′AGCAAGCAGCCAGAAGCAGA3′,
Isg15.R: 5′CCCCTTTCGTTCCTCACCA3′,
Irf7.F: 5′TGCTGTTTGGAGACTGGCTAT3′,
Irf7.R: 5′GGCTCACTTCTTCCCTATTTT3′,
Il1b.F: 5′ACAAGGAGAACCAAGCAACGA3′,
Il1b.R: 5′TGTCCTGACCACTGTTGTTTC3′,
Actb.F: 5′TTCTTTGCAGCTCCTTCGTT3′,
Actb.R: 5′GCACATGCCGGAGCCGTT3′.

2.8. Statistical Analysis. Results are expressed as the mean ± SD. The statistical significance of differences between experimental groups was calculated using the unpaired two-tailed Student's t-test. Results with a p value < 0.05 were considered significant.

3. Results

3.1. In Vivo Antigen Specific CD8 T Cell Proliferation after fdOVA/sc-αDEC Bacteriophage Immunization. The ability of bacteriophage particles to induce a strong immune response was assayed using the double hybrid bacteriophage coexpressing the $OVA_{(257–264)}$ antigenic peptide and anti-DEC-205 scFv *in vivo* in a mouse model. We inoculated subcutaneously the recombinant fdOVA/sc-αDEC bacteriophage particles into C57BL/6 mice that had been adoptively transferred with purified, CFSE-labeled, $CD8^+$ T cells derived from $OVA_{(257–264)}$ specific OT-I transgenic mice 24 hours before the immunization. We measured the ability of fdsc–αDEC bacteriophage carrying $OVA_{(257–264)}$ to induce an antigen specific immune response, 96 hour after the immunization, by measuring the $OVA_{(257–264)}$ specific $CD8^+$ T cell proliferation as CFSE reduced fluorescence by FACS analysis on $CD8^+$ $V\alpha2^+$ gated cells. A group of mice immunized with PBS alone was used as control.

As reported in Figure 2 the fdOVA/sc-αDEC induced a strong proliferative response of the OVA-specific $CD8^+$ T cells, in absence of exogenous adjuvant.

3.2. RNA-Seq Analysis of BMDC Treated with Bacteriophage fdsc-αDEC. To gain insights into the molecular mechanisms through which fdsc–αDEC induces a strong cell-mediated immune response, we used RNA-Sequencing to analyze the transcriptional profiles of BMDCs *in vitro* challenged with fdsc–αDEC phage particles. The gene expression pattern of these cells was compared to the one of control untreated BMDCs (i.e., cells treated only with PBS). Two technical replicates for each condition were performed. Approximately 55 million reads (95% of them uniquely mapped on the reference genome) per replicate were produced. Expression values for both control and fdsc-αDEC-treated DCs were measured as FPKM (fragments per kilobase of transcript per million mapped reads). Technical replicates revealed a very highly correlation. Using RNA-Sequencing we could simultaneously measure gene expression levels of (virtually) all genes expressed in mouse DCs. Setting an arbitrary threshold (FPKM = 1) for gene expression, we found about ten thousand genes expressed at significant levels in both conditions. Then, we compared gene expression levels between the two conditions. This analysis revealed that approximately 3800 genes (FDR < 0.01) were differentially expressed (DE) in DCs after exposure to fdsc-αDEC compared to control cells (Figure 3(a)). As reported in Figures 3(a) and 3(b), we selected different FDR intervals. Genes with a FDR value between 0.05 and 0.005 are classified in the first group and represent the 30% of the DE genes (in red in Figures 3(a) and 3(b)); the second group includes the 18% of the total DE genes with FDR between 0.005 and 0.0005 (in green in Figures 3(a) and 3(b)), while the most significant DE genes, with FDR under 0.0005, represent the 52% of the total DE genes (in grey in Figures 3(a) and 3(b)). Among them, we further selected DE genes with a fold change (FC) > ±2 in DCs exposed to fdsc-αDEC *versus* control cells. All further analyses were performed on this group of DE genes named DEG (differentially expressed Genes, shown in blue in Figures 3(a) and 3(b)). Most of these DEG were significantly upregulated in DCs upon treatment with the fdsc-αDEC, whereas only very few of them were downmodulated. To understand if these genes with a significant upregulation were related to specific cells function and/or pathways, we interrogated the Database for Annotation, Visualization and Integrated Discovery (DAVID). The most enriched biological pathways (using KEGG database) are shown in Figure 3(c). Interestingly, the exposure of DCs to fdsc-αDEC significantly upregulated many genes involved in inflammatory pathways linked to innate immunity.

The pathways with the larger number of upregulated genes were the "NOD-like receptor signaling," the "cytokine-cytokine receptor interaction," the "toll-like receptors," the

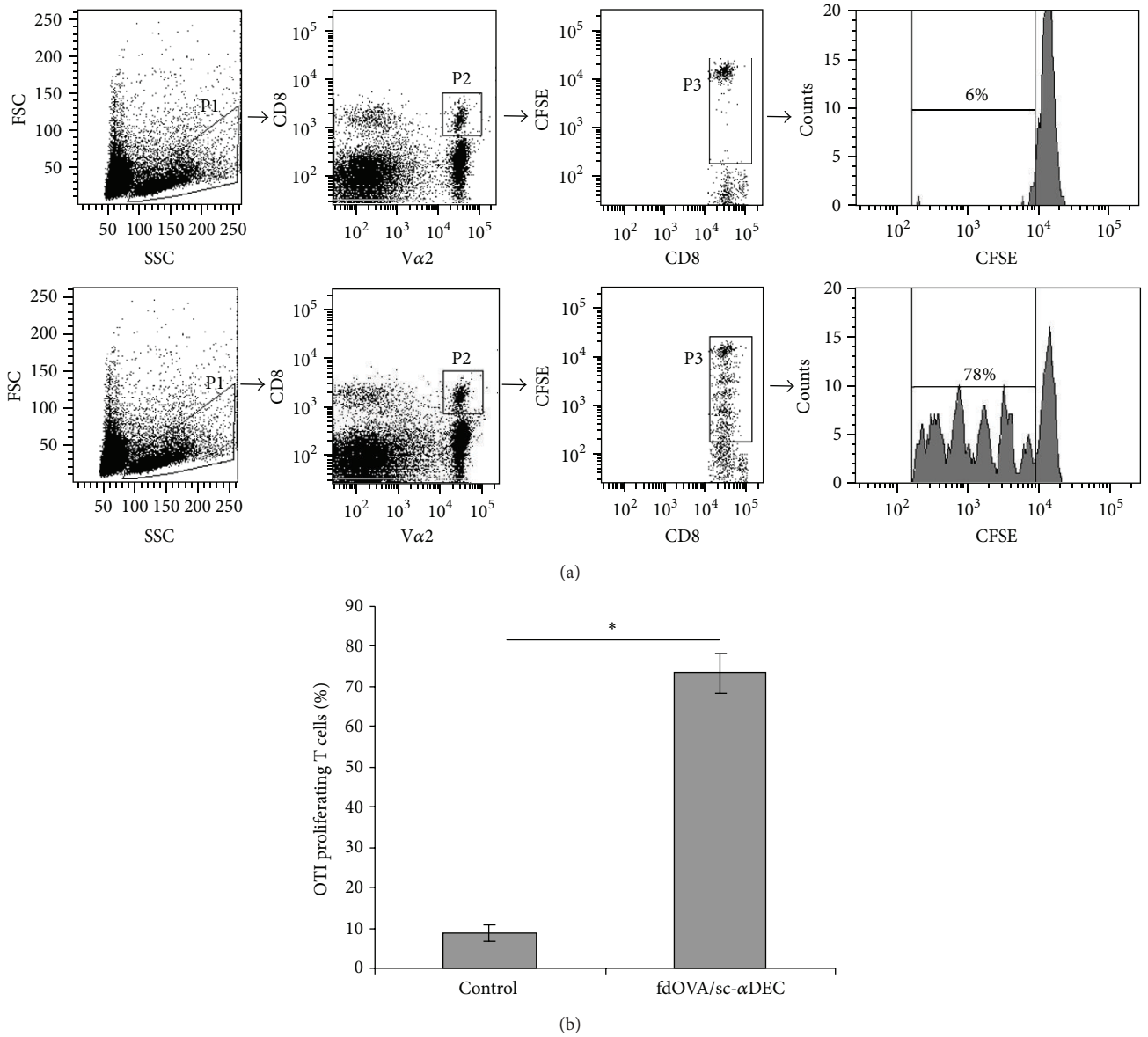

Figure 2: OVA specific OT-I CD8 T cell proliferation. (a) Flow cytometry strategy analysis of OT-I T cells proliferation. One representative sample per group is reported. P1: live cells, P2: OT-I CD8+ Vα2+ T cells, and P3: OT-I CD8+ Vα2+ proliferating cells with CFSE fluorescence intensity $<10^4$ and $>10^2$. The CFSE fluorescence intensity of the P3 population is reported in the histograms and numbers represent the percentage of proliferating cells. Peaks represent the cell division. (b) Mean ± SD percentage of proliferating T cells from adoptively transferred mice ($n = 5$) immunised with PBS or fdOVA/sc-αDEC bacteriophage is shown. $^*p < 0.01$.

"cytosolic DNA sensing," the "chemokine signaling," and the "RIG-I-like receptor signaling" pathways (Figure 3(c)).

To better dissect our results, we compared them with the already published data [23] describing mouse DCs treated with phylogenetically different organisms, such as bacteria, helminths, and parasites as a paradigm of how DCs undergo marked reprogramming during infection with live pathogens. Our RNAseq data show that in agreement with data obtained with the live pathogens, our procaryotic virus is able to activate specific classes of genes such as the CXCL1 (growth-related oncogene 1 (GRO1)), CXCL2 (GRO2), CCL2, CCL7, and genes encoding the proinflammatory mediators tumor necrosis factor α (TNF-α) and interleukin (IL-1 β) (see Table 1). Moreover, we also found the significant upregulation (more than 19-fold) of CXCL10 (IFN-inducible protein 10); importantly, this chemokine is essential for the generation of protective $CD8^+$ T cell responses and it is produced by dendritic cells following CpG-ODN stimulation [24]. Measuring the expression levels of the antiviral genes Oas3, Oas2, and Eif2ak2 we found that they were 18-, 16-, and 13-fold change increased, respectively, in bacteriophage-treated DCs (Table 1). Finally, expression data revealed a significant upregulation of Interferon-Stimulated Genes (ISG) and in particular of *Isg15* gene that was upregulated more than

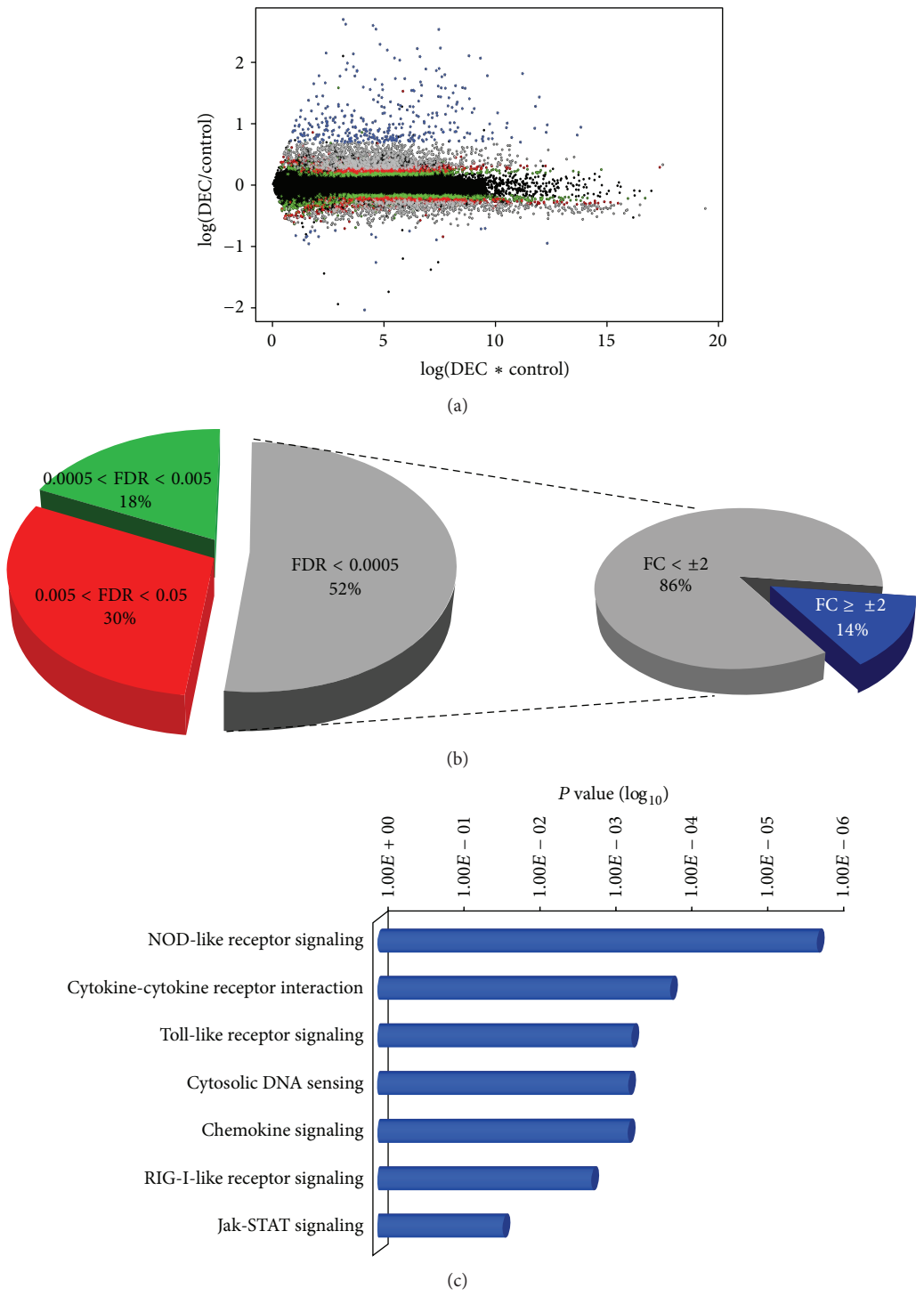

Figure 3: RNA-Seq data analysis of BMDC in presence and absence of fdsc-αDEC. MA plot of expressed genes (a) and pie chart (b) of the differentially expressed genes classified according to their FDR value. Genes with FDR < 0.0005 and fold change > ±2 are shown in blue. For these genes, pathway analysis is reported in (c).

twentyfold, similarly to *Irf7* gene (Table 1). The expression of these genes was assessed also by quantitative real-time PCR showing a fold change of 8.6 for *Isg15* and 6.6 for *Irf7* gene in DC treated with the engineered bacteriophage (Figure 4). Also the *Il1b* gene expression was measured by real-time PCR and showed a twofold increase of mRNA in fdsc-αDEC treated DC.

3.3. Interferon Signature on Differentially Expressed Genes. The genes whose expression was significantly modulated

Table 1: Selection of most significant IFN-related upregulated genes in mouse BMDCs treated with fdsc-αDEC bacteriophage vaccine.

Official gene symbol	Gene ID	Description
Chemokine and cytokine		
Il1b	NM_008361	Interleukin-1 beta
Ccl12	NM_011331	Chemokine (C–C motif) ligand 12
Ccl2	NM_011333	Chemokine (C–C motif) ligand 2
Ccl7	NM_013654	Chemokine (C–C motif) ligand 7
Cxcl1	NM_008176	Chemokine (C–X–C motif) ligand 1
Cxcl10	NM_021274	Chemokine (C–X–C motif) ligand 10
Ccr2	NM_009915	Chemokine (C–C motif) receptor 2
Ccr5	NM_009917	Chemokine (C–C motif) receptor 5
Transcriptional factors		
Irf7	NM_016850	Interferon regulatory factor 7
Batf2	NM_028967	Basic leucine zipper transcription factor, ATF-like 2
Znfx1	NM_001033196	Zinc finger, NFX1-type containing 1
Zbtb38	NM_001033196	Zinc finger And BTB domain containing 38
Stat1	NM_009283	Signal transducer and activator of transcription 1
Stat2	NM_019963	Signal transducer and activator of transcription 2
ISGilation and ubiquitination		
Isg15	NM_015783	Interferon-stimulated exonuclease gene 15 kDa
Isg20	NM_020583	Interferon-stimulated exonuclease gene 20 kDa
Usp18	NM_011909	Ubiquitin specific peptidase 18
Usp25	NM_013918	Ubiquitin specific peptidase 25
Herc6	NM_025992	HECT domain and RLD 6
March1	NM_175188	Membrane-associated ring finger (C3HC4) 1
Nucleic acid binding proteins		
Ddx58	NM_172689	Dead box polypeptide 58/RIG-1
Ddx60	NM_001081215	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
Zbp1	NM_021394	Z-DNA binding protein 1
Eif2ak2	NM_011163	Eukaryotic translation initiation factor 2-alpha kinase 2/PKR
Antiviral proteins		
Gbp2	NM_010260	Guanylate binding protein 2
Gbp3	NM_018734	Guanylate binding protein 3
Gbp4	NM_008620	Guanylate binding protein 4
Oas2	NM_145227	2′-5′-Oligoadenylate synthetase 2
Oas3	NM_145226	2′-5′-Oligoadenylate synthetase 3
Oasl1	NM_145209	2′-5′-Oligoadenylate synthetase-like 1
Oasl2	NM_011854	2′-5′-Oligoadenylate synthetase-like 2
Mx1	NM_010846	Myxovirus (influenza virus) resistance 1
Mx2	NM_013606	Myxovirus (influenza virus) resistance 2
Rsad2	NM_021384	Radical S-adenosyl methionine domain containing 2

after fdsc–αDEC treatment in dendritic cells were analyzed using the INTERFEROME 2.0 database. The analysis revealed the presence of a clear transcriptional interferon signature. Notably, 183 genes out of 361 significantly (FDR < 0.0005) DEG (about 50%) are modulated by Interferon Regulated Factors (IRFs) or by the NF-kB transcription factors and possess Interferon-Stimulated Response Elements (ISREs). The Venn diagram in Figure 5(a) illustrates how many DE genes are regulated *per* interferon type. In detail, 73 out of 183 genes are IFN Type-I dependent, 26 are type-II dependent, and 84 are regulated by both interferons. Expression levels of interferon-regulated genes in DCs, in presence or absence of fdsc-αDEC, are shown in the heatmap of Figure 5(b). In addition, we decided to assess whether genes that are affected by fdsc-αDEC treatment in DCs have a specific transcription factor binding sites (TFBS) signature within their promoters. To this aim, using Pscan we found that most of upregulated genes have ISREs and binding sites for IRF1, IRF2, and IRF7 molecules (Figure 5(c)). Interestingly, among them are included genes coding for cytokines and chemokines, transcriptional regulators, DNA binding proteins, proteins involved in ISGylation and ubiquitination,

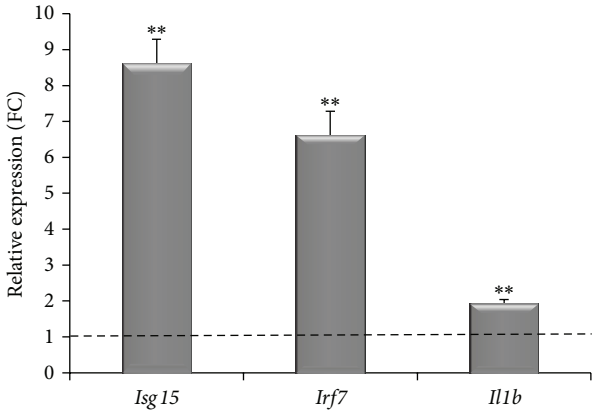

Figure 4: Real-time PCR validation of RNA seq analysis. The *Isg15*, *Irf7,* and *Il1b* gene expression in BMDCs *in vitro* challenged with fdsc-αDEC phage particles was measured by real-time PCR. The dashed line corresponds to the mean value of gene expression of PBS treated DCs. Graph shows the fold change (FC) (mean ± SD). $^{**}p < 0.05$.

and proteins with a known activity in the innate anti-viral response and immune activation (details in Table 1).

4. Discussion

It is a current opinion that vaccines should activate the innate immune system in order to start a rapid response to pathogens and sustain at the same time the development of the adaptive immune response. Thus, in order to choose the best vaccine formulations able to stimulate both innate and adaptive immune response, one of the more recent approaches is to take advantage from the analysis of gene expression using the efficient high throughput whole-genome screening. Moreover, using this approach, it is possible to have an overview of how the immune system attacks invading microorganisms, maintains tolerance, and creates a memory of past infections. Up to a decade ago this analysis was based on microarrays, but the development of RNA-Seq methodologies has opened a new era of investigations to identify genes that are differently expressed when samples are treated with different compounds. Moreover, this discovery-based research provides the opportunity to characterize both new genes with unknown functions and genes not previously known to be involved in a particular biological process [25].

In this paper we take advantage from the RNA-Seq with its massive data output in order to elucidate the mechanism by which filamentous bacteriophage antigen delivery system targeting dendritic cells *via* the DEC-205 receptor is able to induce a strong and sustained antigen specific immune response as previously described and represented in Figure 2 [11, 13]. The importance of dendritic cells in initiating immune responses was the key reason for us to select this cell type as target and to investigate at a genetic level how DCs sense this procaryotic virus. DCs reside in an immature state in most tissues, where they recognize and phagocytose pathogens and other antigens. Direct contact with many pathogens leads to the maturation of DCs, which is characterized by an increase in antigen presentation, expression of costimulatory molecules, and subsequent stimulation of naive T cells in lymphoid organs [26].

As a very powerful research tool, the RNA-Seq method shows as a drawback the production of impressive amount of data, and it is up to the researchers to select among them only the more reliable. To be more confident in our study, we analysed only genes with a FDR < 0.0005 and with a fold change in expression level >2; using these genes we started to dissect the gene expression changes in dendritic cells exposed to our vaccine candidate.

Following analysis using DAVID bioinformatics resources, we were able to classify DE genes in pathway categories and we found that the more transcribed genes, after DC challenge, were the ones involved in pathogen recognition for innate immunity activation: genes from the NOD-like receptor and toll-like receptor signaling pathway were upregulated, and the evocated innate immune response started the production of cytokines and chemokines and the up regulation of their receptors. In particular, we found that NOD1 and NLRP3 are upregulated more than 7-fold in DC treated with anti-DEC-205 bacteriophage fd. NOD1 is one of the nucleotide-binding oligomerization domain-like receptors (NLRs), a family of intracellular receptors that detects PAMPs and endogenous molecules; at the same family belongs NLRP3 that is involved in the formation of multiprotein complexes termed inflammasomes that mediates the caspase-1-dependent conversion of pro-IL-1β and pro-IL-18 to IL-1β and IL-18 [27].

Toll-like receptors belong to the family of pathogen recognition receptors that are triggered by PAMPs expressed by bacteria, viruses, fungi, and protozoa and their stimulation contributes to the induction and maintenance of innate and adaptive immune pathways as well as memory function. In our analysis we registered the upregulation of genes encoding the endosomal toll-like receptors TRL3, TLR7, TLR8, and TLR9.

We previously described that DCs pulsed with bacteriophage fd *via* anti-DEC-205 are able to produce type I IFN [11, 13]; now we found by RNA-Seq analysis that 50% of the reported DE genes are regulated by Interferon Regulated Factors (IRF) and possess Interferon-Stimulated Response Element (ISRE) as reported in Figure 5(c). However, we were not able to detect the upregulation of interferon genes by RNA-Seq analysis, due to the gene organization (only one exon) and high conservation among sequences of the different genes encoding Interferon type I. Since the RNA-Seq method is unable to match the reads on the reference genome, in order to better identify the genes induced by interferon molecules, we used the INTERFEROME bioinformatics tool. From this analysis the interferon signature is evident (see the heatmap in Figure 5(c)), and it is also clear that either type I or type II is involved (see Venn diagram in Figure 5(a)). The type I interferons signal through the interaction with the interferon alpha receptor (IFNAR). Interaction between IFN-I and IFNAR triggers signaling cascades which culminate in the transcriptional regulation of hundreds of IFN-stimulated genes (ISG). One of the first pathways activated *via* IFN-I

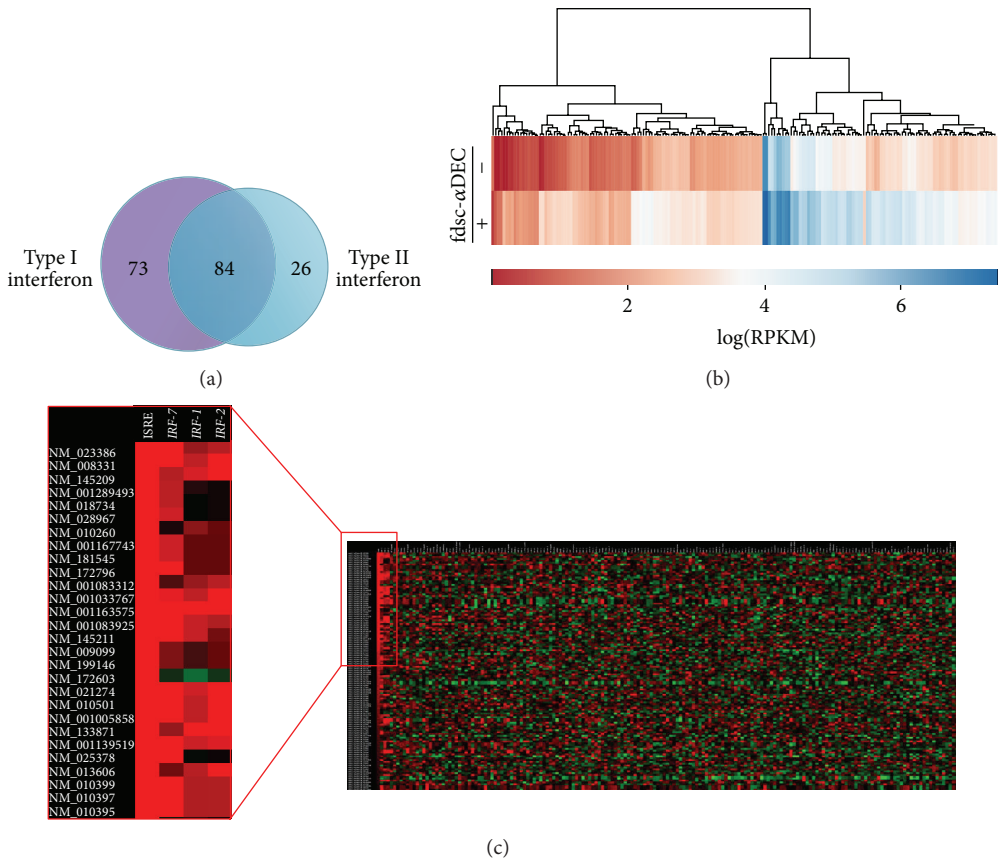

Figure 5: Interferon signature in genes modulated by fdsc-αDEC. (a) Venn diagram of the genes analyzed by INTERFEROME 2.0 database. (b) Heatmap of 183 "interferon-related" genes modulated upon fdsc-αDEC treatment. Expression values are indicated as logRPKM (reads per kilobase of transcript per million of mapped reads). (c) Heatmap obtained using the online Pscan tool indicating the presence (in red) of transcription factors binding sites (TFBS) within promoters of a selected gene list. In the red box on the left, a detail of such computational analysis is shown.

is the JAK-STAT pathway. STAT1 and STAT2 form dimers and enter into the nucleus to form ISGF3 transcription factor complex, which binds to IFN-stimulated response elements, driving the expression of Isg genes. In our RNA-Seq analysis in BMDC treated with anti-DEC-205 phage particles, we found upregulation of both *Stat1* and *Stat2* (10- and 13-fold, resp., by fd exposure) as well as other genes like *Irf7, Isg15* (confirmed also by real-time PCR, see Figure 4), and *Rig-1* genes, all of which contribute to the positive feedback regulation of the IFN pathway. Moreover, we found upregulation of transcripts of many interferon inducible and anti-viral genes like *Oas, Eif2ak2, Mx1, and Mx2*.

By comparing our results with the already published data on the same subject, in particular in paper describing how host cells undergo marked reprogramming of their transcriptome during infection with live pathogens, we observed a common core of the host DCs-transcriptional program.

In agreement with these data [23], we also observed the upregulated expression (more than 20-fold) of the *Igs15* gene. The function of Isg15 has been recently elucidated: it is ubiquitin-like protein and its activity, named ISGylation, is a modification of target protein and after modification these proteins are conjugated to Isg15. The conjugation of ISG15 to target proteins is reversible and is mediated by conjugating/deconjugating enzymes. ISG15 is conjugated to lysines on numerous target proteins through the action of specific IFN-inducible E1/E2/E3 enzymes. We show here that bacteriophage fd particle uptaken by dendritic cells induced gene expression of *Isg15* and both its conjugating and deconjugating enzymes. In fact, beyond the upregulation of *Isg15*, we found increased levels of transcripts of other factors that regulate ISG15-mediated ISGylation like HECT domain and RCC1-like domain containing protein 6 (HerC6) that represents the main ISG15 E3 ligase, essential for global ISG15 conjugation in mice [28]. Currently, the number of identified ISGylated proteins is increasing, and although the functional role of protein ISGylation is not yet completely understood, there are increasing bodies of evidence to suggest a role in mediating an innate antiviral response. It has been recently demonstrated [29, 30] that the key regulators of signal transduction, such as phospholipase Cγ1, Jak1, and ERK1, are modified by ISG15, as the transcription factor STAT1, an immediate substrate of Jak1 kinase.

In summary, all our findings indicate the ability of bacteriophage fd to upregulate *via* DEC-205 in the targeted DCs several genes involved in triggering an innate

immune response. These findings are in agreement with the already published data on the same subject, in particular in papers describing how dendritic cells undergo marked reprogramming of their transcriptome during infection with live pathogens [23].

5. Conclusions

Overall, filamentous bacteriophage fd expressing antigenic epitopes and modified to target the DEC-205 dendritic cell receptor is a valuable vaccine candidate able to induce dendritic cells activation and to trigger antigen specific T cell response.

Here we provide the evidence that bacteriophage fdsc-αDEC particles can activate antiviral innate immune responses *via* induction of type I interferons, which in turn orchestrate activation of dendritic cells, enhancing their antigen presenting functions.

From these results we can conclude that DEC-205 fd-targeted bacteriophages have *per se* adjuvant properties and that the fd antigen delivery system combines the safety and capability to trigger a strong cellular antigen-specific immune response even without administering exogenous adjuvants.

Conflict of Interests

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

Acknowledgments

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Research Article

Targeted Collection of Plasmid DNA in Large and Growing Animal Muscles 6 Weeks after DNA Vaccination with and without Electroporation

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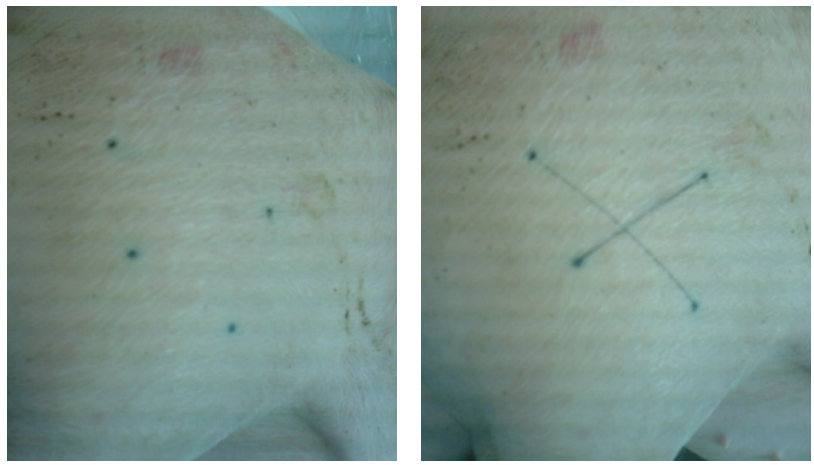
DNA vaccination has been developed in the last two decades in human and animal species as a promising alternative to conventional vaccination. It consists in the injection, in the muscle, for example, of plasmid DNA encoding the vaccinating polypeptide. Electroporation which forces the entrance of the plasmid DNA in cells at the injection point has been described as a powerful and promising strategy to enhance DNA vaccine efficacy. Due to the fact that the vaccine is composed of DNA, close attention on the fate of the plasmid DNA upon vaccination has to be taken into account, especially at the injection point. To perform such studies, the muscle injection point has to be precisely recovered and collected several weeks after injection. This is even more difficult for large and growing animals. A technique has been developed to localize precisely and collect efficiently the muscle injection points in growing piglets 6 weeks after DNA vaccination accompanied or not by electroporation. Electroporation did not significantly increase the level of remaining plasmids compared to nonelectroporated piglets, and, in all the cases, the levels were below the limit recommended by the FDA to research integration events of plasmid DNA into the host DNA.

1. Introduction

DNA vaccination is widely studied to develop new and alternative vaccines for humans and animals. DNA vaccines are circular plasmid DNA molecules that encode the vaccinating antigens, these antigens being synthesized inside cells of the injected body. Many efforts have been made to increase the immunising potential of these vaccines. For example, plasmids encoding cytokines or copresentation molecules as well as toll-like receptors agonists were successfully used as adjuvants in various models (for a review see [1]). Other strategies were based on the route of injection, the controlled release of the plasmids, and/or the forcing of the entrance of the plasmids in the cells at the injection point. Among the strategies that force the entrance of the plasmids, electroporation has a promising future [2]. Electroporation consists in the application of an electric current on both sides of the injection point. Cells at the injection site are thereby temporarily permeabilized, promoting the entry of plasmids conveyed by the electric current into the cells. This results in many cases in the improvement of DNA vaccine efficacies [3–6]. In particular, electroporation has been demonstrated as a powerful technique also in large animals, including pigs [7–9].

DNA vaccination is generally well tolerated, even when electroporation is applied [9]. No adverse reactions and changes in metabolic activity were observed in numerous animal and human clinical trials upon DNA vaccination [10]. Histological damage has been hardly observed, with the exception, for example, of one study in rats shortly after the injection, but this was associated with the route of injection [11].



(a) (b)

Figure 1: Identification of the injection point. (a) Four dots were tattooed with Indian ink on the skin of the left *biceps femoris* muscle 2 to 3 weeks before the injection. (b) The injection site of the plasmids was located at the intersection of the two lines passing through these dots. These two lines were drawn on the skin just before the injection.

Due to the fact that DNA vaccines are composed of DNA, close attention on the fate of the plasmid DNA upon vaccination has to be taken into account. In general, most plasmids remain at the site of injection (for a review see [12]) for, in certain cases, up to several months [13]. Shortly after injection, small amounts of plasmids spread throughout the body and are detected in remote organs [14]. DNA vaccination involves the introduction of small amounts of plasmid DNA into the nucleus of host cells. It is then conceivable that there is a potential risk of partial or complete integration of plasmid DNA into the host cell genome. Therefore, this potential risk should be examined carefully. Furthermore, it is also conceivable that for techniques allowing an entrance increase of the plasmids in the cells, as it is the case for electroporation, these potential risks should be even more deeply taken into consideration. Usually, plasmids are quantified in the injected tissue samples by PCR-based methods (as, e.g., in [15]). It is admitted that if there are no plasmids detected, plasmid integration event may not have occurred. To unambiguously characterize integration events, Wang et al. developed in mice the PCR-based repeat anchored integration capture-PCR (RAIC-PCR) [16]. Four integration events have been identified four weeks after intramuscular injection of the DNA vaccine followed by electroporation.

When small animal models like mice are used, the fate of DNA plasmids can be studied on whole injected muscle homogenates. To apply these PCR-based tests for large and growing animal models (e.g., pigs), it is essential to recover the injection point, especially several weeks after injection. In fact, the muscle and skin surfaces of the animals are large and growing. Therefore, precise benchmarks are essential to identify the injection points. In the present study, we developed a strategy to be able to localize precisely the injection point in muscles of growing piglets at least 6 weeks after DNA vaccination. With our strategy, the benchmarks are not located directly within the injection point; therefore there is no disturbance of the injection point due to the presence of these benchmarks. With this technique, we compared the concentration of remaining plasmids 6 weeks after a single DNA vaccination of piglets accompanied or not by electroporation. The model used here is a DNA vaccine against pseudorabies virus infection. The electroporation conditions used here were previously shown to be efficient to induce a significant increase of immune responses due to the DNA vaccine.

2. Materials and Methods

2.1. Plasmids. The endotoxin free pcDNA3 plasmids encoding or not pseudorabies virus glycoprotein B (PrV-gB-pcDNA3) were produced and purified as previously described [9, 17].

2.2. Pig Experiments. The experimental protocol was approved by the ethic committee for animal experimentation of ANSES/National Veterinary School of Alfort/University of Paris-Est Créteil (France) (Notice number 10/04/13-05). Pigs were housed and treated in accordance with the requirements of the local veterinary authority. Four groups of four specific pathogen-free eight-week old pigs were used. The injection site of the plasmids was identified through four dots tattooed with Indian ink on the skin of the left *biceps femoris* muscle, the injection site being located at the intersection of the two lines passing through these dots (Figure 1). All pigs were anesthetized with an auricular intravenous injection of thiopental (1 g/50 kg body weight). The first and second group were injected with 2.5×10^{14} copies of PrV-gB-pcDNA3 prepared in 600 μL PBS. The third one received 2.5×10^{14} copies of pcDNA3 and the last group was injected with PBS. 0.45 mm × 12 mm needles were used. Eighty seconds later [18], electroporation which consists of 5 pulses of 150 V and 20 ms with a 200 ms interval between each pulse [7] was applied through stainless-steel electrodes (0.2 mm wires, 1 cm

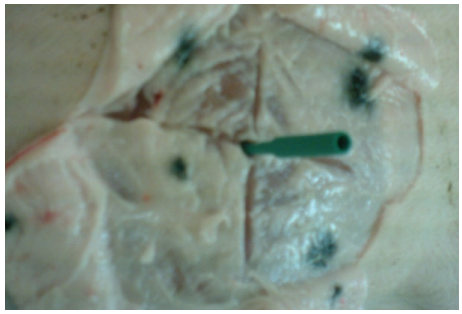


Figure 2: Sampling of the injection point 6 weeks after injection. Six weeks after injection, the injection point was localized as done at the injection time (Figure 1). The two lines were drawn thanks to the four tattooed points. After removing the skin and the fat layer, the portion of the injected muscle was sampled using a disposable 2 cm long biopsy punch that was horizontally applied on the muscle surface.

long, and 10 mm apart) introduced on either side of the injection point of pigs of groups 2 to 4. The electric current was applied with a BTX ECM 830 pulse generator (Harvard Apparatus, Holliston, MA, USA). Pigs were observed daily. Body temperature and body weight were measured daily and weekly, respectively. Pigs were sacrificed six weeks after injection. The muscle injection site identified through the tattooed dots was sampled using a disposable 2 cm long and 0.8 cm diameter biopsy punch (Figure 2), frozen in liquid nitrogen, and stored at −80°C until DNA extraction.

2.3. DNA Extraction and Quantification of Plasmids by Quantitative PCR (qPCR). Prior to DNA extraction, the 2 cm long pieces of muscle excised were divided into six equal samples (from the superior to the inferior part of the muscle). Each muscle fraction was weighed and resuspended in PBS buffer according to the measured mass. Then homogenization was carried out using a Teflon pestle at 30 Hz for 1 min or until all major tissue clumps were dispersed. Host DNA extractions were performed on 30 mg of the homogenized tissue sample using the QIAamp DNA Mini Kit (Qiagen) after overnight proteinase *K* digestion according to the manufacturer's instructions. Thereafter, plasmid DNA concentration was measured in each muscle sample by quantitative PCR (qPCR). The target of the qPCR is a 92 nucleotides sequence located in the neomycin gene of the plasmids. Primers, probes, and qPCR conditions were those previously described [19]. Measurements were performed in triplicate. Experimental data were analyzed using the nonparametric Mann-Whitney test [20] included in SYSTAT 9 software (SYSTAT Software, Inc., Point Richmond, CA, USA).

3. Results and Discussion

Four points were tattooed with Indian ink on the skin of the left *biceps femoris* muscle of 5-week-old piglets (as shown in Figure 1). When the piglets were eight-week old, they were i.m. injected either with PrV-gB-pcDNA3 followed or not by electroporation, with pcDNA3 and electroporation, or with PBS and electroporation. The injection site was located at the intersection of the two lines passing through these dots (Figure 1). The mean weight of the animals at the vaccination time was of 24.7 ± 2.1 kg. No adverse reactions, no fever, and no delay in the growth of the pigs were observed during the 6-week period following these injections. The mean weight of the animals 6 weeks after injection was 66.5 ± 5.4 kg. This means that the piglets gained 41.8 ± 4.0 kg during the whole experimental period (i.e., about 170% of their initial weight). In a previous report we showed that plasmid injection coupled with electroporation applied exactly in the same manner as here increased the production of specific antibodies against PrV and peripheral blood mononuclear cells proliferated in response to stimulation with PrV glycoproteins [9]. This means that the electroporation conditions used here are effective ones. At week 6 after injection, 2 cm long muscle samples were collected exactly at the injection site using the dots tattooed on the skin (Figure 1). This time-point was the same as used in the study describing the research of integration events by RAIC-PCR [16]. Furthermore, this time-point seemed to us realistic since we detected small amounts of plasmids in the injected muscle 21 days after DNA vaccination, without electroporation, with much less plasmids injected, and without identifying precisely the injection point as done here [14]. At this time-point, the four tattooed dots were still strongly marked. The two lines delineating the injection site were drawn again on the skin (as shown in Figure 1). After removing the skin and the fat layer, the portion of the injected muscle was sampled using a disposable 2 cm long and 0.8 cm diameter biopsy punch that was horizontally applied on the muscle surface (Figure 2), frozen in liquid nitrogen, and stored at −80°C until DNA extraction. In preliminary experiments, the injection point was found to be located approximately in the middle of the muscle sample (data not shown). No plasmids were detected in pigs injected with PBS. The fractions containing the highest concentration of plasmids (around 3,000 to 14,000 copies/μg of host DNA) are located around the middle of the muscle samples, between fractions F2 and F5, with 6 out of 8 pigs within fractions F3 or F4 (Figure 3). The concentrations were higher in F2–F4 (1 pig in F2, 2 pigs in F3, and 1 pig in F4) when electroporation was applied and in F4-F5 (3 pigs in F4 and 1 pig in F5), that is, deeper, in the other case. When taking into account the mean values for each fraction, the concentrations of plasmids within the electropored muscle fractions were not significantly higher than in the nonelectropored ones ($P > 0.05$) (not shown). But these observations have to be taken with caution since the pressure we applied to the device was not controlled, although we tried. Nevertheless this seems consistent with the fact that less diffusion of the plasmids and better precision of injection are observed with electroporation [21]. Importantly, the fraction which is at the end of the needle is restricted to a small area, at least in depth, which shows the usefulness of precise benchmarks. Finally all the electropored and nonelectropored muscle samples have a concentration of plasmid DNA inferior to 30,000 copies/μg of host DNA. If we take into account the recommendations of the FDA [22], it is not necessary to perform additional integration analyses of plasmid DNA into

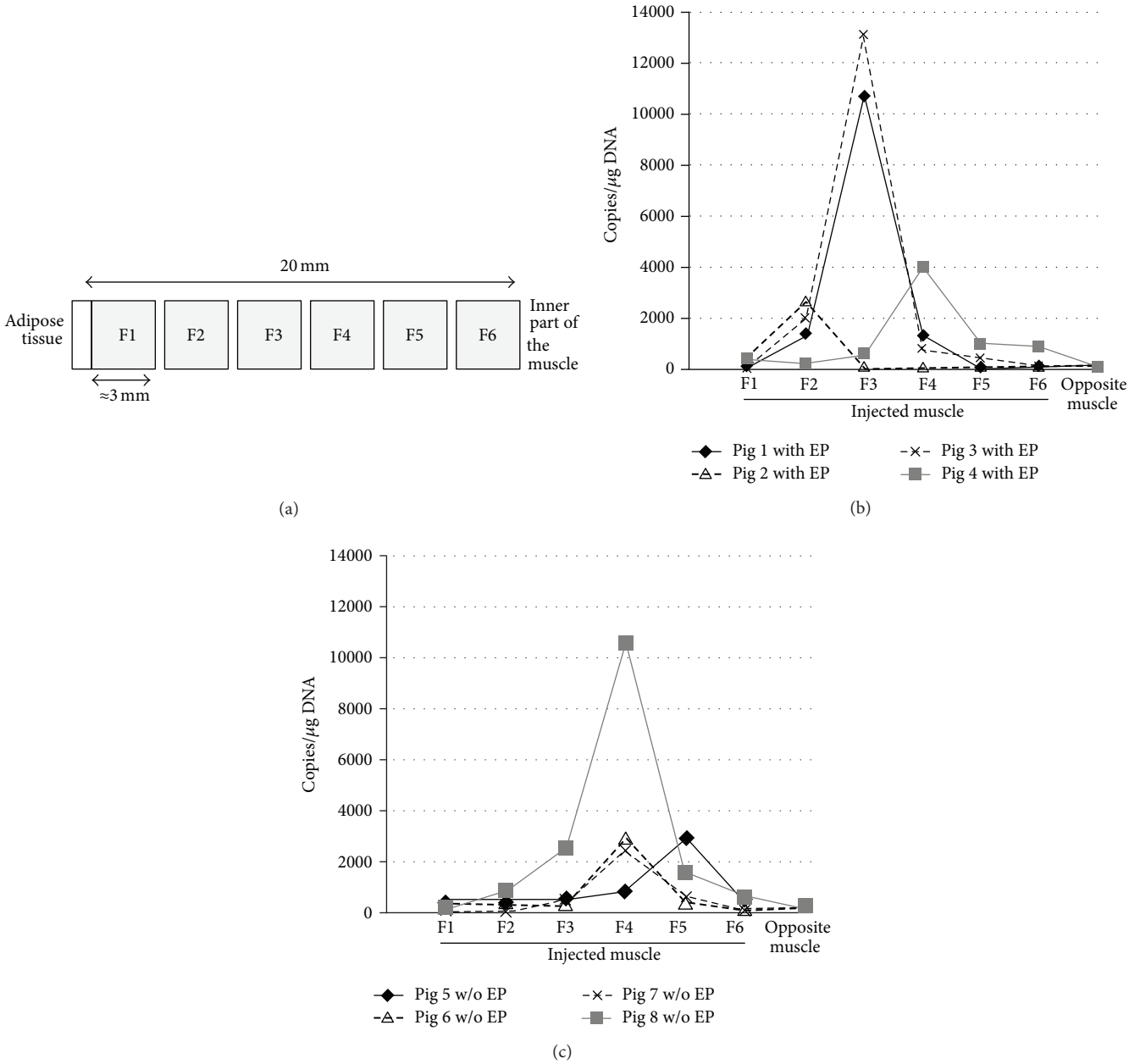


Figure 3: Plasmid concentrations in the different muscle fractions six weeks after injection. Two groups of four pigs were intramuscularly injected with 2.5×10^{14} copies of PrV-gB-pcDNA3 with or without electroporation, respectively. Six weeks later, excision of the muscle injection site was performed using a disposable 2 cm long and 0.8 cm diameter biopsy punch. (a) The excised muscle portion was divided into six fractions, F1 to F6. Fraction F1 represents the most external part of the muscle (i.e., under the skin) and F6 the most internal part. Thereafter plasmid DNA concentration was measured in each fraction after DNA extraction. Levels (in number of plasmid copies per μg of total DNA) of PrV-gB-pcDNA3 present in injected or opposite (noninjected) *biceps femoris* were quantified by real-time qPCR. Individual plasmid concentrations in each muscle sample for each pig injected with (b) or without (c) electroporation are presented. When taking into account the mean values for each fraction (not shown), the differences between both groups were not statistically significant ($P > 0.05$, nonparametric Mann-Whitney test).

host DNA since the probability of integration is low (<30,000 copies of plasmid/μg of host DNA).

In conclusion, a method to recover at least in depth the DNA vaccine injection area 6 weeks after injection in growing piglets was developed. At the time of vaccination, the mean weight of the piglets was 24.7 ± 2.1 kg and at the sampling time it was 66.5 ± 5.4 kg. This means that between these two time-points the piglets gained around 40 kg (+140%). Even if electroporation enables significant increases of immune responses levels [9], no significant enhancement of remaining

plasmids was observed when electroporation was applied compared to nonelectropored piglets. Furthermore, as electroporation consists in the forcing of the plasmid entrance in cells, special attention has to be paid on the potential risk of integration of plasmid DNA in host DNA. This method described here will be useful to obtain porcine muscle fractions to further study the fate of the plasmids upon DNA vaccination in evaluating their integration within host DNA if the level of remaining plasmids is above 30.000 copies/μg of host DNA (according to the FDA recommendations) [22].

Conflict of Interests

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

Authors' Contribution

Daniel Dory and Vincent Le Moigne contributed equally to the study.

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Review Article

Application of "Systems Vaccinology" to Evaluate Inflammation and Reactogenicity of Adjuvanted Preventative Vaccines

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Advances in "omics" technology (transcriptomics, proteomics, metabolomics, genomics/epigenomics, etc.) allied with statistical and bioinformatics tools are providing insights into basic mechanisms of vaccine and adjuvant efficacy or inflammation/reactogenicity. Predictive biomarkers of relatively frequent inflammatory reactogenicity may be identified in systems vaccinology studies involving tens or hundreds of participants and used to screen new vaccines and adjuvants in *in vitro*, *ex vivo*, animal, or human models. The identification of rare events (such as those observed with initial rotavirus vaccine or suspected autoimmune complications) will require interrogation of large data sets and population-based research before application of systems vaccinology. The Innovative Medicine Initiative funded public-private project BIOVACSAFE is an initial attempt to systematically identify biomarkers of relatively common inflammatory events after adjuvanted immunization using human, animal, and population-based models. Discriminatory profiles or biomarkers are being identified, which require validation in large trials involving thousands of participants before they can be generalized. Ultimately, it is to be hoped that the knowledge gained from such initiatives will provide tools to the industry, academia, and regulators to select optimal noninflammatory but immunogenic and effective vaccine adjuvant combinations, thereby shortening product development cycles and identifying unsuitable vaccine candidates that would fail in expensive late stage development or postmarketing.

1. Introduction

As preventive vaccines are typically administered to healthy people including infants, children, and persons with comorbidities, there is particular emphasis on safety, with an expectation of high benefit-risk ratio. This is especially the case with vaccines administered during pregnancy, where enhanced regulatory concerns have been expressed [1]. Modern vaccines therefore frequently make use of recombinant technology to manufacture purified pathogen subunit molecules, an approach that has allowed rational vaccine design, cGMP manufacture, and reproducibility of immune responses and acceptable adverse events profiles. A significant benefit of recombinant subunit vaccines, especially bacterial, is that the exclusion of nonantigenically relevant pathogen cellular components with inherent immunostimulatory properties greatly reduces activation of innate immune pathways and consequent inflammation, thereby reducing undesirable reactogenicity. A downside of this minimalist approach, seen, for example, with the switch from whole cell to acellular subunit pertussis vaccines, is that it may lead to reduced immunogenicity of the vaccine and coadministered vaccines, especially at the extremes of age. Therefore, to preserve efficacy without reactogenicity, various selective vaccine adjuvants have been developed with the aim of restoring immune activation to retain potency, but without inducing unacceptable inflammation. While the mode of action of adjuvants is diverse and remains unknown for even some licensed adjuvants, many typically harness components of the innate immune system [2–4], such as pattern recognition receptors (PRR), that detect infection by mimicking toll like receptor (TLR) agonists [5]. While immunostimulatory adjuvants offer the potential to beneficially modulate the immune response to antigens, their use may raise safety

concerns amongst the general public and regulators, related to speculation that they may induce overproduction of inflammatory and pyrogenic molecules [6], especially in outbred human populations where genetic or environmental factors may amplify or modify innate and adaptive immunity [7] in potentially unpredictable ways.

Furthermore, while rodent species are often employed in basic immunology research and preclinical toxicology testing, marked differences between murine and human innate and adaptive immunity exist [8, 9]. This is in part due to species-specific differences in receptor responses and the tissue distribution of innate system molecules and pathways [10]. The situation may be further compounded for some species such as ferrets, used in models of influenza infection and immunization, where suitable immunology reagents may be totally unavailable. It is therefore increasingly acknowledged that traditional toxicological approaches to evaluate adjuvanted vaccines in preclinical models [8, 11] may fail to identify an increased risk of adverse events following immunization (AEFIs) [12], which may emerge in clinical development [13] or even postmarketing [14]. Additionally, given the wide variety of vaccine adjuvants and their targets or modes of action and combinations with different antigens in a variety of formulations that may modulate the adjuvant effect, predictive immune parameters and toxicology readouts from preclinical studies for a particular adjuvanted vaccine may not be predictive across animal species or different adjuvanted vaccines.

While the occurrence of relatively frequent immediate inflammatory reactogenicity such as fever and injection site reactions may be possible to model in preclinical models, the risks of rare but serious AEs associated with vaccines that could occur in certain at-risk populations (as seen with initial introduction of rotavirus vaccine [15]) are unlikely to be revealed in preclinical studies or during clinical development and may require long follow-up of subjects in clinical trials. Recent late stage clinical trials [16] which have shown either a complete or a partial lack of efficacy or raised safety concerns, despite encouraging preclinical data, have reinforced the need for reliable predictive biomarkers of safety and efficacy that could be used in preclinical studies to prioritize available candidates and in early clinical development to avoid failure during lengthy and costly Phase 2b/3 clinical trials.

There is therefore an urgent unmet need to develop new technologies to identify novel biomarkers of adjuvant toxicity, especially experimental medicine models harnessing advances in human immunology, for subsequent validation in clinical trials, which will enhance preclinical and clinical safety evaluation of products containing existing and novel vaccine adjuvants.

2. Systems Vaccinology: Biomarkers of Vaccine Safety and Efficacy

A biomarker is a characteristic measured objectively at a single time point and evaluated as an indicator of a physiological or pathological process or pharmacological response(s) to a therapeutic intervention such as vaccine-induced protective immunity [12]. Several published studies have used vaccines in translational studies in which multiparametric technologies such as transcriptomics, metabolomics, and proteomics are used to dissect out fundamental mechanisms of reactogenicity and efficacy in human and animal models, in which whole blood or separated cell population gene expression, cytokine responses, and cellular and humoral immune responses are integrated [17, 18]. One benefit of the systems approach is that observations from small number of clinical samples can be further explored in animal models [19]. A systems-wide analysis has been used to identify novel mechanisms regulating vaccine responses [20, 21], now often referred to as "systems vaccinology" [22]. While a number of publications have demonstrated underlying mechanisms possibly associated with vaccine efficacy (as measured by immunogenicity), there are often conflicting outcomes due to a lack of standardization of systems biology techniques and bioinformatics analyses [23, 24]. Furthermore, different vaccine antigens or adjuvant systems are likely to induce different innate and adaptive responses, making extrapolation from different trials challenging.

While a number of research projects and consortia have been initiated to identify biomarkers of vaccine *efficacy* (such as the European Commission-Funded High Impact Project ADITEC, http://www.aditecproject.eu/ [25]), much less has been carried out in the area of vaccine *safety* and *reactogenicity*. Wang et al. indirectly observed that upregulation of genes associated with innate immunity, cytokine production, and responses to virus infection, particularly IFN-inducible genes, observed in nonhuman primates did correlate with adverse events seen in human trials [26]. An adverse reaction not uncommonly seen after immunization is fever. Activation of innate immunity and inflammation induces a febrile response [27], probably via the action of pyrogenic cytokines such as IL-1β, IL-6, and TNF-α [28] or prostaglandins such as PGE2 synthesized by liver Kupffer cells. Despite being normally an unwelcome but tolerable reaction to immunization, the association of febrile convulsions in infants with some vaccines such as whole cell pertussis is a safety concern. As a result, attempts to model the cytokine and prostaglandin profile after immunization in preclinical toxicology assays employing rabbits and human cell lines have been undertaken with some success [29]. Indeed, a systems approach was successfully applied to retrospectively identify underlying factors (i.e., biomarkers) responsible for the unexpected increase in febrile seizures in children associated with a specific trivalent influenza vaccine, by combining human, animal, cell line, and primary cell culture experiments with gene profiles and cytokine readouts [30]. This opens the possibility to screen vaccine antigen combinations or production methods for reactogenicity before release, using systems vaccinology. A recent meeting on the use of biomarkers for assessment of vaccine safety concluded that while the integration of high throughput multiparametric data from *in vitro*, preclinical, and clinical evaluations of vaccines and adjuvants in systems analyses was a powerful tool to identify basic mechanisms involved in vaccine and adjuvant reactogenicity and efficacy, considerable effort is still required to simplify, harmonize, and standardize these approaches if the data are to be practically applicable to

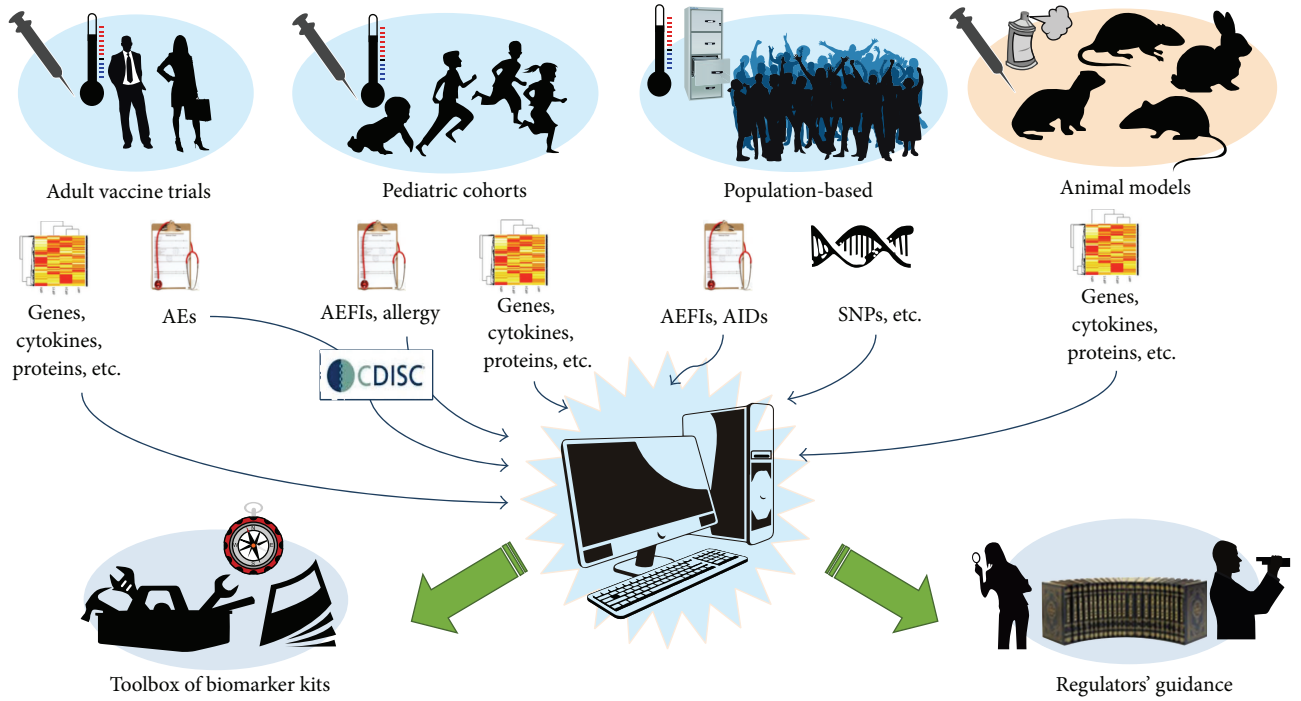

Figure 1: Integration of multiparametric "omics" data with clinical events from human clinical trials, together with population genetics, pediatric cohorts, and animal models in the BIOVACSAFE project.

vaccine and adjuvant development and safety monitoring [31].

3. BIOVACSAFE: Biomarkers of Vaccine Immunosafety

In 2011, the 5-year BIOVACSAFE project initiated, for the first time, a program of activities that integrate a systems biology approach with animal models and established clinical evaluation of reactogenicity after immunization or natural infection, and population-based genetics, to identify biomarkers of vaccine safety and reactogenicity (see Figure 1). The 30M€ project, coordinated by the University of Surrey, UK, and Novartis Vaccines, Italy, and funded by the Innovative Medicine Initiative, is a unique public-private partnership involving four EFPIA member pharmaceutical companies (GSK Bio, Sanofi Pasteur, Novartis Vaccines, and deCODE, AmGen) with 17 academic organizations, SMEs, and public institutions. Organized into work packages, it is the first truly systematic approach to apply systems vaccinology to vaccine and adjuvant safety rather than efficacy.

3.1. Human Experimental Medicine Studies of Systems Vaccinology. The BIOVACSAFE project has significantly refined the previous approach of systems vaccinology in which a "training set" of data are generated from small clinical studies with around 15 healthy adult subjects per vaccine group, to identify putative correlations or biomarkers associated with a desired outcome (such as immunogenicity) which are then confirmed in larger "confirmatory studies" typically involving over 100 participants. For example, unlike previous efforts in which different vaccines are evaluated in separate trials (in which many variables may be different such as population, environment, and methodology), BIOVACSAFE has taken a highly structured approach to perform head-to-head comparisons in naive or immune populations using prototypic vaccines and adjuvants (see Figure 2) performed as separate groups within the same protocol at the same clinical site. Both adjuvanted (MF59C, AS04C, and alum) and unadjuvanted vaccines are compared head-to-head in primed (influenza and booster immunization with hepatitis B vaccines) and naive subjects (priming immunization with hepatitis B vaccine). Alongside these subunit adjuvanted vaccines, live viral vaccines were tested in naive (yellow fever) and immune (varicella) populations. This structure is unique to BIOVACSAFE and will allow biomarkers unique to each vaccine-target combination to be discriminated from more generalized biomarkers common to a number of vaccines or target populations.

Furthermore, in the typical systems vaccinology scenario, clinical samples are taken on days 0, 3, 5, and 7 and at weekly intervals thereafter to characterize immune responses in an outpatient setting, with the schedule being highly influenced by convenience of study organization, which misses very early time points when innate immune cells may be most active in setting the direction of subsequent immune response and reactogenicity. In contrast, the BIOVACSAFE clinical "training trials" were conducted in an *inpatient setting* in which diet, exercise, sleep, alcohol, and tobacco were strictly regulated to ensure minimal background variability that could interfere with subtle physiological events after immunization. This allowed very subtle changes to be detected as

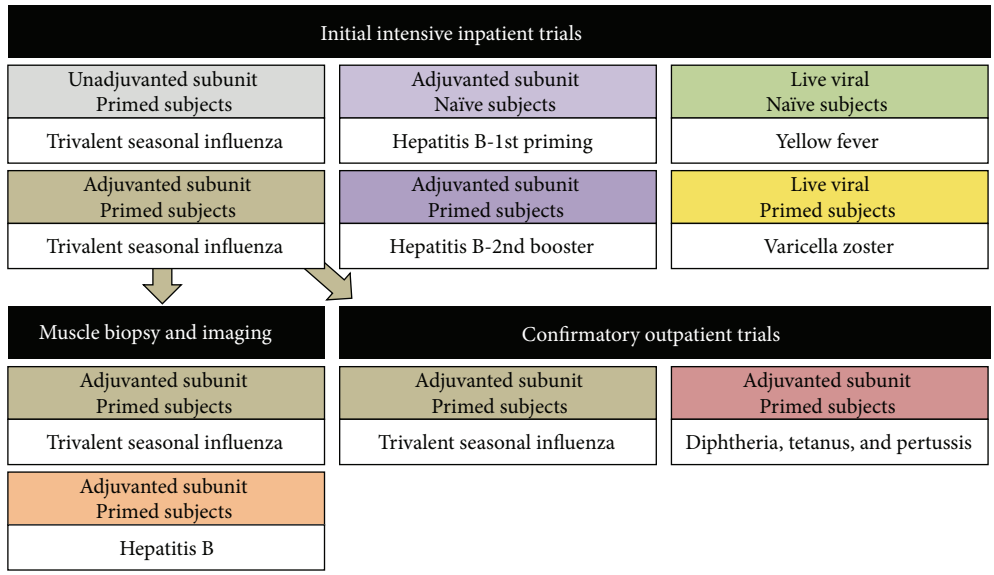

Figure 2: Organization and sequence of head-to-head comparison of adjuvanted and unadjuvanted vaccines, with live vaccines in experimental medicine studies within the BIOVACSAFE project.

a signal in proteomic and transcriptomic readouts without background noise that would be expected in an outpatient setting. In addition, as most immediate inflammatory reactogenicity to vaccines or adjuvants occurs within the first few days (see Figure 3), this setting allowed samples to be taken extremely frequently in the first 72 hours, to permit unique characterization of very early innate immune activation (see Figure 3), both at the transcriptomic and the proteomic level (acute phase proteins, cytokines, and chemokines).

3.2. The "Incarceration Effect". Inpatient confinement marked a significant departure from previous applications of systems vaccinology. However, it introduces an unexpected but well recognized problem: the "*incarceration effect*" seen typically in variations in physiological parameters and safety laboratory readouts in healthy subjects who are confined for long periods, for example, in Phase 1 drug trials, where pharmacokinetics or specific restrictions mandate an inpatient regime with controlled diet and so forth. In such settings, changes in laboratory parameters may be observed within normal laboratory ranges and typically dismissed as "not clinically significant." For example, in the BIOVACSAFE trials, a very distinct trend was seen in plasma proteins albumen and total protein with a gradual fall over time during the inpatient stay, followed by a marked jump between days 5 and 7 to a new set-point that was maintained at least 28 days. In contrast, acute phase proteins showed no baseline variation but were able to discriminate adjuvanted from nonadjuvanted vaccines in their response.

These observations raise an important question: if biomarkers are to provide useful alternatives to the standard clinical practice of identifying unacceptable levels of significant clinical reactogenicity (e.g., pain, fever, and redness measured in clinical trials) and identify subtle levels of reactogenicity that may only manifest a clinically significant problem late in clinical development or postmarketing, or in special groups (infants, pregnancy, and comorbidity), how will we distinguish these novel but subtle biomarkers from what has previously been dismissed as "not clinically significant"?

To do so will require a paradigm shift in thinking around preclinical toxicology and clinical pharmacovigilance and resultant regulatory guidance, especially for adjuvanted vaccines. As novel adjuvants are introduced, as personalized medicine becomes more common, and as vaccines are introduced for high-risk groups, these questions will have to be addressed if novel biomarkers of adjuvant reactogenicity are to shorten the cycle of discovery of unacceptable reactogenicity. The BIOVACSAFE program will set the limits of reactogenicity using safe licensed vaccines and adjuvants; further studies will have to employ more reactogenic or novel molecules that may manifest early or more frequent side effects.

3.3. Confirmation and Validation of Biomarkers Are Essential. With these considerations in mind follow-on clinical trials involving over 200 participants will be carried out by BIOVACSAFE, for example, to further characterize a biomarker profile identified with an adjuvanted influenza vaccine (Figure 2). A particular feature of this follow-on trial is that not only will whole blood be collected for RNA extraction and transcriptomics, but also cells will be separated into monocyte and granulocyte fractions. This results from initial observations in the inpatient trials that lymphocyte subpopulations have marked kinetics with differences between adjuvanted and nonadjuvanted as well as live vaccines (Figure 4), with peaks and troughs that are sometimes concordant and at other times discordant. Although a typical systems vaccinology study of adjuvant effect may separate peripheral blood mononuclear cells (PBMCs) in an attempt to control for

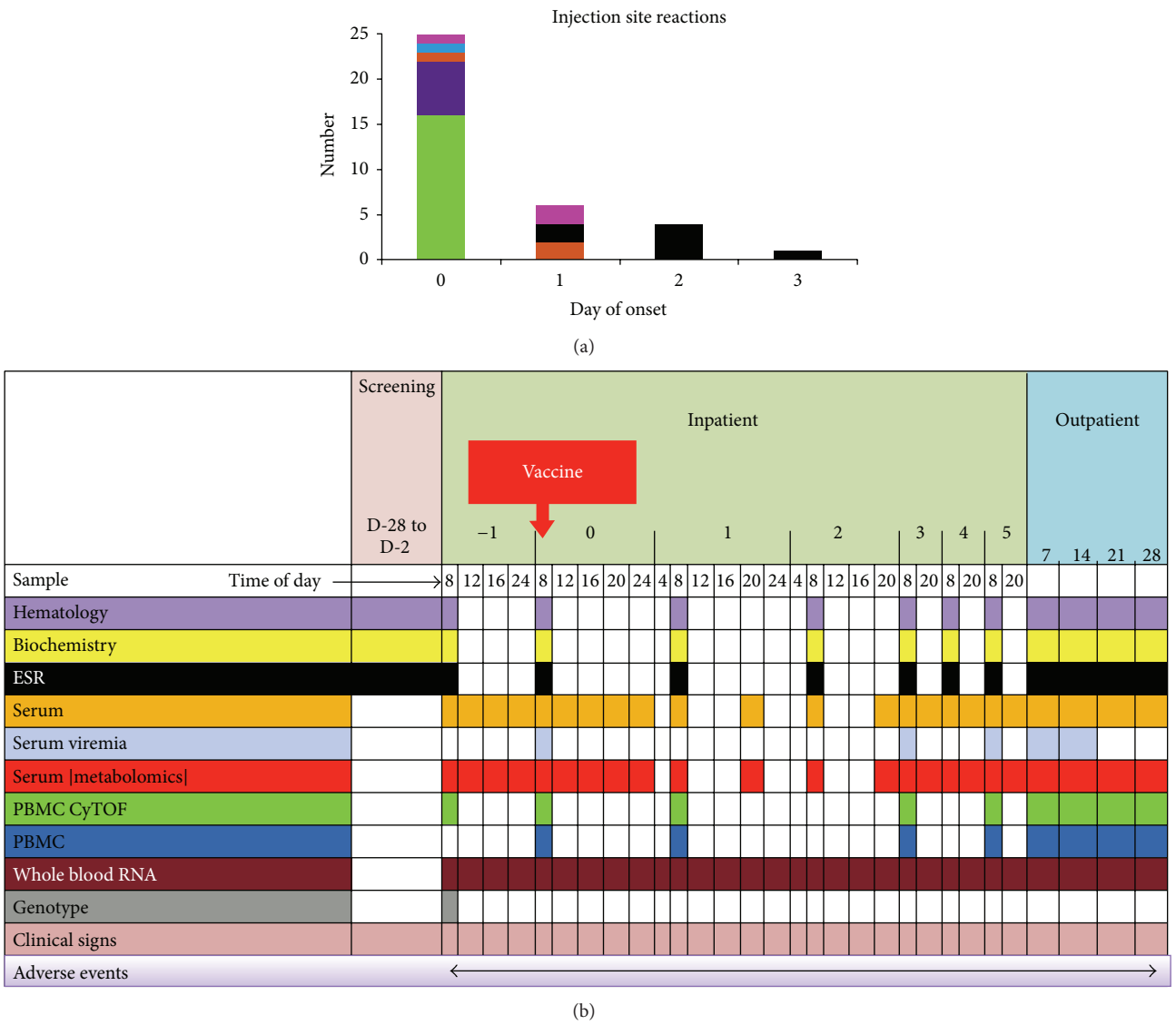

Figure 3: (a) Day of onset for injection site reaction for five different vaccines plus saline placebo (blue), (b) schedule of sampling for systems vaccinology parameters during intensive inpatient studies.

cellular kinetics, without these pilot data characterizing the exact time course, the wrong time points may be selected. In addition, granulocyte populations are generally overlooked as standard gradient centrifugation preparations will not easily isolate these cells, while data from the BIOVACSAFE intensive inpatient trials highlights significant activation of neutrophils by adjuvanted vaccines (Figure 4). By careful observations of cellular kinetics following adjuvanted or nonadjuvanted immunization, the BIOVACSAFE project has guided the design of trials which can better address the innate/inflammatory axis and focus on population such as neutrophils that are central actors in innate responses.

3.4. Setting the Limits of Inflammatory Reactions to Vaccines and Adjuvants. A second problem with many systems vaccinology trials to date is that only nonreactogenic vaccines have generally been studied. To compensate for this, a large outpatient trial involving healthy adults having a booster immunization with a dTaP vaccine (diphtheria, tetanus, and acellular pertussis) has been organized to compare the transcriptomics, metabolomics, and proteomics profiles of subjects who may experience slightly more reactogenicity, within ethically acceptable parameters, largely as a result of diphtheria toxoid boosting. This will begin to explore the higher end of the scale of biomarkers of reactogenicity and act as validation for discriminatory biomarkers already observed.

3.5. Beyond the Clinical Trial: Validating Biomarkers against Natural Infections. One of the most significant features of a benefit-risk evaluation of a new vaccine or adjuvant is to contrast any adverse reactions from the immunization with those experienced during the infection that has been averted. The BIOVACSAFE consortium therefore includes pediatric cohorts in Ecuador and Germany: the former is

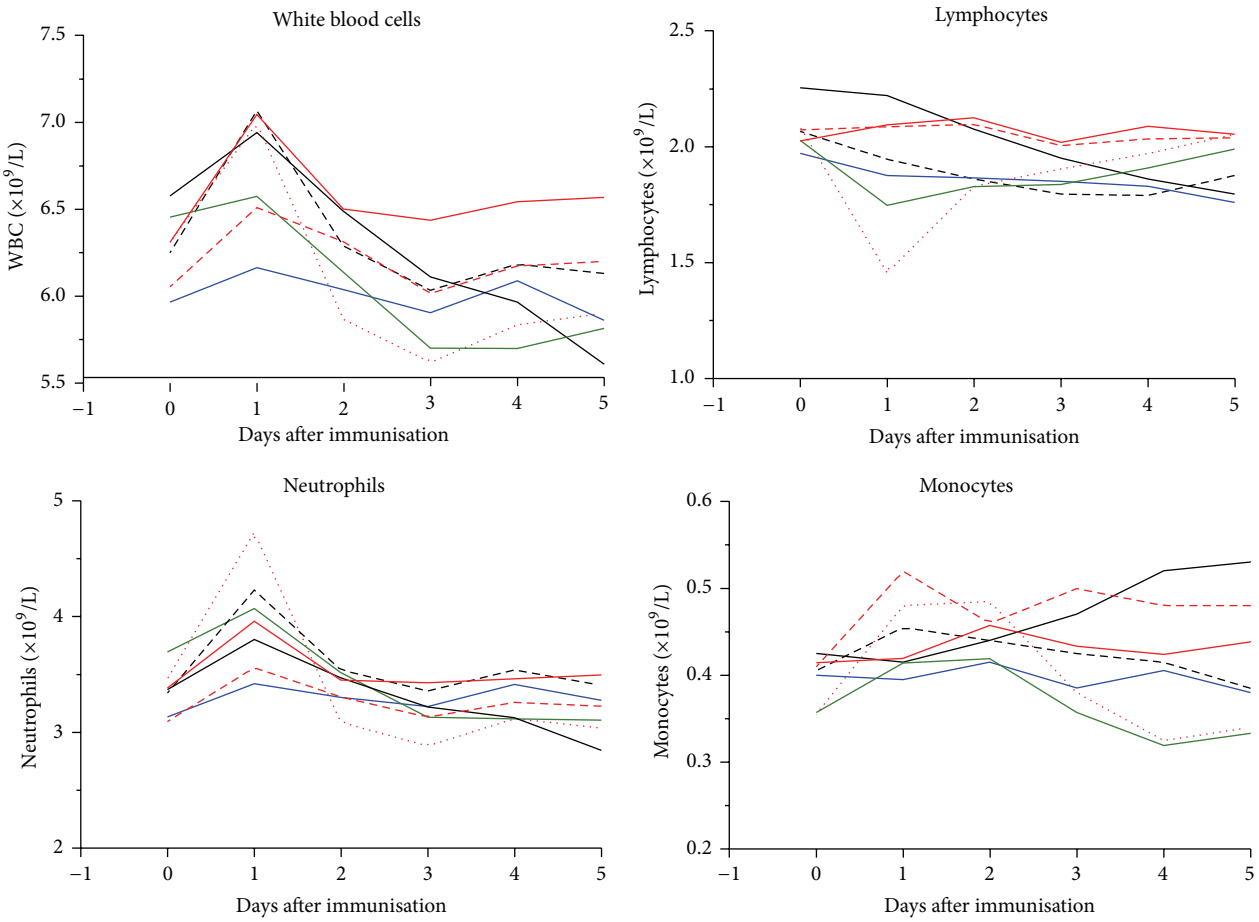

FIGURE 4: Differential kinetics of white blood cell populations enumerated by Coulter Counter at frequent time points following immunization with adjuvanted (red), unadjuvanted (green), or live (black) vaccines, or saline placebo.

collecting clinical samples from children being immunized with whole cell pertussis vaccines which are the standard EPI vaccines in Ecuador, to characterize the "omics" response to vaccines no longer routinely used in the EU; the latter is characterizing inflammatory responses in children presenting with natural infections. These data can act as a "positive" control to benchmark and place in context any subtle changes seen after immunization with licensed vaccines. By uniquely integrating data from transcriptomics and proteomics generated within a single core facility (Max Planck Institute for Infection Biology, Berlin) collected from adults and children undergoing immunization with adjuvanted and unadjuvanted vaccines or experiencing acute infection, the project is generating human data of unparalleled harmonization and direct comparability.

3.6. Beyond the Blood: Exploring Innate Immune Responses to Vaccine Adjuvants at the Site of Immunisation and Beyond. Integration and systems analysis of the trials is progressing, but initial analysis of whole blood transcriptomics changes from the intensive inpatient trials has already identified discriminatory transcriptomics profiles between adjuvanted and nonadjuvanted vaccines, with peaks at various times

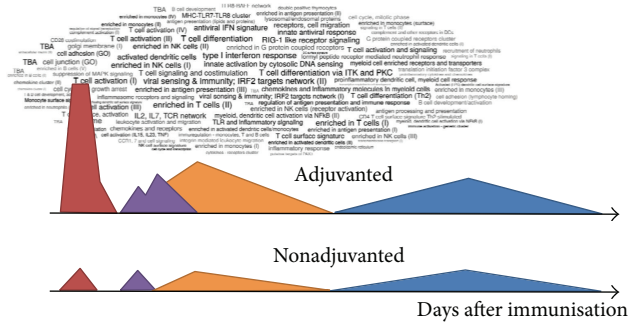

FIGURE 5: Cartoon illustrating relative expression of related genes over time for an adjuvanted and unadjuvanted vaccine, with the names of the associated overrepresented gene clusters in the first peak (red) for the adjuvanted vaccine identified in the cloud.

after immunisation in which pathways were active (*personal communication*, January Weiner, Max-Planck Institute For Infection Biology, Berlin, Figure 5). Interestingly, there was no obvious "incarceration effect" on transcriptomics patterns (in contrast to blood chemistry and some hematology parameters).

Table 1: Examples of hierarchy of selected medDRA terms used to describe AEFIs in BIOVACSAFE clinical trials.

Preferred term	Higher level term	Higher level group
Abdominal distension	Flatulence bloating and distension	Gastrointestinal signs and symptoms
Abdominal discomfort	Gastrointestinal signs and symptoms not elsewhere coded	
Abdominal pain	Gastrointestinal and abdominal pains	
Nausea	Nausea and vomiting symptoms	
Neutrophil count decreased	White blood cell analyses	
Neutrophil count increased		

While systems vaccinology using whole blood or separated PBMCs has greatly advanced our understanding of the mechanisms of vaccine and adjuvant immunogenicity, events in the bloodstream are remote from the sites of immune activation such as the site of injection and draining lymph nodes. While these can be studied in animal models, there is an increased desire to undertake safe and ethical human immunology studies to generate data from humans. In a further refinement of the systems vaccinology model, BIOVACSAFE has embarked on a study in which a head-to-head comparison of adjuvanted vaccines for influenza (MF59C squalene microemulsion adjuvant) and hepatitis B sAg (ASO4C adjuvant, alum with TLR-agonist 3-*O*-desacyl-4′-monophosphoryl lipid A) is done, in which subjects will have a muscle biopsy at the site of injection, and from the contralateral leg as control, at different time points after immunisation: +3 hours and 1, 3, 5, and 7 days. RNA extraction and analysis of muscle transcriptomics will be compared with simultaneous whole blood. This is of particular relevance to adjuvanted vaccines as animal models have shown that while certain adjuvant systems (e.g., AS03, AS04) induce localised but also draining lymph node innate activation, associated with immunogenicity [32–34], others (e.g., MF59) induced a localized immunostimulatory environment in the muscle but did not modulate the transcriptome in the draining LN and do not induce any antigen-independent activation of B and T cells [35]. This unique direct comparison will enhance parallel studies in animal models including nonhuman primates.

The Bergström needle technique has been in use for many decades as a safe and effective way to obtain muscle including gene expression [36]. While techniques of fine needle aspiration of pathological or enlarged lymph nodes are also ethically possible, they give only a very limited amount of tissue from one site. BIOVACSAFE will take advantage of observations from the cancer literature that radiolabelled glucose Positron Emission Tomography (^{18}F-FDG-PET) used in clinical practice to identify tissue with raised metabolism (glucose uptake) can image immune activation at both site of injection and draining lymph nodes in humans over the first 7 days after adjuvanted vaccines [37]. By combining a PET scan immediately prior to the muscle biopsy with characterization of muscle and blood transcriptomics profiles, BIOVASCAFE will characterize both the extent (distribution) and the intensity of draining lymph node activation after these two adjuvants and lay the foundation for future studies in which radiolabelled cytokine-specific ligands can be used to further dissect out immune responses at site of immunization with different adjuvants [38].

3.7. A New Language to Describe Adverse Events following Immunization (AEFIs) with Adjuvanted Vaccines for Systems Vaccinology. Another distinctive feature of the BIOVACSAFE collaboration is that all clinical and safety laboratory data have been harmonized to CDISC standards (http://www.cdisc.org/) at all clinical sites, making integration into centralized databases efficient and reliable for systems vaccinology analysis. This ensures that all data points are correctly and uniformly identified allowing portability and interoperability of data exchange across collaborators and external users and over time.

In order to systematically record and analyze AEFIs, various standard lexicons are used such as medDRA, developed by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) as "a rich and highly specific standardized medical terminology to facilitate sharing of regulatory information internationally for medical products used by humans" (http://www.meddra.org/). This takes a free text description of an adverse event by an investigator (e.g., pain, swelling, or inflammation at an injection site) and progressively translates it to a standardized term of wider scope. However, systems biology requires a standardized approach to data grouping that can be used to differentiate different participants, for example, "infected" or "uninfected." Experience generated in the unique BIOVACSAFE clinical studies has revealed that it is difficult to select a single level of medDRA coding to direct the computer algorithms when comparing groups, as the level at which useful specificity may converge varies from event to event.

Table 1 illustrates the process for some AEFIs recorded during the BIOVACSAFE clinical trials of adjuvanted vaccines. As can be seen, *abdominal bloating*, *abdominal discomfort*, and *abdominal pain* remain split right up to the *higher level term*, leading to too few episodes in each to reach significance, when "discomfort" and "pain" are clearly closely related and could potentially be grouped during analysis. However, if integration takes place against the *higher level group*, they are combined with unrelated symptoms such as *nausea*. Similarly, as has been shown, significant changes in lymphocyte populations occur (Figure 4), and yet medDRA groups *increased* and *decreased neutrophil count* together with *any other white blood cell abnormality* at the *higher level term*. Furthermore, many linguistic synonyms such as "*reduced*"

or "*low*" occur at the level of *preferred term* leading to possible splitting of related events at the clinical site, thereby reducing the power of the systems biology analysis. It is therefore extremely difficult, if not impossible, to direct the systems biology algorithms to a single preferred level in the medDRA hierarchy across all parameters. To compensate for this, significant postprocessing of AEFIs may be required to achieve a consensus level of precision, leading to potential bias or error. Finally, the *a priori* definition of reserved terms to apply only to immunization and not other study specific procedures such as phlebotomy may be required, to avoid mixing AEFIs with other procedures. In the BIOVACSAFE studies, the intensive inpatient trials were used to identify a lexicon of *preferred terms* most frequently observed that were incorporated in a drop-down menu on the electronic data capture forms to guide investigators to the most efficient way to classify AEFIs in follow-on trials. If systems biology is to be applied routinely in the clinical evaluation of adjuvanted vaccines, a major overhaul of how we report, classify, and grade AEFIs will be required, as is being pioneered in the BIOVACSAFE project.

3.8. Genetics of Adverse Reaction to Immunisation. As personalized medicine advances and adjuvanted vaccines are increasingly applied to populations that may be at risk of hyporesponsiveness or severe or autoimmune reactions, the genetic factors affecting immune responses may become important. This requires huge data sets at the population level to identify infrequent gene associations or adverse events such as autoimmunity. The involvement of deCODE in BIOVACSAFE, with access to the immunisation histories and clinical outcomes of thousands of Icelanders who have been genotyped and chip-typed, provides a powerful tool to answer such questions.

3.9. Animal Models of Reactogenicity to Immunization with Adjuvanted Vaccines. While a great deal can be achieved in human experimental medicine studies, and although it is increasingly accepted that animal models do not always reliably mimic the clinical experience, there are experiments that cannot ethically be conducted on humans; and rodents and rabbits remain the standard models for preclinical toxicology evaluation of adjuvanted vaccines. In addition, by studying only ethically acceptable and generally nonreactogenic vaccines in humans, it is difficult to know where the threshold of acceptability lies for any identified "biomarkers" (see Figure 6), in comparison, for example, with the inflammation induced by natural infection, which is assumed to be far greater than after immunisation. BIOVACSAFE will uniquely address this by integrating into the same data set human and animal data of transcriptomics and proteomics. Comparison will be made between human samples from the clinical trials and mice, rats, rabbits, and ferrets (blood, injection site, draining lymph nodes, spleen, liver, thymus, and bone marrow) immunized with the same adjuvanted and unadjuvanted vaccines or TLR-agonist positive controls, using a harmonized set of immune readouts (Table 2). Many bespoke assays have been created, particularly qPCR regents for ferret

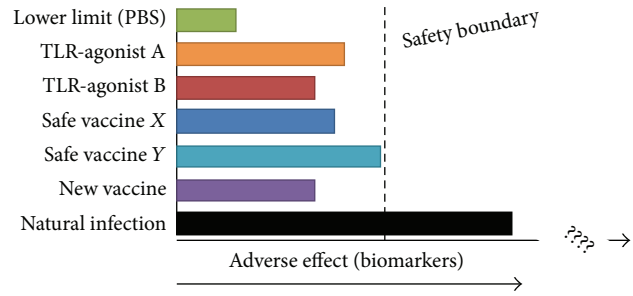

Figure 6: Illustration of need to identify a "safety boundary" on the sliding scale of adverse effects or biomarkers observed in response to "known to be safe" vaccines, immune agonists, and natural infection in animal models and human experimental medicine.

studies that are not commercially available, even though this species is an important influenza infection model. All these data will be integrated into a single integrated systems biology database for querying and analysis. Outputs may guide the use of appropriate preclinical toxicology models for novel adjuvants.

3.10. Putting It All Together: Integrated Database for Systems Vaccinology. Effective application of systems vaccinology requires the retrieval and integration of data from many different sites and assays, including preclinical and clinical data as well as complex laboratory and systems biology (or "omics") data (Figure 7). BIOVACSAFE has developed a bespoke annotated large data warehouse using the open access tranSMART platform, including the provision of database hosting and curation as well as data mining capabilities. The use of the tranSMART platform allows the collection of data in a format that will be compatible with other international projects and consortia. Clinical data will meet CDISC-CDASH, CDISC-SDTM, and BRIDG UML standards to ensure seamless comparisons between trial protocols within BIOVACSAFE and externally or in the future. The shared database will enable partners to conduct exploration and analysis using a systems biology approach leading to biological interpretation, while preserving high standards of data protection and confidentiality. Data inventory requirements will be served by a standards compliant data repository that will store project data and metadata according to the list above. Once cleaned and curated, data will be accessed via a warehouse based on tranSMART for data mining and analytical processes. Data will then be accessible for export to specific systems biology and statistical tools for the analysis and correlation, after selection within the database on specific criteria. Statistical Analysis Plans will ensure that appropriate biological questions are framed in advance. This unique combination of adverse reactions, safety laboratory variables, and "omics" data from human and animal models will be an invaluable resource.

However, as with many fixed-term public-funded initiatives, a significant risk to the project remains the ongoing funding and availability of this resource after the project ends. It is to be hoped therefore that further public-private

Table 2: Harmonized minimum data set of immune parameters for cross species evaluation of responses to adjuvanted vaccines and TLR agonists.

Vaccine or TLR agonist	Commercially available Luminex assays	Bespoke Luminex assays to mirror clinical studies	Bespoke qPCR plasmid calibrants for ferrets
Pentavalent/whole cell pertussis	IL-1α	C-reactive protein	IL1β
Trivalent influenza	IL-1β	Serum amyloid A	IL6
Trivalent influenza + MF59	IL-2	a2-macroglobulin	IL8
Engerix B	IL-3	LPS binding protein	CXCL10
Varicella vaccine	IL-4	Procalcitonin	CCL2
Poly I: C	IL-5	PTX3 pentraxin	IFNα
Lipopolysaccharide	IL-6	TREM-1	TNFα
Incomplete Freund's adjuvant	IL-9		Serum amyloid A
	IL-10		GAPDH
	IL-12 (p40)		RPL32
	IL-12 (p70)		
	IL-13		
	IL-17A		
	KC (CXCL1)		
	MCP-1 (CCL2)		
	MIP-1α (CCL3)		
	MIP-1β (CCL4)		
	RANTES		
	IFN-γ		
	TNF-α		
	GM-CSF		
	G-CSF		
	Eotaxin		

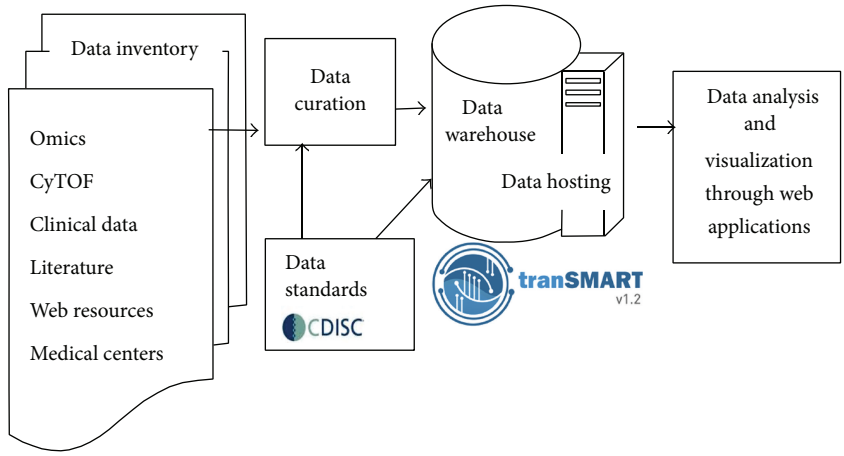

Figure 7: Integrated database for systems vaccinology.

funding may invest in follow-on projects that capitalize on the information being generated using systems biology to investigate efficacy and reactogenicity of adjuvants and vaccines.

Conflict of Interests

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

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Review Article

Extracellular Vesicles: Role in Inflammatory Responses and Potential Uses in Vaccination in Cancer and Infectious Diseases

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Almost all cells and organisms release membrane structures containing proteins, lipids, and nucleic acids called extracellular vesicles (EVs), which have a wide range of functions concerning intercellular communication and signaling events. Recently, the characterization and understanding of their biological role have become a main research area due to their potential role in vaccination, as biomarkers antigens, early diagnostic tools, and therapeutic applications. Here, we will overview the recent advances and studies of Evs shed by tumor cells, bacteria, parasites, and fungi, focusing on their inflammatory role and their potential use in vaccination and diagnostic of cancer and infectious diseases.

1. Introduction

Extracellular vesicles (EVs) are particles of 20 nm up to 5 μm in diameter composed of proteins, nucleic acid, and lipids that are found in body fluids such as plasma, serum, saliva, urine, breast milk, ascites, and cerebrospinal fluids [1]. These particles are involved in intercellular communication, modulating a wide range of signaling events during innate and acquired immune responses (Figure 1 and Table 1) [2–4]. EVs are secreted during health conditions or upon inflammation during the course of diseases by all mammalian cells types [2, 3, 5].

EVs include different types of particles and may be named or classified depending on the cell type or function. They can be derived from dendritic cells (dexosomes), prostate tissue (prostasomes), bone, cartilage and atherosclerotic plaques (matrix vesicles), neurons (synaptic vesicles), apoptotic blebs or apoptotic bodies (microparticles, exosomes, and apoptotic vesicles), shed vesicles, shedding microvesicles or microparticles (ectosomes or microvesicles), and membrane fragments of virus infected cells, protozoa, fungi, and bacteria outer membrane vesicles [1, 2, 4, 6–10].

The vesicles derived from mammalian cells contain a family of integral membrane proteins that cross four times the lipid bilayer and are called tetraspanins [11], including the surface markers of lymphocytes and antigen-presenting cells such as CD37, CD9, CD53, CD63, CD81, and CD82. EVs also contain molecules of the major histocompatibility complex (MHC classes I and II) (http://www.exocarta.org/) [11, 12]. EVs derived from normal cells cause either suppression or activation of the immune response by modulating the production of inflammatory mediators. For example, T-cells and monocytes secrete vesicles that contain FasL on the surface that modulate apoptosis of the other cells (Figure 2) [13]. Vesicles isolated from monocytes deliver proinflammatory mediators that activate endothelial cells [14, 15]. Tumor cells secrete EVs that are able to downregulate the immune system, allowing the escape from the immune system. Furthermore, these vesicles can control tumor development and growth, by decreasing the expression and release of IL-2 reducing

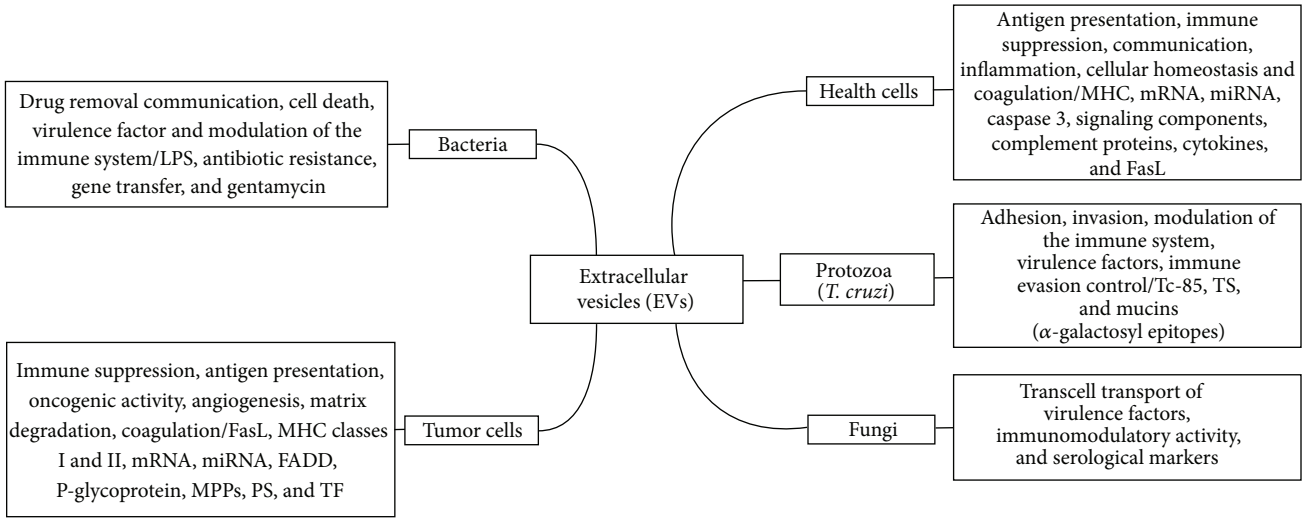

Figure 1: Schematic review of the origin function and markers (molecules to delivery) of EVs normal and tumor cells, parasites, fungi, and bacteria.

Table 1: Major components of extracellular vesicles and their functions described.

Origin	Molecule	Function	Reference
Tumor	MHC I and II	Antigen presentation	[18]
	miRNA and mRNA	Oncogenic activity, drug resistance, and metastasis angiogenesis	[19–22]
	CXCR4 and MMP-9	Invasion and migration	[23]
	TrkB, EGFR, and TES complex	Angiogenesis	[24]
	Rab22A, Pabp1, and PSA	Metastasis	[25–27]
	CD40, CD80, CD86, and CD54	Immunity	[28]
	GTPase and Rab27a	Upregulated immune system and inhibited tumor growth	[29]
Bacteria	OmpQ and pertactin	Immunogenic	[30]
	Gene transfer	Communication	[31]
	Gentamycin	Cell death	[32]
	RNAs	Communication	[33, 34]
Protozoa	tGPI-mucin	Activation	[35]
	Tc85	Invasion/adhesion	[36]
	gp63 and LPG	Virulence factor	[37–39]
	TS	Virulence factor	[40, 41]
Fungi	α-gal	Immunogenic	[42]
	GXM and GlcCer	Virulence factor	[43–45]
Eukaryotic mammalian cells	MHC I and II	Antigen presentation	[2]
	FasL	Immune suppression	[13]

the proliferation of natural killer (NK) cells [14, 15]. Therefore, EVs are potential biomarkers and antigens for vaccination, with potential uses for early diagnostic, and therapeutic applications in several diseases.

The purpose of this review is to provide an updated overview of the vesicles released by distinct pathogens and mammalian tissues, highlighting their potential use in vaccination and diagnostic of cancer and infectious diseases.

2. Extracellular Vesicles in Cancer

EVs derived from tumors may be involved in tumor growth control and in the communication events between tumor and normal cells by delivering oncogenic proteins and growth factors [16, 17]. In some cases, EVs suppress tumor growth by exposing dendritic cells MHC classes I or II molecules, peptides, and costimulatory molecules for the immune

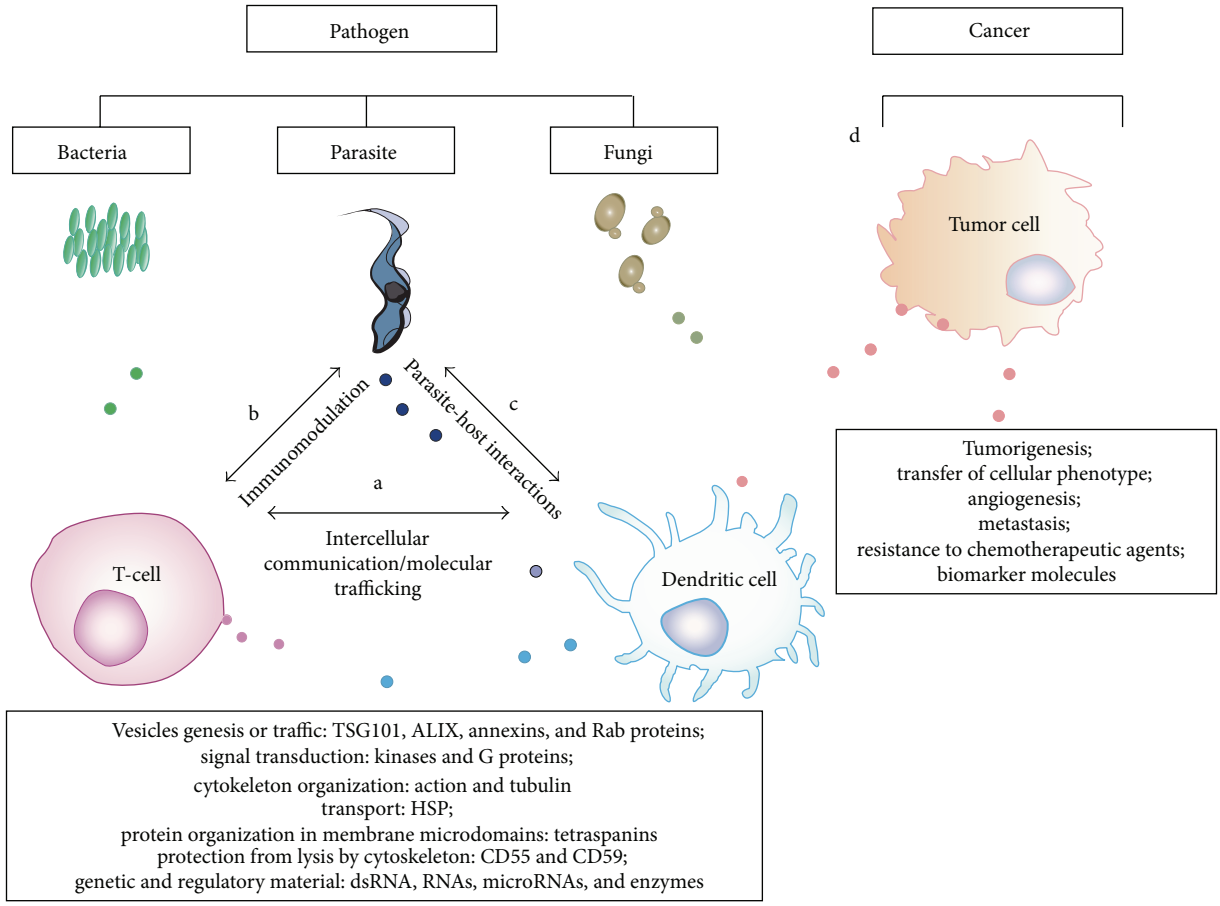

Figure 2: EVs released from all cell types. These particles are involved in physiologic and pathologic processes: (a) intercellular communication and molecular trafficking delivering regulatory signal molecules and (b) and (c) parasite-host interactions and immunomodulation in pathologic conditions; (d) drug resistance, cancer progression, angiogenesis, and metastasis are some functions of exosomes in cancer [1].

system. This amplifies the immunological response, preventing tumor growth [18]. EVs can also stimulate the resistance to chemotherapeutic agents. Moreover, EVs contain proteins and genetic material from the originating tumor cells that can be used as diagnostic biomarkers. In this regard, recent efforts to elucidate different roles and signaling pathways of EVs have been conducted.

A pivotal role of EVs during cancer cell migration and invasion has been reported in different cell types. For instance, EVs derived from 786-0 renal tumor cells enhance their migration and invasion properties [23]. This occurs through induction of type 4-chemokine receptor (CXCR4) and matrix metalloproteinase-9 (MMP-9) expression by EVs. In addition, adhesion and invasion of the gastrointestinal interstitial stroma are enhanced by the oncogenic protein tyrosine kinase (KIT) present in tumor cell EVs [46]. More importantly, those structures have also been involved in drug resistance. Tamoxifen-resistant breast tumor cells release exosomes that contain microRNAs (miR221/222) and promote drug resistance in naive cells [19]. Similarly, resistance to docetaxel in breast tumors and prostate cancer, as well as cisplatin in human lung cancer line (A549 cells), was associated with the content of vesicular microRNAs transferred to susceptible cells [47–49]. Moreover, EVs from A549 cells containing TrkB, EGFR, and sortilin receptors (TES complex) were related to angiogenesis induction through endothelial cells [24]. In hepatocellular carcinoma (HCC), one of the most lethal cancers, the tumor becomes more resistant to TGFβ-dependent chemotherapy through long noncoding RNAs (lncRNAs) obtained from EVs [50]. Therefore, the extracellular communication through EVs is an important mechanism to activate/deactivate certain crucial events in tumor cell biology.

Other studies have evidenced the role of microRNAs present in EVs in cancer establishment. For instance, miR-105, detected in EVs from breast tumor, is associated with metastasis formation via destruction of endothelial monolayers. Interestingly, it is possible to detect miR-105 in the blood circulation before the metastasis establishment reinforcing its potential role as a diagnostic biomarker [20]. Likewise, gastric cancer stromal cells deliver exosomes to gastric tumor cells. Expression of miR-214, miR-221, and miR-222 present in these EVs is related to lymph node metastasis, venous invasion, and tumor development [21]. In some cases, miR-containing EVs repress proangiogenic events and impair tumor development on a bone cancer model [22].

The study of biogenesis of stress-induced vesicles also becomes crucial to understand the development of metastasis. For example, the elevated expression of *RAB22A* gene in breast tumor cells induced by hypoxia, common in advanced tumors, increases the shedding of vesicles, and the Rab protein colocalizes with the sites of budding EVs. Moreover, the knockdown of *RAB22A* prevents metastasis, supporting the idea that Rab is involved in the generation of EVs [25]. EVs released from heat-stressed tumors in a mouse model can induce antitumor immunity [51]. These vesicles showed chemotactic effects on CD4+ and CD8+ T-cells, efficiently activating dendritic cells (DC). Another study showed that EVs derived from breast cancers can alter the tumor microenvironment and promote tumorigenesis of normal cells via induction of autophagy, response to DNA damage repair (DDR), and induction of reactive oxygen species (ROS) in normal breast epithelial cells [52].

EVs also carry potential cancer biomarker molecules, as reported by several groups. This includes the polyadenylate-binding protein 1 (Pabp1), predominant in EVs from metastatic duodenal tumor cell lines [26], prostate-specific membrane antigen (PSA) related to prostate cancer progression, angiogenesis, and metastasis [27], miR-21 and miR-146a in cervical cancer [53], and finally lncRNAs in skin cancer (secreted into the blood or urine through EVs) [54]. All the above-mentioned microRNAs are proposed as potential biomarkers for cancer noninvasive diagnosis. It was also shown that EVs from pancreatic tumor cells contain fragments of double-stranded genomic DNA (dsDNA), suggesting that mutations may be identified in this dsDNA as predictors of cancer and streamline therapeutics [55]. Based on these findings, it is clear that new biomarkers, once optimized, could be used in therapeutic conducts, offering great advantage over other established methods.

In cancer therapy, EVs can also be employed as vehicles to deliver drugs. EVs from tumor cells are able to associate better with their recipient cells than liposomes (>10-fold), due to their lipid and protein composition [56]. In addition, microRNAs can be delivered to tumor cells and interfere with cancer progression and metastasis. In this logic, synthetic miR-143 was introduced into mesenchymal stem cells, and the secreted exosomes containing miR-143 was transferred to osteosarcoma cells to reduce the migration of the latter cell [57]. Interestingly, a feedback regulatory mechanism for controlling exosome release was suggested, in which exosomes derived from normal human mammary epithelial cells could impair the release of exosomes from breast tumor cells [58]. These authors suggest that this may be used as a novel therapeutic approach, attenuating carcinogenic effects of tumor exosomes. Another interesting strategy is to use a synthetic structure based on tumor-derived exosomes and staphylococcal enterotoxin B to induce apoptosis in breast tumor cells [59, 60]. The vesicles could be used as a diagnostic, because tumor cells release vesicles in biologic fluids like urine, blood, ascites, and pleural fluids. For example, patients with ovarian cancer shed vesicles derived from tumor cells in the circulation. These vesicles are enriched up to 4-fold more in patients with cancer than healthy controls. Therefore, they can be used as biomarkers to identify early cancers in asymptomatic patients that will potentially develop malignancy. In addition, specific miRNAs are found in extracellular vesicles from patients with lung cancers [61].

DC have been widely used in the research of therapeutic cancer vaccines. For example, DC were primed with interferon-gamma (IFN-γ) to induce the expression CD40, CD80, CD86, and CD54 in exosomes, endowing a potent CD8+ T-cell-triggering potential *in vitro* and *in vivo* [28]. Yao et al. [62] compared the antitumor immunities between EG7 tumor cell-derived exosomes [EXO (EG7)] and EXO-(EG7-) targeted dendritic cells [DC (EXO)]. They showed that the latter DC (EXO) was more effective in inducing antitumor immunity, and this was independent from the host DC, emphasizing the role of the host DC in tumor cell-derived exosomes (TEX) vaccines. In contrast, CD8+ T-cell responses could be induced *in vivo* when mice were immunized with protein-loaded instead of peptide-loaded dexosomes. Recently, protein-loaded dexosomes were used to protect against tumor growth, whereby CD8+ T-cell responses occurred *in vivo* [63, 64].

Purified MHC classes I and II inserted in exosomes and delivered to melanoma were recognized by specific T-cells. This was used to transfer functional MHC/peptide complexes to antigen-presenting cells [65]. In this way, antitumor response could be elicited as these complexes may stimulate CD8+ and CD4+ T-cell responses in an "acellular" immunotherapy approach. In another study, exosomes from Rab27a overexpressing cells increased significantly CD4+ T-cell proliferation *in vitro* because these exosomes upregulated MHC class II, CD80, and CD86 molecules in DC. Moreover, exosomes containing a small GTPase protein involved in secretion of exosomes also were capable of retaining tumor growth *in vivo* [29].

Plasmid DNA vaccines encoding EV-associated antigens were recently used as vaccines in mice in order to produce ovalbumin containing-EV antigens *in vivo*, either exposed on the surface of vesicles or incorporated inside membrane-enclosed virus-like particles [66]. In both cases, these vaccines were able to induce specific T-cell responses and efficiently prevent the growth of ovalbumin-expressing tumors *in vivo*, showing that immunotherapy based on EVs may be a valuable method to promote tumor control and other diseases.

3. Bacterial Vesicles

Bacteria release vesicles sizing from 20 to 250 nm [33, 67] are named outer membrane vesicles (OMVs) for Gram-negative and membrane vesicles, or blebs, for Gram-positive bacteria [68, 69]. EVs are required for the exchange of genetic information between bacteria such as *Bacillus anthracis*, *Staphylococcus aureus*, *Mycobacterium ulcerans*, *Bacillus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Helicobacter pylori*. Additionally, EVs contain toxins and deliver virulence factors to host cells [32, 70–80]. Bacterial EVs are composed of cytosolic and membrane proteins, lipoproteins, phospholipids, glycolipids, and nucleic acids [31, 32, 81–84]. Detailed composition analysis and biogenesis of OMVs from different Gram-negative bacteria are available [33]. For example,

OMVs from *Bordetella parapertussis* contain surface immunogenic molecules, porin, outer membrane protein OmpQ, and pertactin that were used in a murine model to assess the protection against infection [30]. In the same way, OMVs of *Pseudomonas putida* KT2440 have outer membrane proteins such as OprC, OprD, OprE, OprF, OprH, OprG, and OprW which can serve as adjuvants or vaccine [85]. *Vibrio cholerae* OMVs contain several proteins that contribute for the virulence and are essential for cell growth and colonization *in vivo* [86]. Another interesting aspect of OMVs is their role in delivering endotoxins to host cells as demonstrated for enterogenic and uropathogenic *Escherichia coli* ((ETEC) and (UPEC)), the causative agents of traveler's diarrhea and human urinary tract infections. Both ETEC and UPEC strains are able to produce many virulence factors including the heat-labile enterotoxin (LT), homologous to cholera toxin, and cytotoxic necrotizing factor type 1 (CNF1). These toxins are released from bacteria in OMVs and delivered to host cells through vesicle internalization [74, 87]. In particular, LT also acts as a ligand for vesicle binding, which is internalized via lipid rafts. Once inside the cell, the toxin is trafficked via retrograde transport through the Golgi and the endoplasmic reticulum [74].

An outstanding role of OMVs in biotechnology is their use as general vehicles to deliver human, heterologous, or viral antigens [33, 88]. *Neisseria meningitidis* serogroup B OMVs showed remarkable adjuvant properties for anti-HIV-1 antigens and induced a production of IFN-γ and IL-4 [89]. Vesicles isolated from DC infected with *Mycobacterium tuberculosis* were able to induce a protective host immunity response [90, 91]. There are also potential uses of these EVs as cancer vaccines through immune stimulation [92]. OMVs from different species of Gram-negative bacteria contain lipopolysaccharide (LPS), proteins, and nucleic acids, which are strong agonists in the modulation of inflammatory reactions through the activation of Toll-like receptors (TLRs). These activations require the action of LPS, which is sensed by Toll-like receptor 4 (TLR4) on host cells, and induce an innate immune response to Gram-negative bacteria leading to inflammatory cytokine production [93–96].

In the case of *Pseudomonas aeruginosa*, OMVs appear to deliver virulence factors to distant locations by fusing with lipid rafts of several host cell membranes [97]. Proteins present in secreted vesicles released from *P. aeruginosa* also seem to play important roles in pathogenesis. This is the case of the inhibitory factor of the cystic fibrosis transmembrane conductance regulator, which promotes changes in the epithelium, allowing reduced clearance of *P. aeruginosa* toxin A that hijacks the host ubiquitin proteolytic system [97]. Therefore, *P. aeruginosa* EVs have the potential to protect the immunized host against subsequent infection and for this reason they have been proposed as vaccines candidates against infection. Another interesting example is OMVs isolated from *Haemophilus influenzae*, which increases the expression of CD69 and CD86 and activating of the humoral response. In addition, they induce TLR9 signaling through bacterial DNA, which causes a significant proliferative response of inflammatory cells [98].

Vesicles from Gram-negative bacteria are released naturally as blebs of the outer membrane through bulging and "pinching off." Alternatively, vesicles can be prepared from the detergent-treated bacteria either from normal or from bacteria carrying genetic modifications such as the generalized modules for membrane antigens (GMMA) to induce a strong immune response [99]. All these vesicles are called OMVs, but it is important to note that they have different composition and properties. Naturally shed blebs are almost free of cytoplasmic and inner membrane components and maintain lipophilic proteins, unlike detergent extracted OMVs derived from bacteria. These differences are relevant when considering the use of vesicles for immunization or diagnostic purposes [67]. Several vaccines are prepared based on OMVs isolated from Gram-negative bacteria [100]. One example is the case of *Neisseria meningitidis*-OMVs vaccine, named Bexsero (Novartis) [67]. These particles activate the immune response and protection against a challenge with bacteria in murine models [82, 101–107]. There are, however, several cases that vaccination with OMVs requires further developments to improve better antigenicity, manufacturability, and reduction of pyrogenicity, detergent extract, and toxicity via LPS detoxification [82].

The mechanism of how Gram-negative bacteria-derived OMVs elicit a vaccination effect, for example *E. coli* used as a model to study the effect of the adaptive immune response decrease against bacteria-induced lethality. However, with high doses these OMVs induced systemic inflammatory, characterized by hypothermia, tachypnea, and leukopenia (sepsis) [108].

Because of the thick cell wall of Gram-positive bacteria, extracellular vesicle secretion has been less studied in these bacteria. Nevertheless, it has been reported that *S. aureus* and *Bacillus subtilis* secrete membrane vesicles to the extracellular milieu. Proteomic analysis revealed that vesicles derived from *S. aureus* harbor several pathogenic components [109]. Furthermore, *S. aureus* extracellular vesicles induce atopic dermatitis-like skin inflammation in mice. These observations provided hints on the possible roles of Gram-positive secreted vesicles. Recently, a study on the immune activating role of Gram-positive bacteria-derived EV has been published [110]. The Gram-positive *Bacillus anthracis*, the agent of the Anthrax d isease, also shed membrane-derived vesicles. These EVs are formed by a double membrane and have a spherical shape sizing from 50 to 150 nm [83]. They are enriched by molecular chaperons and molecules of the cell wall involved in the cellular architecture and include the lethal toxin (LeTx) and the antholysin (ALO). BALB/c mice immunized with these EVs were able to produce more protective IgM to the toxin in comparison with the isolated toxin, prompting to further use these preparations to elaborate vaccines. The protection induced by vesicles obtained from Gram-positive bacteria was not as effective when compared to Gram-negative bacteria OMVs indicating that further work might be necessary to improve their potential.

In summary, OMVs include multiple virulence factors, overcoming the limitation of a single antigen immunization. Furthermore, OMVs can act as adjuvant and antigen carrier.

4. Parasite Vesicles

Cultured protozoan parasites release EVs that contain several molecules that might affect the host (Figure 1). They are composed of membrane fragments and cytosolic components, including proteins, lipids, and nucleic acids that accumulate in the supernatant of the protozoan cultivated in the presence or absence of host cells [40, 41]. When injected in animal models or added to *in vitro* systems, these EVs were found to affect the course of infection and alter the disease progression caused by the parasite, through the modulation of the host innate and acquired immune response. EVs are described in many protozoa such as *Leishmania* spp. [37, 38, 90, 111, 112], *Trypanosoma cruzi* [40, 41, 113–115], *Trypanosoma brucei* [116], *Plasmodium* spp. [117, 118], *Trichomonas vaginalis* [119], *Toxoplasma gondii* [120–122], and *Eimeria* parasites [123]. Helminthes have also released EVs in *Dicrocoelium dendriticum* [124].

Trypanosoma cruzi is a flagellate protozoan that causes Chagas disease. It is acquired by humans either by the insect vector, blood transfusion, or through maternal transmission during new born delivery [125]. When *T. cruzi* enters the host, the first line of defense is the innate immune response, which initiates when receptors that recognize microbial products are activated [126]. This occurs through Toll-like receptors (TLR) signaling and macrophage activation by mucin-like glycoproteins, which corresponds to 60–80% of the parasite surface molecules [35, 127], resulting in the increased production of IL-12, IFN-γ, and nitric oxide (NO) [128]. The production of these proinflammatory cytokines leads to the activation of several kinds of cells such as natural killer (NK) typical of the acute phase of Chagas disease [129]. Very little is known about how mucin-like glycoproteins and other surface components are presented to the host.

Infective parasites obtained from cultured mammalian cells shed large amounts of EVs that are rich in these surface molecules [40]. EVs isolated from infective *T. cruzi* forms promote macrophage activation with an increase in parasitemia levels and amastigotes nests in the heart tissue [41]. These effects are caused by parasite surface glycoproteins present in the vesicles that attenuate the host immune system. *T. cruzi* EVs are enriched in α-gal containing glycoconjugates, found preferentially in the mucin-like molecules [35], and several surface glycoproteins, known as members of a *trans*-sialidase (TS) family that participate in adhesion and invasion of host cells [40, 41, 114, 130]. Mucins containing α-gal residues elicit high titers of IgG antibodies decreasing parasitemia during the chronic phase [131]. Therefore, the production and release of EVs might have a key role in the establishment of infection and may be considered a platform to develop preventive or prophylactic vaccines for Chagas disease [132].

Leishmania genus encloses protozoan species that cause visceral, cutaneous, and mucocutaneous leishmaniasis in humans. The disease is transmitted by sandfly vectors (*Lutzomyia* and *Phlebotomus*), which inject parasites into the host during the insect blood meal [39]. In culture, the insect stages of several *Leishmania* species release EVs containing parasite antigens, such as the surface glycoprotein of 63 kDa (gp63) that has a strong suppressive effect on host macrophages [37, 38]. However, a missing step in *Leishmania* EVs biogenesis is whether those structures also contain the major surface lipophosphoglycan (LPG), a multivirulence factor involved in the interaction with the vertebrate and invertebrate host [39].

EVs derived from *Leishmania donovani* are involved in immune response evasion mechanisms, enabling parasite survival in the host [37, 133, 134]. In contrast, EVs derived from macrophages infected with *Leishmania amazonensis* induce proinflammatory response *in vitro* by stimulating the production of proinflammatory cytokines TNF-α, IL-12, and IL-1β [112]. These host-derived vesicles have been characterized and contain both parasite and host components [37, 38], which indicates that a cross talk of signaling events occurs during infection. Indeed, the immunization of mice with dexosomes derived from DC pulsed with *Leishmania major* antigen was able to provide protection against the parasite [90]. This finding could help to improve the available canine vaccines, used to stop transmission [135], and eventually develop a preventive prophylactic human therapy for leishmaniasis.

Trichomonas vaginalis, a flagellated protozoan that colonizes human vaginal and urethral epithelia, also secretes vesicles that act at the host-parasite interface. *T. vaginalis* EVs stimulate the immune response by increasing the production of IL-6 and IL-8 [15] and promote greater adherence of less adherent strains of the parasite to the epithelium [15]. *T. vaginalis* EVs fuse with and deliver their contents to host cells [136] and are clearly involved in the colonization of the genital host's tract. It is also possible that EVs from this parasite could provide a more suitable environment to other sexually transmitted diseases such as HIV or HPV.

There are several studies about EVs of *Plasmodium* spp., Apicomplexa parasites that cause human and animal malaria focusing mainly in the immunization alternatives. For example, EVs derived from reticulocytes infected with *Plasmodium yoelii*, a rodent malaria, induce protection to infection in mice [137]. *Plasmodium berghei*, another rodent malaria, secretes microparticles in the plasma of infected mice that induce an intense macrophage activation, which results in inflammatory reaction [138] via TLR4 and MyD88 [12]. Therefore, these EVs are key components in the modulation and communication between the parasite and the host [118]. However, one of the main difficulties in working with human *Plasmodium*, especially *Plasmodium vivax*, is the availability to have enough amounts of EVs.

Toxoplasma gondii is another intracellular Apicomplexa protozoan that causes Toxoplasmosis. The disease is usually transmitted by eating contaminated meat, accidental ingestion of cat feces with oocytes, and congenital contact. It may cause abortion in pregnant women [139]. The infection is severe in immune-compromised individuals. EVs derived from DC incubated with *T. gondii* antigens induce an intense immune response, increasing the levels of MHC class II and the specific production of T-cells and cytokines [140]. Studies of immunization with these DC are promising alternatives in promoting protection against *T. gondii* [141, 142]. *Eimeria tenella*, *Eimeria maxima*, and *Eimeria acervulina* are also coccidian parasite of chickens that also release EVs, which

confer protective immune response against the parasite [123, 143, 144].

In summary, EVs isolated from several parasites or from infected cells have major effects on the immune response and are also potential candidates for immunoprevention of parasitic diseases.

5. Fungal Vesicles

Fungi have the capacity to cause devastating human diseases, some of them with high mortality rates, in both immunocompetent and immunocompromised individuals [145]. Pathogenic fungi exhibit a singular genetic flexibility that facilitates rapid adaptation to the host or environment [146]. However, there are several open questions of how these pathogens colonize and cause morbidity.

As other eukaryotic organisms, fungi use membrane trafficking to connect intracellular and extracellular compartments allowing sorting of protein and lipids to their final cellular sites [147]. For a variety of proteins, the extracellular milieu is the final destination of the cell wall components, digestive enzymes, and, in the pathogenic species, virulence factors [148]. In fungi, the cell wall represents the final step of secretion, an event that brings additional complexity to the secretory mechanisms used by these cells [147]. The cell wall is a complex and rigid structure basically composed of chitin, chitosan, β-1,3-glucan, β-1,6-glucan, mixed β-1,3-/β-1,4-glucan, α-1,3-glucan, melanin, and glycoproteins as major constituents [149].

EVs are now recognized as important structures for transcell transport of virulence factors that modulate host immune responses [147, 148, 150, 151], suggesting the importance of these structures in the pathogenesis of many fungal diseases. The production of fungal EVs was initially characterized in the pathogenic yeast *Cryptococcus neoformans* [152]. Currently, EVs were identified in several pathogenic fungi such as *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *Candida albicans*, *Candida parapsilosis*, *Malassezia sympodialis* [42, 78, 152, 153], and nonpathogenic yeast *Saccharomyces cerevisiae* [154]. Different proteins, sterols, phospholipids, polysaccharides, and pigments have been characterized in these fungal EVs isolated from culture supernatants [42, 78, 150–158]. Many of these molecules have been identified as known virulence factors or inducers of host humoral responses.

For example, in *C. neoformans* the most important virulence factor and immunomodulator, the glucuronoxylomannan (GXM) [43], was detected in vesicles released during *in vitro* macrophage infection [150]. In *P. brasiliensis*, similar GXM that interacts with α1,3-glucans was detected in EVs [159]. GXM acts differently on the host immune response, depending on its specific molecular characteristics [44, 45] making it a possible target for antifungal therapy or vaccination [45].

Another key molecule in fungal infection is glucosylceramide (GlcCer), a glycolipid component of the fungal cell wall [160], which has been detected in EVs of *C. neoformans* [152, 158], *P. brasiliensis* [155], and *C. albicans* [151]. Fungal GlcCer is an antigenic glycosphingolipid that elicits antibody responses in experimental infection models [161] and in patients affected by some mycoses, such as cryptococcosis [162]. GlcCer is described as a virulence regulator of *C. albicans* and *C. neoformans* [163, 164]. Furthermore, GlcCer from *P. brasiliensis*, *Aspergillus fumigatus*, and *S. schenckii* inhibited T-cell proliferation *in vitro* [165]. The GlcCer from *A. fumigatus* was able to activate *in vitro* mouse and human NK cells and to induce airway hyperreactivity in mice [166]. These findings indicate that fungal GlcCer may influence both humoral and cellular responses and that inhibition or blocking the GlcCer action can be a therapeutic approach [160].

Other studies have evidenced that vesicles isolated from *C. neoformans* culture supernatant were able to melanize after incubation with L-DOPA [158], a substrate for melanization [167]. Melanin has been identified in several pathogenic fungi [168]. Although it is immunologically active, little is known about its role in the immune response. Melanin protects fungal cells from phagocytosis by macrophages, a key step in the host defense against these pathogens [169]. It also reduces proinflammatory cytokines [170] and decreases their susceptibility to antifungal drugs [148], mainly to amphotericin B and caspofungin, and is less evident or absent in ketoconazole, fluconazole, or itraconazole [171, 172]. Therefore, it seems that melanization is a distinguished feature observed in EVs released during fungal infections and its role should be further explored in the fungal pathogenesis.

Many studies indicated that acquired immunity against EVs is observed during fungal infections. Vesicular components reacted with immune serum from patients with cryptococcosis, histoplasmosis, and paracoccidioidomycosis (PCM) [42, 78, 153] or with serum from *C. albicans*-infected mice [151]. Particularly, EVs of *P. brasiliensis* transport components carrying α-galactopyranosyl (α-gal) epitopes, a highly immunogenic molecule, which were efficiently recognized by anti-α-gal antibodies from patient with PCM [42]. These data showed that the fungal vesicular products might be important serological markers produced during this disease.

The immunomodulatory activity of fungal EVs is still poorly understood. *In vitro* studies have demonstrated that mammalian macrophages can incorporate fungal EVs, resulting in increased levels of both pro- and anti-inflammatory cytokines [150, 151]. Specifically, in *C. neoformans*, the exposure of macrophages to EVs resulted in their internalization and production of IL-10, TGF-β, and TNF-α, while for *C. albicans*, the production of IL-10, IL-12, and TGF-β was observed. In both studies, fungal EVs stimulated murine macrophages to produce higher levels of NO [150, 151]. This effect probably occurred due to the fungal EVs preparations, which were composed of heterogeneous populations of different size and probably content [148, 150]. *M. sympodialis* releases EVs carrying allergen, which induce high levels of TNF-α and IL-4, suggesting that vesicles have multiple immunoregulatory functions in atopic eczema. Despite this controversy in host immune response, fungal EVs were capable of stimulating a protective response against infection. Recently, Vargas et al. [151] showed that inoculation of *Galleria mellonella*, a larvae model, with EVs followed by challenge with *C. albicans* reduced the number of recovered viable yeasts in comparison

to infected larvae control. Moreover, these authors also observed immunomodulation of DC after internalization of EVs from *C. albicans*. The synthesis of IL-12, IL-10, TGF-β, and TNF-α was also significantly increased in comparison to nonstimulated DC [151].

Proteomic-based approaches have been used to characterize *C. neoformans*, *P. brasiliensis*, *H. capsulatum*, and *C. albicans* and *S. cerevisiae* EVs [78, 150, 151, 153, 156]. Interestingly, most of the identified proteins in *P. brasiliensis* and *C. neoformans* lacked the characteristic signal peptide required for conventional secretion [78, 156], suggesting that fungal vesicles can also be derived from unconventional secretory mechanisms, as observed in mammalian cells [173]. These proteomic analyses also revealed a large complexity of proteins with diverse biological functions in fungi EVs. Remarkably, we notice the presence of four proteins repeated in all EVs analyzed as follows: glyceraldehyde-3-phosphate dehydrogenase (GADPH), phosphoglycerate kinase, elongation factor 1-alpha, and 6-phosphogluconate dehydrogenase. Thus, it is possible to consider the potential of these molecules as biomarkers of fungal EVs.

6. Concluding Remarks

EVs are remarkable structures found in all biological fluids in mammals. The major reported functions of EVs are highlighted in Figure 1. In normal and tumor cells, they affect the following: antigen presentation, immune suppression, intercellular communication, inflammation, cellular homeostasis, and coagulation. In pathogens, they are considered virulence factors and are involved in the following: cell adhesion and invasion, evasion and modulation of the immune response, and drug resistance.

There are many molecules in EVs. The EVs from mammalian cells contain molecules such as MHC classes I and II, mRNA, miRNA, caspase 3, signaling factors, structural proteins, and cytokines. The EVs isolated from tumor cells express, for example, FasL, MHC classes I and II, mRNA, miRNA, FADD, P-glycoprotein, MMPs, PS, and TF. In protozoan, EVs are formed by key membrane components involved in host-parasite interaction. OMV or EVs from bacteria have antigenic material providing gene transference of resistance to antibiotics and adaptation factors. Fungal EVs are structures for transcell transport of virulence factors, immunomodulatory molecules, and serological markers. Therefore, EVs extend the cell-to-cell communication between host and pathogens. By preventing this communication, EVs can be used as targets for vaccination. In addition, the presence of EVs and the characterization of their composition can provide new diagnostic information on several diseases. Furthermore, studies on EVs in the different situations can be useful to understand the intimate mechanisms of pathogenesis. In conclusion, EVs represent a rich and challenging subject for basic and applied research enabling the understanding of a plethora of different mechanisms and opening new tools to combat diseases (Figure 2).

Abbreviations

ALIX:	ALG-2-interacting protein X
AP-1:	Activator protein 1
CD55:	Complement decay-accelerating factor
EGFR:	Epidermal growth factor receptor
FADD:	Fas-associated protein with death domain
FasL:	Fas ligand
HSPs:	Heat shock proteins
MHC:	Major histocompatibility complex
MyD88:	Myeloid differentiation primary response gene (88)
NF-kB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
RAB GTPases:	Ras-related in brain GTPases
TGF-β:	Transforming growth factor beta
TLR4:	Toll-like receptor 4
TNF-α:	Tumor necrosis factor
TrkB:	Tropomyosin related kinase B
TSG101:	Tumor susceptibility gene 101.

Conflict of Interests

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

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Review Article

Are the Two Human Papillomavirus Vaccines Really Similar? A Systematic Review of Available Evidence: Efficacy of the Two Vaccines against HPV

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Background. When the bivalent and the quadrivalent HPV vaccines were marketed they were presented as having comparable efficacy against cervical cancer. Differences between the vaccines are HPV types included and formulation of the adjuvant. *Method*. A systematic review was conducted to assess the efficacy of the two vaccines against cervical cancer. Outcomes considered were CIN2+, CIN3+, and AIS. *Results*. Nine reports (38,419 women) were included. At enrolment mean age of women was 20 years, 90% had negative cytology, and 80% were seronegative and/or DNA negative for HPV 16 or 18 (naïve women). In the TVC-naïve, VE against CIN2+ was 58% (95% CI: 35, 72); heterogeneity was detected, VE being 65% (95% CI: 54, 74) for the bivalent and 43% (95% CI: 23, 57) for the quadrivalent. VE against CIN3+ was 78% (95% CI: <0, 97); heterogeneity was substantial, VE being 93% (95% CI: 77, 98) for the bivalent and 43% (95% CI: 12, 63) for the quadrivalent. VE in the TVC was much lower. No sufficient data were available on AIS. *Conclusions*. In naïve girls bivalent vaccine shows higher efficacy, even if the number of events detected is low. In women already infected the benefit of the vaccination seems negligible.

1. Introduction

In the seventies, Dr. Harald Zur Hausen firstly postulated the link between human papillomaviruses (HPVs) and cervical cancer: studies to develop an anticancer vaccine followed. Approximately 70% of cervical cancers worldwide are associated with two high-risk HPV types (16/18) [1, 2] and almost 90% of genital warts are associated with two low-risk HPV types (6/11). Each year around 500,000 women develop invasive cervical cancer worldwide, with 83% of new cases and 85% of deaths occurring in developing countries [3, 4]. Risk factors associated with HPV infection are younger age at first coitus, higher number of sexual partners, smoking cigarettes, and history of herpes simplex virus infection [5, 6]. Some 75% of sexually active women develop a HPV infection [7], more frequently soon after their sexual debut: the majority of these infections (between 70% and 90%) spontaneously clear [8–10]. A minority progress from acute infection to cervical cancer, a process taking decades and going through precancerous lesions named cervical intraepithelial neoplasia (CIN) of increasing severity, from CIN1 to CIN3; spontaneous regression of the lesions is possible at any point [4, 8]. Incidence of genital warts is less precisely known, due to lack of data on the general population, but it is estimated to be around 1% [8].

Based on the results of five randomized controlled trials (RCTs) involving 40,000 women [11–17] two HPV vaccines entered the market. The quadrivalent vaccine against HPV6/11/16/18 was approved by the FDA and the EMA in 2006 [18, 19], whereas the bivalent vaccine against HPV16/18 was first approved in Europe in 2007 [20] and then in the USA in 2009 [21]. Soon after, several western countries such as the USA, Australia, and five European states [22] started national immunization campaigns targeting adolescent girls. The number of countries adopting the vaccines has since increased: in April 2014, 23 out of 29 European countries were reported to have implemented it [23], budgetary constraints being one relevant obstacle for the remaining countries. The primary target of HPV vaccination is adolescent girls aged 11 to 13 years, with some minor differences in national recommendations: in the USA routine vaccination is recommended at age 11 or 12 years with quadrivalent or bivalent vaccine for females and with quadrivalent vaccine for males in a 3-dose schedule during a 6-month interval [24]; in the UK HPV vaccine is recommended for girls under 15 years of age and consists in two injections spaced at least six and not more than 24 months apart. The vaccine is also recommended for men who have sex with men [25]. No prior assessment with Pap testing or screening for existing HPV infection is required.

Both vaccines contain human papillomavirus L1 self-assembling virus-like particles and are not infectious. Differences between the two vaccines are the number of HPV types included and formulation of the adjuvant (Table 1) possibly leading to different vaccine efficacy (VE) [36–38]. Head-to-head comparisons between the two vaccines are still exclusively based on immunogenicity [38, 39], although an immune correlate of protection has not yet been established [40].

Three meta-analyses, published when vaccines were marketed, showed comparably high efficacy of the two vaccines against precancerous lesions associated with HPV16/18 [30, 41, 42]. The meta-analyses subsequently published [43, 44] have confirmed the high efficacy of the vaccines against lesions associated with HPV16/18, but they did not provide information about VE against any cervical lesions irrespective of HPV type, nor did they discuss possible differences in terms of efficacy of the two vaccines. Thus, our systematic review that includes studies with longer follow-up aims to assess differences between the two vaccines from a public health perspective, considering all cervical lesions.

2. Methods

The systematic review was developed based on a prespecified protocol (protocol number FARM8N2ZFL) funded by the Italian Medicines Agency (AIFA) within a program of independent research on drugs [45]. The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guided the content and reporting of the review [46]. Published and unpublished RCTs comparing any of the two HPV vaccines versus placebo or any other control were considered for inclusion. We exclusively considered studies involving women, irrespective of age at enrolment.

As for protocol, primary outcome measures were cervical lesions (i.e., cervical cancer, CIN2, CIN3, and AIS) associated with any HPV type and cervical lesions exclusively associated with HPV16/18 occurring in three study populations: according to protocol population (ATP), the general population of vaccinated women (total vaccine cohort (TVC)) approximating all women regardless of status of HPV infection at vaccination, and a selected population of women seronegative for HPV16/18 and HPV DNA negative for 14 oncogenic HPV types, approximating the group of young adolescent girls targeted in the national immunization campaigns (TVC-naïve). In this paper only data related to cervical lesions associated with any HPV type occurring in the TVC and in the TVC-naïve population are reported, as the other data are not relevant from a public health perspective.

2.1. Literature Search. Trial identification: we searched the Cochrane Library (to Issue 3, 2014), MEDLINE (to March 2014), and EMBASE (to March 2014) using keywords and MeSH terms as reported in Annex 1 online, in combination with a highly sensitive filter for identifying RCT [47]. There were no language or time restrictions. Reference lists of relevant papers were also examined to identify additional studies. For unpublished RCTs, we searched the Internet for prepublication study presentations at conferences or meetings. Moreover experts in the field and vaccine manufacturers were contacted for further information (unpublished studies and single patient data). Clinical trial registers were searched for ongoing studies. Two review authors independently screened abstracts of potential studies and retrieved full articles for those deemed eligible.

2.2. Study Selection. Two reviewers carried out independent assessment of citations retrieved. When more than one publication reported the same trial, the one with a longer follow-up was selected. Reasons for exclusion were recorded. Quality of trials was assessed using the criteria outlined in the Cochrane Handbook [47] and included the assessment of (i) generation of the randomization sequence, (ii) quality of the allocation concealment, (iii) completeness of follow-up, and (iv) blinding of the outcome assessment. Based on quality assessment the risk of bias of the included trials was defined as low, high, or unclear. Differences in opinion were resolved through discussion involving a third author if needed.

2.3. Statistical Analysis. Two review authors extracted the data independently using a data extraction form. Statistical analyses were carried out using the STATA software version 11. For time to event data the hazard ratio (HR) was used as a measure of association. The results were summarized by using the inverse of variance method and the random effects model. Point estimates as well as their 95% confidence intervals (95% CI) were calculated and represented by the forest plot. Vaccine efficacy (VE) was calculated as $VE(\%) = (RU - RV)/RU \times 100$, where RU is the rate of disease in the unvaccinated and RV is the rate in the vaccinated [48]. The equation can be rewritten to use the HR in the following way: $VE(\%) = (1 - HRv/u) \times 100$, where HRv/u is the HR of the vaccinated

Table 1: Characteristics of bivalent and quadrivalent HPV vaccine.

	Quadrivalent vaccine	Bivalent vaccine
Commercial name	Gardasil/Silgard	Cervarix
Manufacturer	Sanofi Pasteur MSD SNC	GlaxoSmithKline Biologicals S.A.
HPV types	HPV 6 L1 protein 20 μg HPV 11 L1 protein 40 μg HPV 16 L1 protein 40 μg HPV 18 L1 protein 20 μg	HPV 16 L1 protein 20 μg HPV18 L1 protein 20 μg
Common characteristics	L1 protein in the form of noninfectious virus-like particles produced by recombinant DNA technology	
Differences in cellular culture	Yeast cells (*Saccharomyces cerevisiae* CANADE 3C-5 (strain 1895))	Hi-5 Rix4446 cells derived from *Trichoplusia ni* using a Baculovirus expression system
Differences in adjuvant	Amorphous aluminium hydroxyphosphate sulfate adjuvant, 225 μg	AS04 adjuvant system composed of aluminium hydroxide and 3-O-desacyl-4′-monophosphoryl lipid A, 50 μg
Therapeutic indications	Gardasil is a vaccine for use from the age of 9 years for the prevention of (i) premalignant genital lesions (cervical, vulvar, and vaginal) and cervical cancer causally related to certain oncogenic human papillomavirus (HPV) types; (ii) genital warts (condyloma acuminata) causally related to specific HPV types.	Cervarix is a vaccine for use from the age of 9 years for the prevention of (i) premalignant cervical lesions and cervical cancer causally related to certain oncogenic human papillomavirus (HPV) types.
Efficacy data leading to registration	95.2% (87.2, 98.7)[1]	90.4% (53.4, 99.3)
Efficacy data in the latest publication	43% (13, 63)[3]	93% (79, 99)[4]
Mean follow-up of phase III trials	3.6 years[1]	4 years[2]

[1]Vaccine efficacy against CIN/AIS associated with vaccine related HPV in the TVC-naïve from EMA registration data [19].
[2]Vaccine efficacy against CIN2+ associated with vaccine related HPV in the TVC-naïve from EMA registration data [20].
[3]Vaccine efficacy against any CIN3 in the TVC-naïve from Muñoz et al. 2010 [26].
[4]Vaccine efficacy against any CIN3+ in the TVC-naïve from Lehtinen et al. 2012 [27].

versus the unvaccinated. Heterogeneity among studies was assessed using the I^2 statistics [47].

2.4. Subgroup Analyses. The following prespecified subgroup analyses were planned:

(i) Geographical areas (Europe, Africa, Asia, North America, and South and Central America): the literature suggests that prevalence and circulation of HPV high risk types varies according to geographical areas [49–51].

(ii) Vaccine type (bivalent, quadrivalent): data on VE can be influenced by type of vaccine used as differences between the two formulations could be not negligible.

3. Results

3.1. Selection of Studies. Study identification and selection process is outlined in Figure 1. Of the 726 records initially identified, 3 were duplicates and 670 were excluded based on title and abstract assessment. The most common reasons for exclusion were the following: reports were not RCTs, did not assess the outcome of interest, were not related to oncogenic HPV or to vaccine administration, or were studies reporting exclusively laboratory or immunogenicity data. We assessed the full text of 53 articles and excluded 44 (reasons reported in Figure 1). Thus nine reports [26–29, 31–35], corresponding to five registered protocols [52–56], were included in the systematic review: in three trials (20,797 women) the bivalent vaccine was used (PATRICIA trial being the larger study) [27–29] and in two trials (17,622 women) the quadrivalent vaccine was assessed (FUTURE I and FUTURE II trials) [26]. Five publications reporting subset of data of the above-mentioned trials relating to specific geographical areas were also included [31–35].

The data of the phase III trials included in our meta-analysis have a mean follow-up of 4 years for the bivalent trial [27] and 3.6 years for the quadrivalent trial [26], longer than that of the systematic reviews with meta-analysis published up to now.

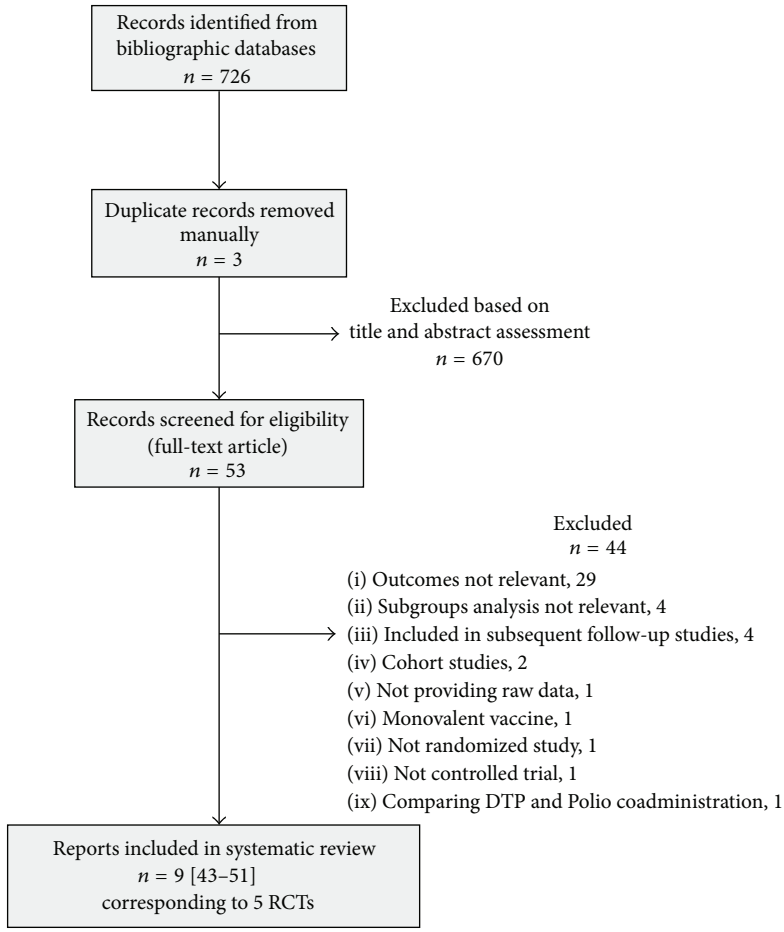


Figure 1: Study selection flowchart.

3.2. Quality of Included Trials and Characteristics of Trial Participants. Characteristics of the trials are summarized in Table 2.

Studies were all double blind RCTs, with an adequate sample size. They were all manufacturer-sponsored. Eligible participants were healthy, not pregnant women aged between 15 and 26 years, with 6 or less lifetime sexual partners and no history of abnormal Pap smear at enrolment. Almost 90% of women had normal cytology at study entry. Risk of bias was low in 4 out of 5 trials: generation of the randomization sequence, quality of allocation concealment, completeness of follow-up, and blinding of outcome assessment were adequate. Risk of bias was unclear (generation of the randomization sequence and allocation concealment were not described) in one smaller trial conducted in Japan [29]; as the Japanese trial provided only data on CIN2+ lesions exclusively associated with HPV16/18, its results are not reported here. Characteristics of the women enrolled in the trials used in this meta-analysis are reported in Table 3.

3.3. Pooling of the Data. Pooling of the data was possible for CIN2+ [26–28], CIN3+ [26, 27], and AIS [26, 28]. Data for each outcome are presented below for the TVC and the TVC-naïve cohorts.

CIN2+. The pooled HRs for CIN2+ lesions associated with any type of HPV in the TVC and in the TVC-naïve are reported in Figure 2. Corresponding values of VE were 26% (95% CI: 11, 39) in the TVC [26, 27] and 58% (95% CI: 35, 72) in the TVC-naïve [26–28]. Results suggested substantial heterogeneity among bivalent and quadrivalent vaccines: I^2 test was 68.7% and 66.4% in the TVC and in the TVC-naïve, respectively.

CIN3+. The pooled HRs for CIN3+ lesions associated with any type of HPV in the TVC and in the TVC-naïve are reported in Figure 3. Corresponding values of VE were 32% (95% CI: <0, 56) in the TVC [26, 27] and 78% (95% CI: <0, 97) in the TVC-naïve [26, 27]. Results suggested substantial heterogeneity among bivalent and quadrivalent vaccines: I^2 test was 86.3% and 90.7% in the TVC and in the TVC-naïve, respectively.

AIS. The pooled HR for lesions associated with any type of HPV was 0.31 (95% CI: 0.14–0.70) in the TVC [26, 27], corresponding values of VE being 69% (95% CI: 30, 86).

AIS cases in the TVC-naïve cohort were zero in the vaccine group and ten in the placebo; thus only an approximate

Table 2: Characteristics of the five trials selected.

Study [reference]	Protocol	Number of study sites	Countries included	Year of study enrolment	Funding source	Inclusion criteria		Exclusion criteria	Vaccine type*	Control	Women enrolled (TVC)	Women assessed (TVC)	Length of follow-up
						Age years	Sexual partners	Any of the following					
GSK [11, 12, 28]	NCT00120848 Phase II trial	27	Brazil, Canada, and USA	2001	GSK	15–25	≤6	Abnormal cervical cytology, HPV16/18 seropositivity, DNA positivity for 14 oncogenic HPV types, history of abnormal Pap test.	B	Aluminium containing placebo	560 V 553 P	505 V 497 P	5.9 years (average) 6.4 years (maximum)
PATRICIA [13, 27]	NCT00122681 Phase III trial	135	14 (Asia-Pacific, Europe, Latin America, and North America)	2004-2005	GSK	15–25	≤6	History of abnormal Pap test, pregnancy or breastfeeding, chronic disease, autoimmune disease, immunodeficiency.	B	HAV vaccine	9319 V 9325 P	8694 V 8708 P	4 years (average)
Konno et al. [29]	NCT00316693 Phase II trial	13	Japan	2006	GSK	20–25	Not specified	History of abnormal Pap test or genital warts, pregnancy, previous vaccination with HPV or HAV vaccine, MPL administration, hepatitis A infection.	B	HAV vaccine	519 V 521 P	501 V 501 P	13.6 months in Konno 2010
FUTURE I [16, 26, 30]	NCT00092521 Phase III trial	62	16 (Asia-Pacific, Europe, and America)	2001–2003	Merck	16–24	≤4	History of abnormal Pap test or genital warts, pregnancy, being not healthy.	Q	Aluminium containing placebo	2723 V 2732 P	7980 V 7236 P Follow-up FUTURE I + FUTURE II trial	3.6 years (average) 4.9 years (maximum)
FUTURE II [17, 26, 30]	NCT00092534 Phase III trial	90	13 (as above)	2002-2003	Merck	15–26	≤4	History of abnormal Pap test or genital warts, pregnancy, being not healthy.	Q	Aluminium containing placebo	6087 V 6080 P	As above	As above

*B: bivalent vaccine; Q: quadrivalent vaccine.

Table 3: Baseline characteristics of women enrolled in the four studies presented in the meta-analysis.

Study [reference]	Age mean (SD)		HPV 16 positivity at enrolment (%)				HPV 18 positivity at enrolment (%)				Lifetime number of sexual partners (median)			Smoking status (%)		*Chlamydia trachomatis* (%)		Hormonal contraceptive use (%)		Cytological abnormality at entry (%)	
			DNA		Serology		DNA		Serology												
	V	C	V	C	V	C	V	C	V	C		V	C	V	C	V	C	V	C	V	C
GSK [11, 12, 28]	20 ± 3	21 ± 3	NR	NR	NR	NR	NR	NR	NR	NR		NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
PATRICIA [13, 27]	20 ± 3	20 ± 3	14	14	2	1	11	10	1	1	*0*[*]	4%	4%	30	30	6	5	59	61	10	9
											1[*]	74%	74%								
											2[*]	14%	15%								
											≥*3*[*]	8%	8%								
FUTURE I [16, 26, 30]	20 ± 2	20 ± 2	9	8	12	12	3	3	3	3		2	2	26	26	4	5	58	57	11	12
FUTURE II [17, 26, 30]	20 ± 2	20 ± 2	9	9	11	11	4	4	4	4		2	2	NR	NR	4	4	59	60	12	11

V: vaccine group; C: control group; NR: not reported.

[*]Results are presented as percentage of women stratified by number of sexual partners.

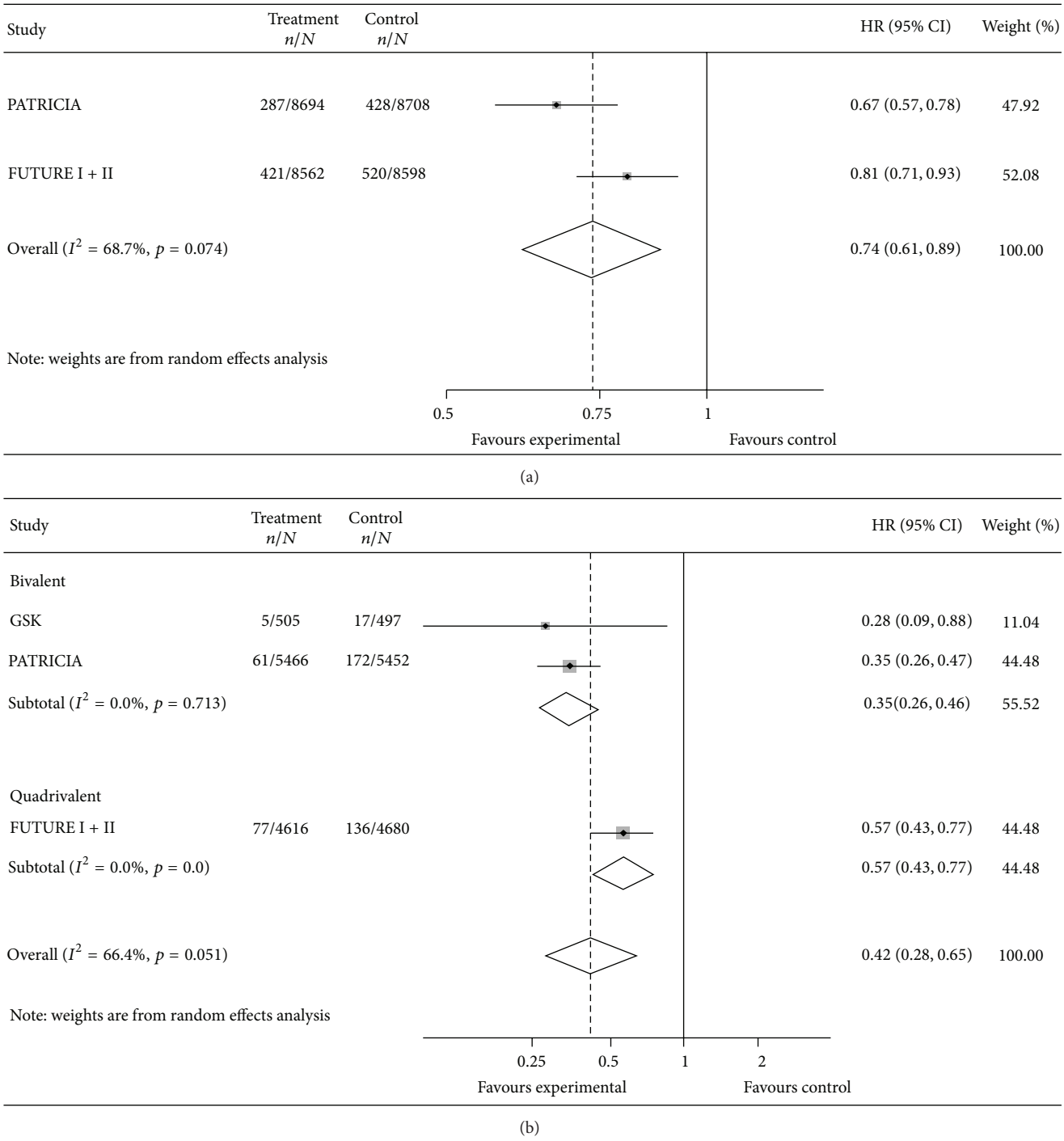
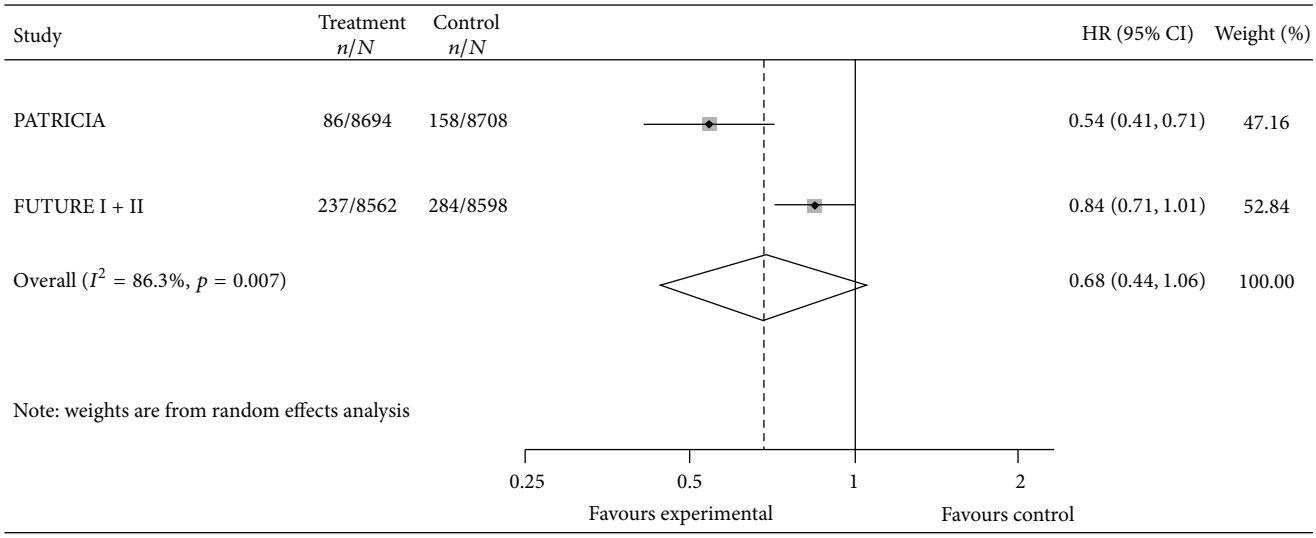


Figure 2: Vaccine efficacy against CIN2+ lesions, in total vaccine (a) and total vaccine naïve cohort (b), any HPV type.

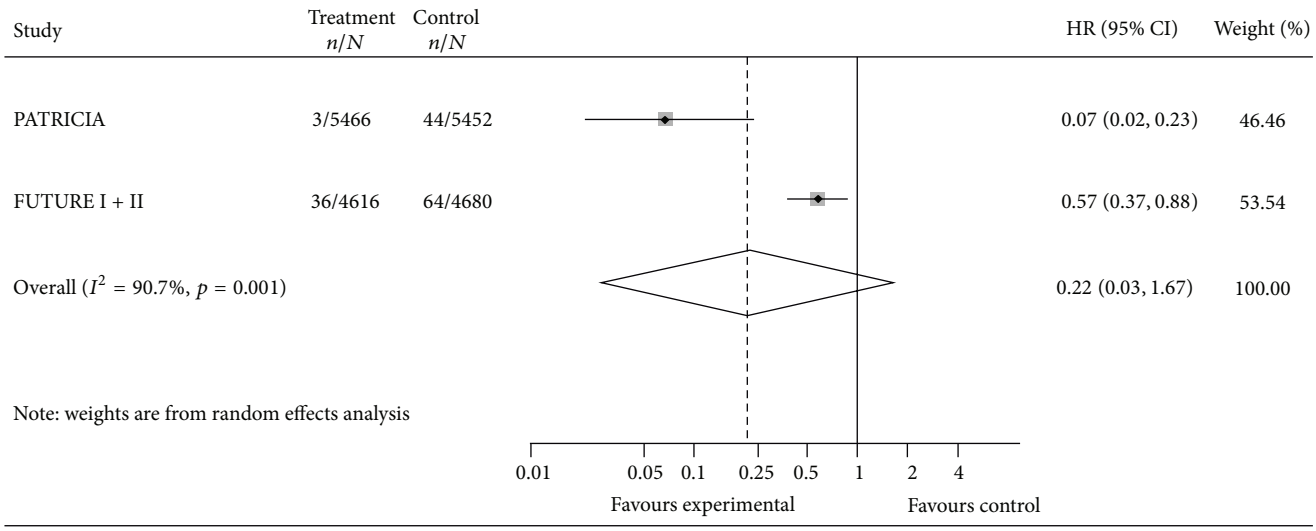
estimate of the efficacy was possible. The pooled HR for lesions associated with any type of HPV was 0.01 (95% CI: 0.01–0.22) resulting in a VE of 99% (95% CI: 78, 99) [26, 27]. For all the comparisons I^2 suggested low heterogeneity.

3.4. Analysis by Geographical Area. Although formally required by the scientific advisory unit of ECDC in Stockholm, the two manufacturers did not provide single patient data (the authors received only partial data unsuitable for the analysis from GSK and no answer at all from Sanofi Pasteur MSD).

Five published papers [31–35] reported data according to geographical areas. Pooling of the data was not appropriate since geographical areas definition differed (Table 4).



(a)



(b)

Figure 3: Vaccine efficacy against CIN3+ lesions, in total vaccine (a) and total vaccine naïve cohort (b), any HPV type.

4. Discussion

Since their introduction into the market, the effectiveness of the two vaccines against cervical cancer, based on first published data [11–17], has been subject of debate. Enthusiastic positions assumed that if vaccines are immunogenic and prevent infections associated with HPV16/18 they also prevent cervical cancer and therefore should be widely used [57–59]. Uncertainty was related to the following issues: correlation between immune response and clinical outcomes, need for a booster dose, replacement with other oncogenic strains, and possible reduction of Pap-test screening among the vaccinated [60–65].

Our systematic review highlights that, for precancerous lesions (CIN), the only available proxy of cervical cancer and heterogeneity among pooled studies is substantial (Figures 2 and 3): the bivalent vaccine shows higher efficacy against precancerous lesions. We focus our comments on the TVC-naïve cohort, as VE in the TVC is confirmed to be much lower and HPV vaccination is not universally offered to women already sexually active and data on AIS are too sparse to make sensible comments. For CIN2+ lesions estimates of efficacy of the two vaccines in the TVC-naïve cohort differ but the wide limits of the confidence intervals partially overlap (VE 65%; 95% CI: 54, 74 for the bivalent and VE 43%; 95% CI: 23, 57 for the quadrivalent), whereas for CIN3+ lesions estimates of efficacy largely differ and the limits of the confidence intervals do not overlap (VE 93%; 95% CI: 77, 98 for the bivalent and VE 43%; 95% CI: 12, 63 for the quadrivalent). However, due to limited number of patients with lesions detected, leading to wide confidence intervals of our estimates of effect, our conclusions should be interpreted with caution. The heterogeneity observed might be due to higher

Table 4: Vaccine efficacy against CIN2+ in different geographical regions (Latin America, Asia-Pacific, and Europe).

Study [reference]	Vaccine type*	Geographical region	Protocols included	Women enrolled	Outcomes assessed	Lesion n/N Vaccine	Lesion n/N Control	VE% (95% CI)
Perez et al. [31]	Q	Latin America	V501-007; V501-0013; V501-0015; V501-0016; V501-0018	6400	CIN2+ HPV 6/11/16/18 in			
					ATP cohort	1/2415	21/2377	95.3 (71.0, 99.9)
					TVC-naïve	3/2671	26/2681	88.5 (62.5, 97.8)
					TVC	45/2718	67/2725	33.1 (1.0, 55.2)
Tay et al. [32]	Q	Asia-Pacific	V501-0013; V501-0015; V501-0016	814	CIN2+ HPV 6/11/16/18 in *ATP cohort*	0/302	5/312	100.0 (−12.4, 100.0)
Majewski et al. [33]	Q	Europe	V501-007; V501-0013; V501-0015; V501-0016	9265	CIN2+ HPV 6/11/16/18 in *ATP cohort*	0/4043	38/4043	100.0 (89.9, 100.0)
					CIN2+ any type in *TVC-naïve*	23/2470	54/2527	56.6 (28.0, 74.6)
Barr et al. [34]	Q	North America	V501-005; V501-007; V501-0013; V501-0015; V501-0016	5996	CIN2+ HPV 16/18 in			
					TVC-naïve	0/2100	35/2116	100.0 (89.0, 100.0)
					TVC	19/2313	57/2356	66.4 (42.7, 81.1)
					CIN2+ any type in *TVC*	72/2313	108/2356	33.0 (8.9, 51.0)
de Carvalho et al. [35]	B	Brazil	NCT00689741 HPV001; NCT00120848 HPV007; NCT00518336 HPV023	431	CIN2+ HPV 16/18 in *TVC*	0/219	3/212	100.0 (−129.8, 100.0)
					CIN2+ any type in *TVC*	5/219	8/212	40.6 (−106.0, 84.7)

CIN: cervical intraepithelial neoplasia; ATP: according to protocol; TVC: total vaccine cohort.
*Vaccine type Q = quadrivalent; B = bivalent.

efficacy of the bivalent vaccine against cervical cancer possibly related to the specific adjuvant used (ASO4 adjuvant system), as suggested in two recent immune response head-to-head studies that consistently showed a higher neutralizing antibody production [38, 39] and a higher CD4+ T cell response [38] in bivalent than in quadrivalent vaccine recipients. Heterogeneity can also be due to baseline differences between the populations enrolled in the two trials, although such differences were not reported in the two trials (Table 3). However, misclassification of naïve women cannot be ruled out since the two manufacturers used different laboratory tests to measure immune response and to identify naïve girls. In fact cLIA test was used in the FUTURE trials whereas ELISA test that has a higher sensitivity than cLIA [66] was used in the PATRICIA trial. Moreover, data are often differently and poorly reported in the published trials [26–29, 31–35]; thus our ability to make meaningful comparisons and further analysis (e.g., assess the possible effect modification by smoking status or age) is hindered. We asked the manufacturers to provide individual patient data, but we did not receive a positive answer.

Another relevant point is that the length of the follow-up in the trials assessed seems insufficient to detect information relevant to the public and to policy-makers: as time interval from HPV infection to cervical cancer development is approximately 20 years, all information gathered in a much shorter period of time is not conclusive. Nevertheless the PATRICIA trial has a planned follow-up of 4 years [27] and longer follow-up data on bivalent vaccine are only available for 436 Brazilian women enrolled in a previous phase II trial [67]. We will have more information in 2020, when the results of the Finnish study that extended the follow-up for Finnish girls enrolled in the PATRICIA study will be published [68] and when the extension studies for the FUTURE II trial assessing the quadrivalent vaccine will also be available. In the meantime an open debate in this respect is urgently needed: national health agencies should set up a surveillance system to provide data on actual vaccines efficacy in the field and to allow international comparisons. At the moment, this comparison is not possible and we still do not know how to choose between the two vaccines [69]. Contrary to what is previously reported in other meta-analyses [30, 41–44], our systematic review suggests that the quadrivalent and the bivalent vaccines differ in terms of efficacy. This could be attributable to the different adjuvants contained in the two vaccines. Such difference in efficacy has not been widely recognized by national health agencies. For example, in Italy HPV vaccines are chosen and purchased through tendering schemes organized by regional health authorities that are based on the lowest price [70].

Apart from cervical cancer prevention, quadrivalent vaccine is known to effectively prevent genital warts [71, 72], whereas the bivalent vaccine can only marginally impact on these benign but distressing lesions [73]. The UK and a few Italian regions have recently substituted the bivalent with the quadrivalent vaccine, assuming comparable efficacy of the two vaccines against cervical cancer and giving an additional value to the activity against genital warts of the quadrivalent vaccine [10, 74]. Unfortunately, it is not possible to anticipate the consequences of the UK's and Italian choice: if different vaccine efficacy of the bivalent and the quadrivalent vaccine is going to be confirmed, lack of equivalence in terms of cervical cancer prevention could become an issue. The availability in the coming years of new broader spectrum HPV prophylactic vaccines could provide more insight into the current debate [75, 76].

5. Conclusions

In conclusion, we acknowledge that this systematic review has some limitations due to a low number of women with events and a high heterogeneity among trials that suggest caution in the interpretation of results. However, our conclusions are consistent with those from recent immunogenicity head-to-head studies [38, 39] and that provides strength to our interpretation. Our systematic review suggests that after nine years since HPV vaccines were introduced, their estimates of efficacy seem to diverge over time. The decision to consider the two vaccines similar in terms of cervical cancer prevention seems challenged by our longer term follow-up analyses. This might have implications for policy and pragmatic choices and deserves an open and comprehensive discussion. Moreover, international comparisons of the actual effectiveness of the two vaccines used in the field can add valuable information.

Finally, regulatory agencies should encourage the pharmaceutical companies to provide data across trials to assess all relevant outcomes in a comparable way, to reduce uncertainty, and to support health policy-makers that have to choose between alternative options [77, 78].

Conflict of Interests

The authors declare that no competing interests exist.

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Research Article

Development of Nonaggregating Poly-A Tailed Immunostimulatory A/D Type CpG Oligodeoxynucleotides Applicable for Clinical Use

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Immunostimulatory CpG ODNs have been developed and utilized as TLR9-dependent innate immune activators and vaccine adjuvants. Four different types of immunostimulatory CpG ODNs (A/D, B/K, C, and P type) have been reported. A/D type ODNs are characterized by high IFN-α production but intrinsically form aggregates, hindering its good manufacturing practice grade preparation. In this study, we developed several D35-derived ODNs (a commonly used A/D type ODN), which were modified with the addition of a phosphorothioate polynucleotide tail (such as dAs40), and examined their physical properties, solubility in saline, immunostimulatory activity on human PBMCs, and vaccine adjuvant potential in monkeys. We found that two modified ODNs including D35-dAs40 and D35core-dAs40 were immunostimulatory, similar to original D35 in human PBMCs, resulting in high IFN-α secretion in a dose-dependent manner. Physical property analysis by dynamic light scattering revealed that both D35-dAs40 and D35core-dAs40 did not form aggregates in saline, which is currently impossible for the original D35. Furthermore, D35-dAs40 and D35core-dAs40 worked as better vaccine adjuvant in monkeys. These results suggested that D35-dAs40 and D35core-dAs40 are two promising prototypes of nonaggregating A/D type ODN with advantages of ease of drug preparation for clinical applications as vaccine adjuvants or IFN-α inducing immunomodifiers.

1. Introduction

Immunostimulatory CpG oligodeoxynucleotides (ODNs) have been developed and utilized as Toll-like receptor (TLR) 9-dependent innate immune activators and vaccine adjuvants for more than 10 years [1]. Based on their backbone and sequence characteristics, immunostimulatory ODNs can be divided into four proposed different types (or classes) [2, 3]: A/D, B/K, C, and P-type ODNs. A/D types ODNs (mostly phosphodiester backbone with poly G tail at 3′ end) mainly stimulate interferon- (IFN-) α production from plasmacytoid dendritic cells (pDCs). B/K type ODNs (all phosphorothioate

backbone) activate B cells and interleukin- (IL-) 6 production, and C type ODNs can stimulate both IFN-α and IL-6 despite IFN-α induction being lower than that by A/D type ODNs. Recently another P-type ODN has been described [4]. P-type ODN (all phosphorothioate backbone) contains two palindromic sequences with a cytokine profile similar to C type ODNs but with higher IFN-α production [4].

Although many different immunostimulatory ODNs have been developed, the most characteristic difference among them is their IFN-α induction profile. In this sense, A/D (high IFN-α profile) type and B/K (low IFN-α, high IL-6 profile) type ODNs are considered as two distinct and typical prototypes of immunostimulatory ODNs [5]. A recent microarray study also supported overlapping but different gene signatures between A/D and B/K type ODNs [6]. A/D type ODNs were dominantly characterized by prolonged induction of type I IFNs while B/K type ODNs induced many genes that were significantly associated with resistance to bacterial infection such as IL-1β and IL-6 [6]. These *in vitro* profile differences might reflect *in vivo* observed differences when these ODNs are utilized as a vaccine adjuvant or a monoimmunotherapeutic drug. In the case of a malaria vaccine, K3 (a B/K type ODN) showed better adjuvanticity for antibody production than D35 (an A/D type ODN), when it was added to the SE36/AHG immunization in cynomolgus monkeys [7]. However, A/D type ODNs induced better protective immune responses with heat-killed *Leishmania*/AHG vaccine compared to B/K type ODNs in rhesus monkeys [8]. Similarly, as a monotherapeutic drug for Leishmaniasis, A/D type ODNs also showed a better potential in both healthy and SIV-infected rhesus monkey models than B/K type ODNs [9]. Interestingly, in this model, B/K type ODN administration promoted the pathology of cutaneous Leishmaniasis [9].

Strong IFN-α induction with A/D type ODNs has been closely linked to the higher order structures of this type of ODN. The poly-G tail of A/D type ODNs forms G-quadruplex DNA structures in a salt solution, resulting in nanoparticle/aggregate formations [10–13]. Similarly, IFN-α inducing P-type ODNs formed dimeric structures or aggregates [4]. It has been repeatedly reported that aggregation formation is necessary for high IFN-α production with A/D type ODNs [12, 13], and this greatly hampers the clinical application of A/D type ODNs because autonomous development of ODN multimerization leads to uncontrolled aggregation and precipitation, resulting in high product-by-product differences and administration difficulties. To overcome such problems, introduction of thermolytic protective groups in A/D type ODNs has been attempted [14, 15]. This modification prevented aggregate formation in saline before administration, but, after *in vivo* administration, temperature-dependent cleavage of the protective groups allows G-quadruplex formation [14, 15]. This thermolytic pro-D type ODN strategy is a promising method for clinically applicable A/D type ODNs, but its feasibility for clinical application needs to be evaluated. Currently, no human clinical trials have been reported.

In this study, we developed nonaggregating A/D type ODNs by modifying original D35 and examined their physical and biological characteristics. We found that a simple modification of D35, such as the addition of a phosphorothioate polydeoxynucleotide at the 3′ end, strongly prevented aggregate formation in saline but maintained its high IFN-α inducing property.

2. Materials and Methods

2.1. Human PBMC Preparation. PBMCs were prepared from Japanese healthy adult volunteers with informed consent. All experiments using human PBMCs were approved by the Institutional Review Board of the National Institute of Biomedical Innovation (Permit number: 44). After preparation of PBMCs using Ficoll-Paque PLUS (GE) and LeucoSep (Greiner), they were plated at a concentration of 2×10^7 cells/mL (96-well flat plates, in a total volume of 100 μL/well) in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 unit/mL penicillin, and 100 μg/mL streptomycin (all from Nacalai, Japan).

2.2. CpG ODNs Stimulation with or without N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium Methylsulfate (DOTAP). The ODNs listed in Table 1 were synthesized by GeneDesign (Osaka, Japan). PBMCs were stimulated with the indicated concentrations of K3, D35, D35-dAs40, and other ODNs (Table 1) for 24 h. Stimulation by ODNs with DOTAP (Roche) was performed according to the manufacturers' instructions. Briefly, ODNs solution in serum-free medium (Opti-MEM; Gibco) and DOTAP solution in Opti-MEM were separately prepared and maintained for 15 minutes at room temperature before ODNs and DOTAP solutions were thoroughly mixed by pipetting. The resultant ODNs/DOTAP mixture was maintained for 15 minutes at room temperature. ODNs/DOTAP mixtures (100 μL) were added to human PBMCs (2×10^6 cells/100 μL/well). 24 hours later, the supernatants were assayed for presence of cytokines.

2.3. Measurement of Cytokines by Enzyme-Linked Immunosorbent Assay. Cytokines in supernatants were measured using a pan-IFN-α enzyme-linked immunosorbent assay (ELISA) kit (Mabtech or PBL) and human IL-6 ELISA kit (DuoSet; R&D Systems) according to the manufacturers' instructions. ELISAs were developed with 3,3′,5,5′-tetramethylbenzidine (TMB; KPL). In some experiments, Milliplex assay (MPXH-CYTO60KPMX26; Millipore) was also performed according to the manufacturer's instruction.

2.4. Dynamic Light Scattering (DLS). DLS measurements were performed with a Wyatt DynaPro PlateReader II (Wyatt Technology, USA). Samples (1 mg/mL in PBS, stored for more than 18 hours) were measured (20 acquisitions at 25°C) in a 384-well plate (20 μL/well). The polydispersity, hydrodynamic radius, and molecular weight were analyzed by Dynamics software v7.1.7.16 (Wyatt Technology, USA).

2.5. Transmission Electron Microscopy (TEM). D35 (1 mg/mL in PBS) was dropped (10 μL) on a Formvar-carbon-coated grid and incubated for 2 hours for adhesion to the grid. For negative staining, samples were washed with distilled water

Table 1: Modified D35 ODNs developed in this study.

D35(CG)G-dAs40*	GsGTGCATCGATGCAGGGGsGsGs-As40		1
D35(GC)G-dAs40	GsGTGCATGCATGCAGGGGsGsGs-As40		2
D35(TG)G-dAs40	GsGTGCATTGATGCAGGGGsGsGs-As40		3
D35(CT)G-dAs40	GsGTGCATCTATGCAGGGGsGsGs-As40		4
D35(CG)A-dAs40	GsGTGCATCGATGCAAAAAsAsAs-As40		5
D35(GC)A-dAs40	GsGTGCATGCATGCAAAAAsAsAs-As40		6
D35(TG)A-dAs40	GsGTGCATTGATGCAAAAAsAsAs-As40		7
D35(CT)A-dAs40	GsGTGCATCTATGCAAAAAsAsAs-As40	Figure 1(a)	8
D35(CG)T-dAs40**	GsGTGCATCGATGCATTTTsTsTs-As40		9
D35(GC)T-dAs40	GsGTGCATGCATGCATTTTsTsTs-As40		10
D35(TG)T-dAs40	GsGTGCATTGATGCATTTTsTsTs-As40		11
D35(CT)T-dAs40	GsGTGCATCTATGCATTTTsTsTs-As40		12
D35(CG)C-dAs40	GsGTGCATCGATGCACCCCsCsCs-As40		13
D35(GC)C-dAs40	GsGTGCATGCATGCACCCCsCsCs-As40		14
D35(TG)C-dAs40	GsGTGCATTGATGCACCCCsCsCs-As40		15
D35(CT)C-dAs40	GsGTGCATCTATGCACCCCsCsCs-As40		16
D35T-dAs40**	GsGTGCATCGATGCATTTTsTsTs-As40		1
D35T-dA40	GsGTGCATCGATGCATTTTsTsTs-A40		2
D35T-dTs40	GsGTGCATCGATGCATTTTsTsTs-Ts40	Figure 1(b)	3
D35T-dT40	GsGTGCATCGATGCATTTTsTsTs-T40		4
D35T-dCs40	GsGTGCATCGATGCATTTTsTsTs-Cs40		5
D35T-dC40	GsGTGCATCGATGCATTTTsTsTs-C40		6
D35-dAs40*	GsGTGCATCGATGCAGGGGsGsGs-As40		
D35T-dAs40**	GsGTGCATCGATGCATTTTsTsTs-As40	Figures 3(a), 3(b), and 3(c)	
D35core	TGCATCGATGCA	Figures 4(a) and 4(b)	
D35core-dAs40	TGCATCGATGCA-As40		
D35coreT-dAs40	TGCATCGATGCATTTTsTsTs-As40		
D35core-dAs10	TGCATCGATGCA-As10		
D35core-dAs20	TGCATCGATGCA-As20	Figures 4(c) and 4(d)	
D35core-dAs40	TGCATCGATGCA-As40		

"s" indicates phosphorothioate backbone.
*D35(CG)G-dAs40 and D35-dAs40 have the same sequence.
**D35(CG)T-dAs40 and D35T-As40 have the same sequence.

three times, and then a drop of 2% (wt/vol) uranyl acetate (pH 4.0) was placed on the grid and left to air dry. The grids were examined at a magnification of ×10,000 by an electron microscope (Hitachi H-7650).

2.6. Complexation of CpG ODN and Schizophyllan (SPG). Alkaline denatured schizophyllan (SPG) (Mw. 150,000) solution (15 mg/mL in 0.25N NaOH) was added to the ODN solution (100 μM in NaH_2PO_4) and then mixed thoroughly. The mixture was kept at 4°C overnight to complete the complexation. The molar ratio (SPG : DNA) was fixed at 0.27. Complexation efficiency between ODN and SPG was estimated from the residual-free ODNs in the mixed solution by using a MultiNA microchip electrophoresis system (Shimadzu, Japan).

2.7. Cynomolgus Monkey Immunization. Cynomolgus monkeys (*Macaca fascicularis*) were obtained and maintained at Tsukuba Primate Research Center in the National Institute of Biomedical Innovation (NIBIO). All experiments were performed under the protocol approved by the Committee on the Ethics of Animal Experiments of NIBIO (Permit number: DS22-4R1), and all efforts were made to minimize suffering. Cynomolgus monkeys (weight: 2-3 kg) were subcutaneously (s.c.) administered influenza split vaccine (SV) (5 μg) (A/New Caledonia/20/99, BIKEN), with or without K3 (4.7 nmol = 30 μg), D35 (4.7 nmol = 30 μg), D35-dAs40 (4.7 nmol = 92 μg), D35core-dAs40 (4.7 nmol = 80 μg), or D35-SPG (4.7 nmol as D35-dAs40 amount) in a volume of 500 μL of saline at days 0 and 14. Sera were collected at 4 and 8 weeks after the first immunization. Anti-SV total IgG in the serum

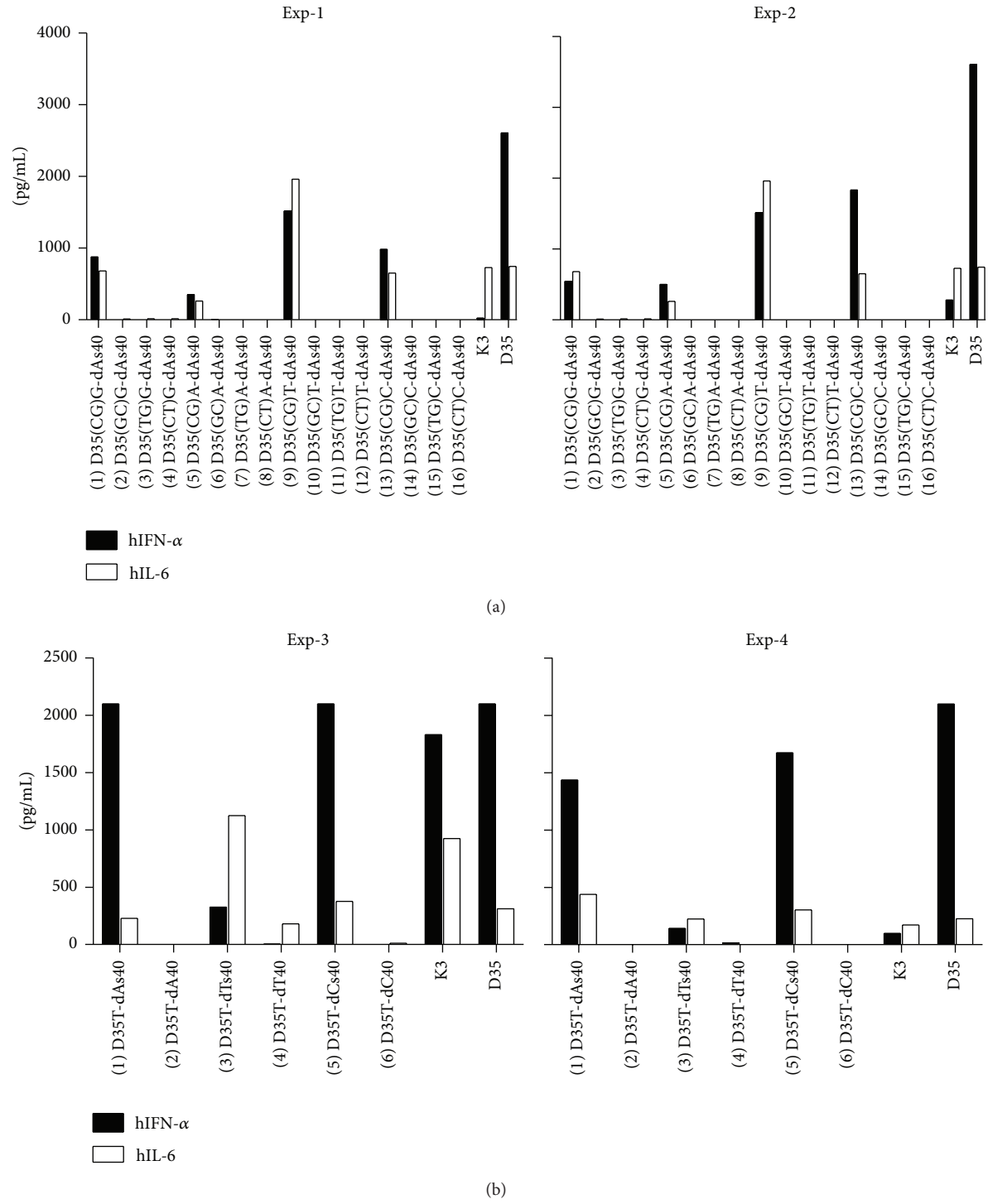


Figure 1: Screening of human PBMC with several different polynucleotide tailed A/D type ODNs. Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) (Table 1) for 24 hours. IFN-α and IL-6 production in supernatants was measured by ELISA. (a) The requirement of CpG-motifs and G-hexamers for cytokine secretion from human PBMCs. Both IFN-α and IL-6 were secreted in a CpG motif dependent but G-hexamer sequence independent manner. (b) Comparison of tail backbones (phosphorothioate or phosphodiester). Cytokine secretion was dependent on phosphorothioate 40-mer tails (1, 3, and 5). ODNs with phosphodiester 40-mer tails showed almost no biological activity (2, 4, 6). The bar graphs indicate the concentrations from a single well of each stimulation. The results are representative of three different experiments consisting of two different donors (Exp-1 and Exp-2, Exp-3 and Exp-4).

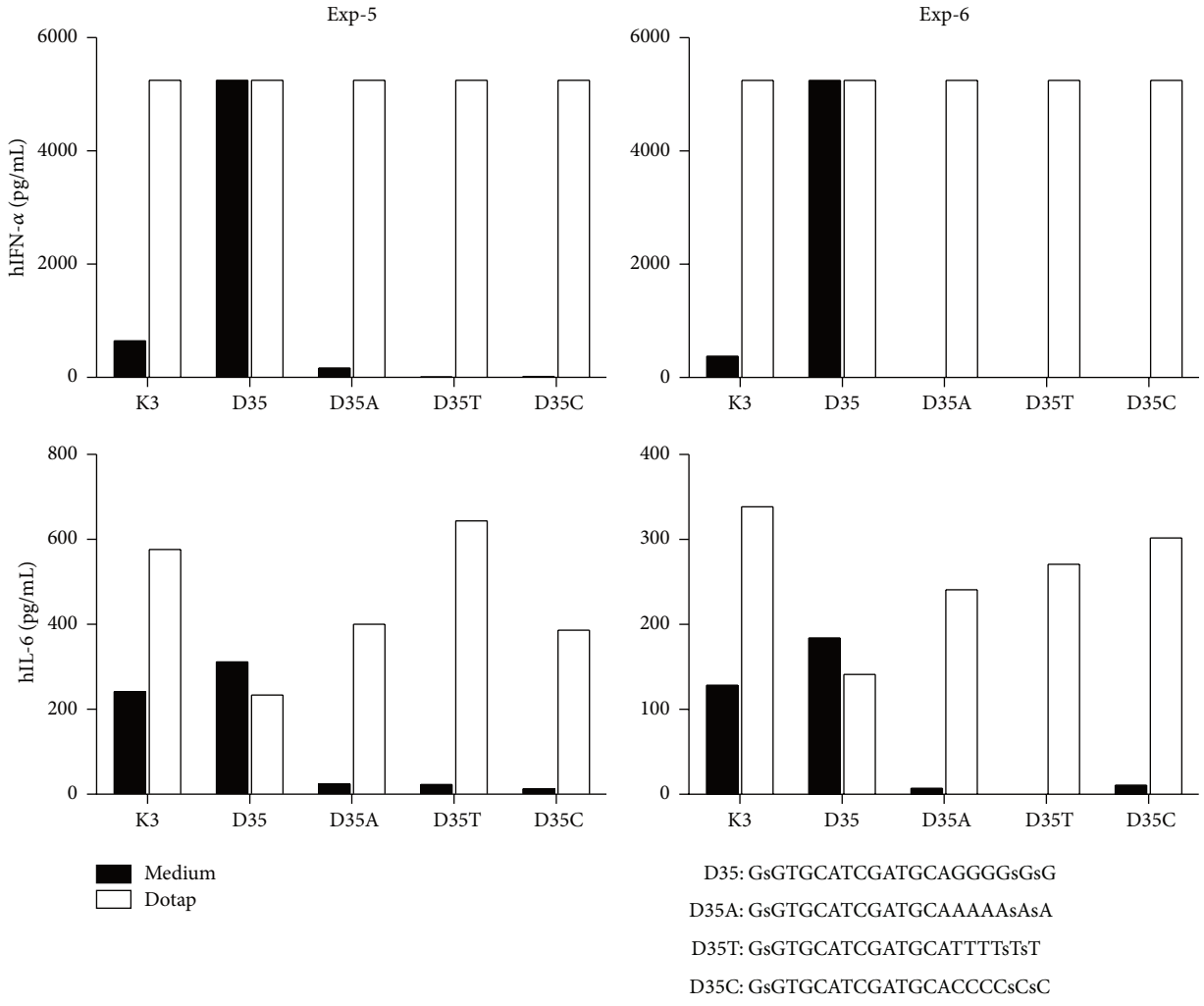


Figure 2: G-hexamer-less D35 ODNs required DOTAP for their immunostimulatory activities. Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) with or without DOTPA for 24 hours. IFN-α and IL-6 productions in the supernatants were examined by ELISA. DOTAP revived the immunostimulatory activities of D35A, D35T, and D35C; those are A/D type ODNs which do not contain G-hexamer. These ODNs did not show any immunostimulatory activities without DOTAP. The bar graphs indicate the concentrations from a single well of each stimulation. IFN-α production overed the ELISA measurement maximum (ca. 5000 pg/mL) with DOTAP in Exp5 and Exp6.

was measured by ELISA. Each serum sample was serially diluted and the antibody titer was calculated as a reciprocal number of the dilution at 0.2 of OD450.

2.8. Statistical Analysis. Statistically significant differences were calculated using Graphpad Prism 5 software. A paired *t*-test was used for cytokine analysis and a two-tail nonparametric Mann-Whitney *U* test was for antibody titer analysis.

3. Results

3.1. Poly-dAs40-Tailed A/D Type ODNs Have Similar Immunostimulatory Potential to Original D35. We previously developed K3-SPG, a second generation B/K type CpG adjuvant, which is a particulate soluble complex of K3 CpG-ODN and schizophyllan (SPG) [16, 17]. To form complexes between K3-ODN and SPG, K3 ODNs have to be modified by adding a phosphorothioate 40-mer of deoxyadenylic acids (dAs40 tail) at the 3′ ends where SPG and dAs40 tails form triple-helix complexes [16]. In parallel with these experiments, we have also synthesized similarly tailed D35 ODNs, and tested their immunostimulatory activities (IFN-α and IL-6 induction) on human PBMCs. Cytokine ELISAs revealed that D35 (A/D type CpG-ODN) with an additional dAs40 tail at the 3′ end was similarly active as the original D35 (Figure 1(a); sample 1 versus D35). Each ODN sequence used in this study is shown in Table 1. Subsequent experiments further revealed that some D35-derived ODNs with a dAs40 tail were also biologically active, even with replacement of the 3′ G-hexamer to A-, T-, and C-hexamer (Figure 1(a); samples 5, 9, and 13). This is relatively unexpected, because the G-hexamer

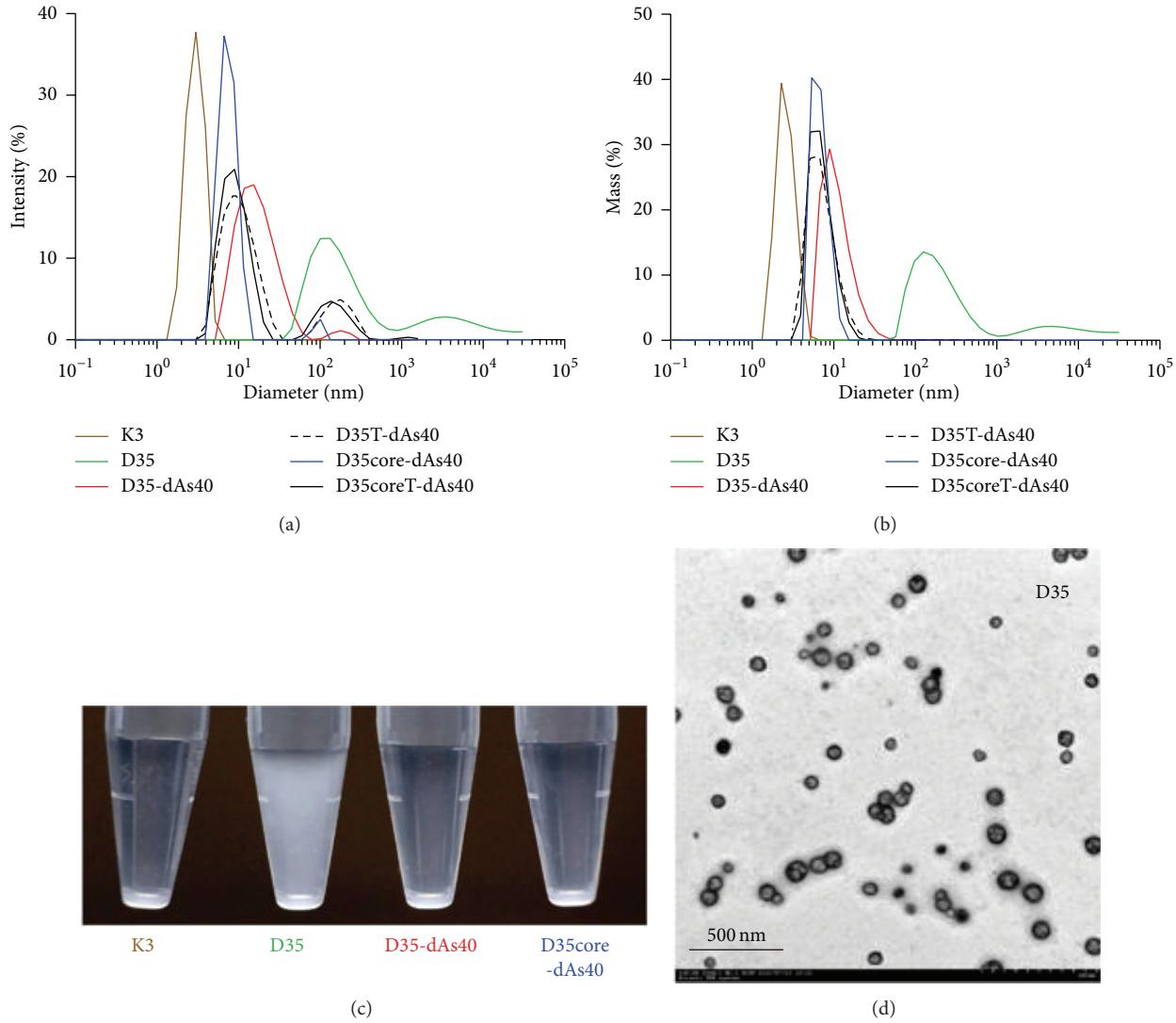


Figure 3: Physical properties of polynucleotide tailed A/D type ODNs in PBS solution. DLS analysis of the indicated ODN solutions (each 1.0 mg/mL in PBS) by % intensity plot (a) or % mass plot (b). See Table 2 for each measurement. (c) Turbidity status of the indicated ODN solution. The indicated ODNs were initially dissolved in distilled water at a concentration of 10 mg/mL (all ODNs were completely solubilized with water and the solutions were clear) and further diluted with PBS at a final concentration of 1.0 mg/mL. Solutions were stored at 4°C for at least 18 hours and then images were captured. D35 developed visible white turbidity during this incubation. In contrast, other ODNs were clear. (d) TEM image of aggregated D35 in PBS similarly prepared as in (c).

Table 2: DLS measurement of each ODN in Figure 3.

	Diameter (nm)	% Pd	Mw-R (kDa)	% intensity	% mass
K3	2.7	26.0	7	100.0	100.0
D35	213.2	74.2	187014	77.1	79.7
D35-dAs40	11.8	48.7	212	97.2	99.9
D35T-dAs40	7.6	43.3	76	80.6	99.6
D35core-dAs40	6.9	23.5	61	96.6	100.0
D35coreT-dAs40	7.3	35.4	70	79.4	99.6

The value of the main peak in each ODN measurement is shown. Pd: polydispersity.

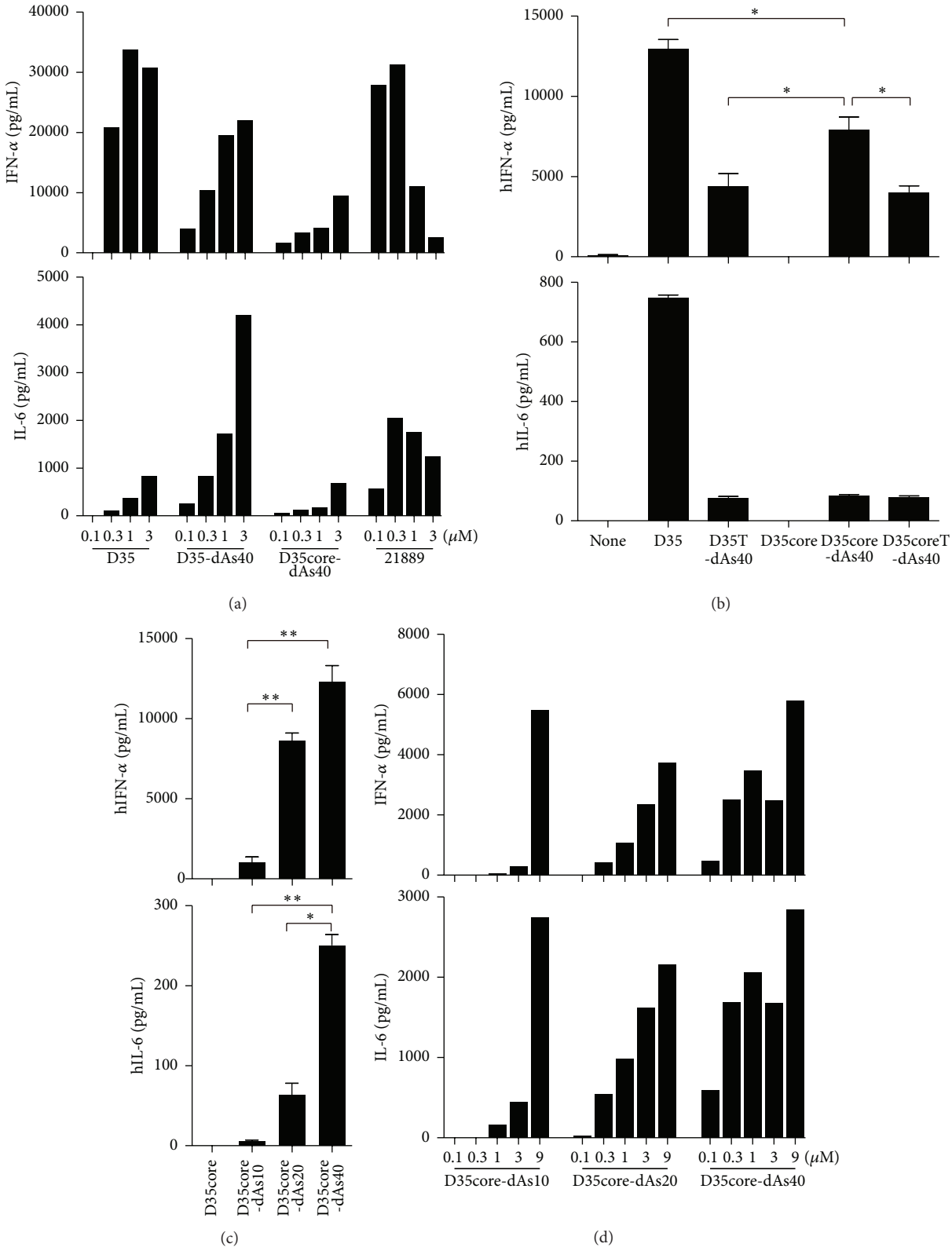


Figure 4: Biological activities of D35-dAs40 and D35core-dAs40. (a) Dose-dependent IFN-α production from human PBMC by the indicated ODNs. (b) Effect of the adjacent sequence of cytokine inducing activity. D35core plus dAs40 is sufficient to induce IFN-α secretion from human PBMC. Bar graph indicates mean ± SEM in triplicate. (c) dAs tail length affects the biological activity of D35core-dAs type ODNs. Human PBMC stimulated with the indicated ODNs (final concentration = 1 μM), and after 24 h cytokine concentration was determined by ELISA. Bar graph indicates mean ± SEM in triplicate. (d) The tail length and ODN dose relations of the indicated ODNs. The bar graph shows dose-dependent IFN-α and IL-6 production from a single well by each stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

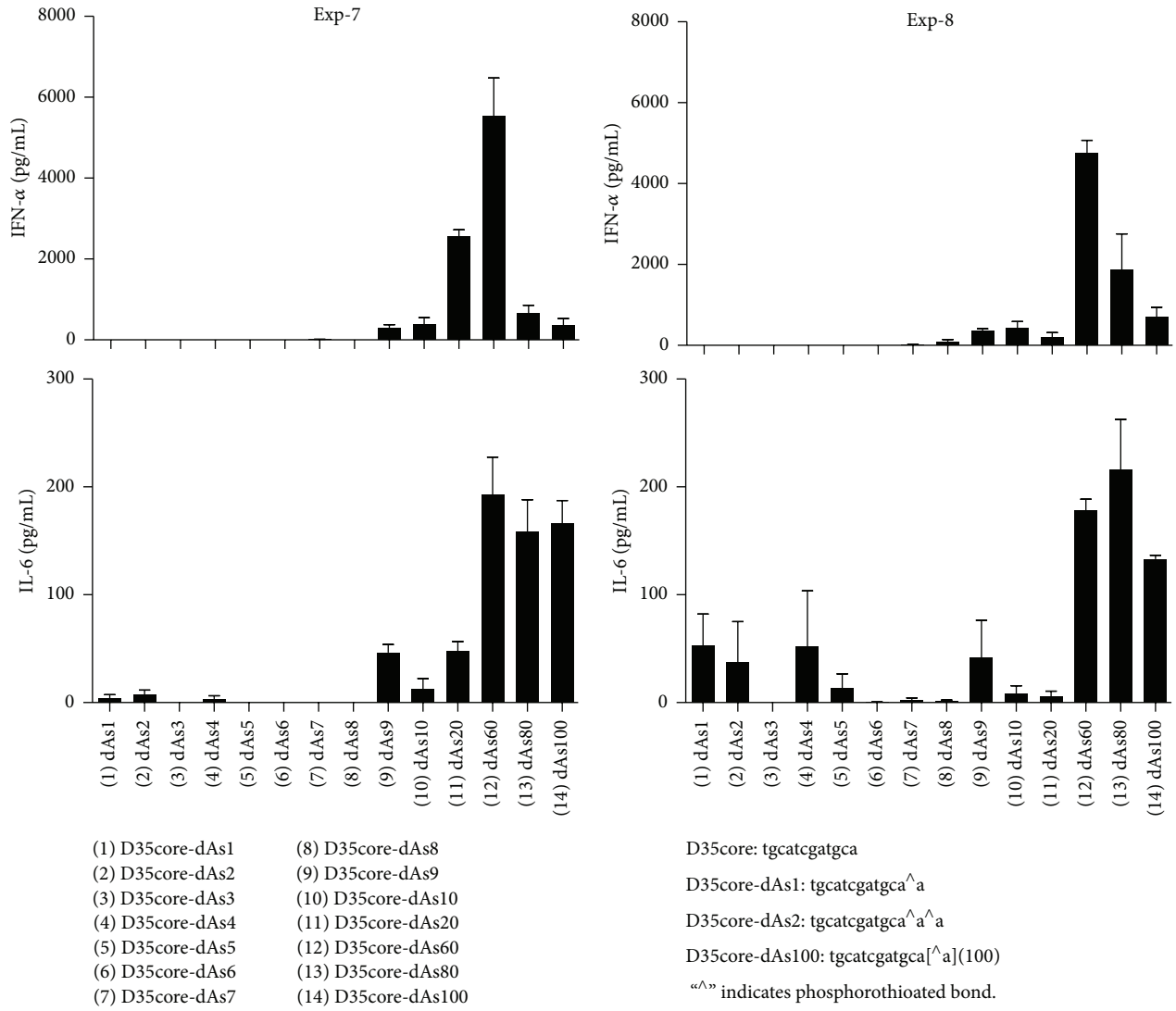


Figure 5: Length of the tail affects ODN's immunostimulatory activities. Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) for 24 hours. IFN-α and IL-6 production in the supernatants were examined by ELISA.

formed quadruplex structures via Hoogsteen base pairing, resulting in self-aggregates [10, 11], and it is believed that G-hexamer-mediated aggregation is an obligatory requirement for the biological activity of A/D type CpG ODNs [5, 12–14]. On the other hand, the immunostimulatory activities of D35-derived ODNs on human PBMCs are totally dependent on the presence of the CpG sequence. Replacement of CpG motif to GC, TG, and CT completely suppressed the ODNs' immunostimulatory activities (Figure 1(a); sample 9 versus samples 10–12), consistent with the unmethylated CpG-motif theory [18]. These results indicated that the presence of the G-hexamer sequence is not a necessary requirement for A/D type ODN immunostimulatory activities, whereas the activities of D35-derived ODNs was dominantly dependent on the presence of the CpG motif sequence.

3.2. Phosphorothioate Polynucleotide Tail Is Required for D35-Derived ODN Immunostimulatory Activity. The addition of a G-hexamer in phosphodiester CpG ODNs has been shown to increase ODNs' stimulatory activities by improving their cellular uptake [19, 20]. It has also been known that phosphorothioate ODNs are bound to proteins nonspecifically, and phosphorothioate CpG ODNs are more efficiently taken up by cells than phosphodiester CpG ODNs [21]. These reports suggested that the addition of dAs40 tails to D35-derived ODNs may improve their cellular uptake by non-specific binding feature of the dAs40 tail's phosphorothioate backbone. Based on this hypothesis, we examined the requirement of the chemical backbone structure, by comparing the immunostimulatory activities of D35T-dAs40 and D35T-dA40 (original G-hexamer replaced with T-hexamer, and the tails were composed of either phosphorothioate or phosphodiester poly-A40) on human PBMCs (Figure 1(b); sample 1 versus sample 2). The biological activities of the ODNs were strongly dependent on the presence of the phosphorothioate tail, and almost no cytokine responses

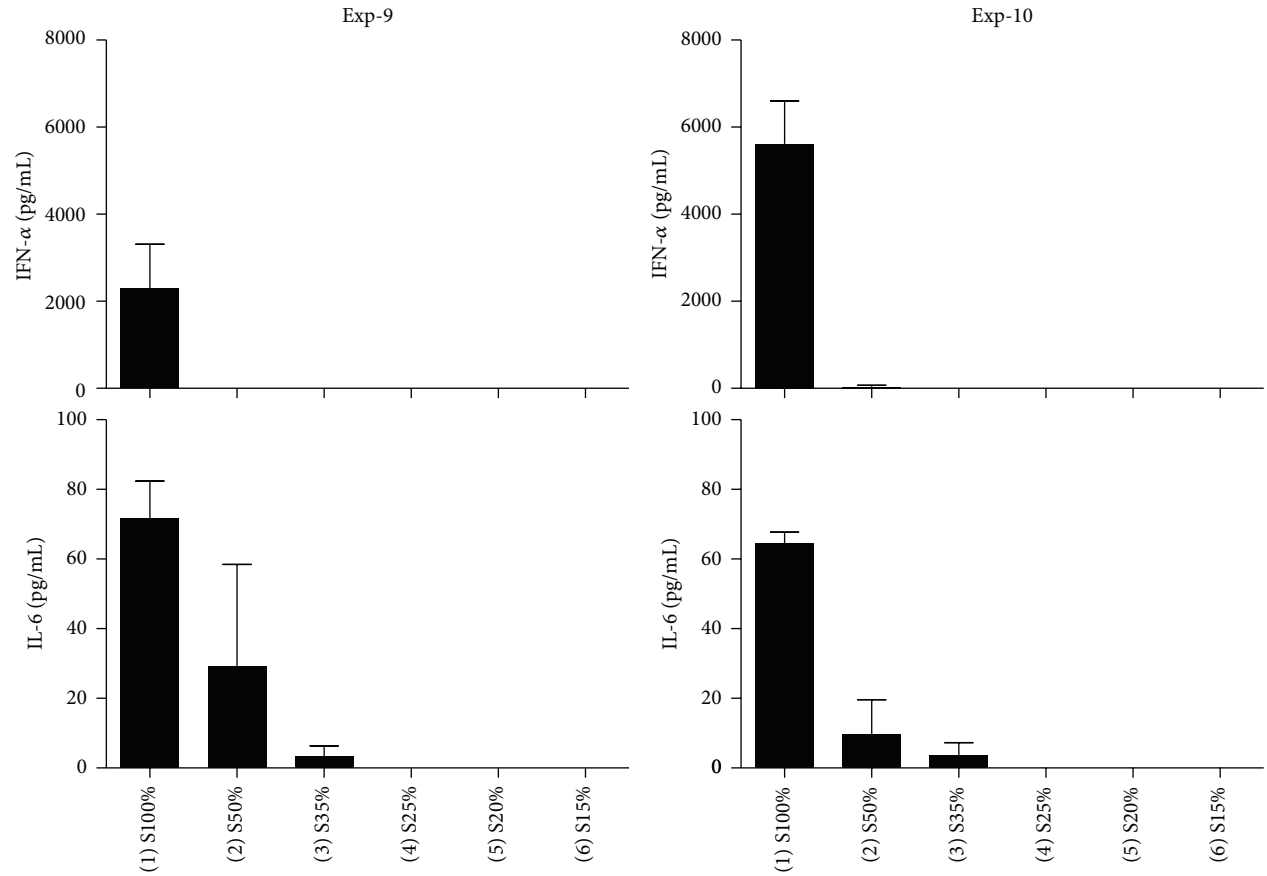
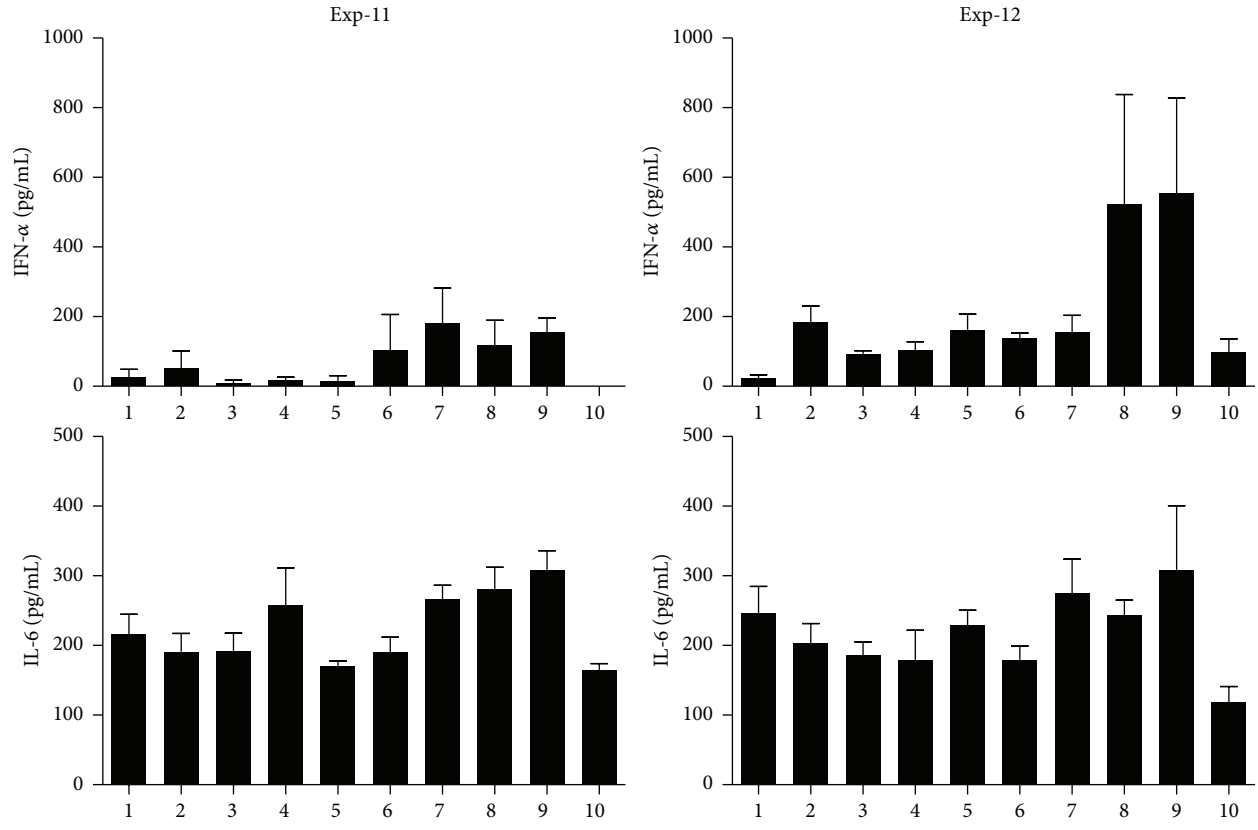


Figure 6: Phosphorothioation amount of the tail affects ODN's immunostimulatory activities. Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) for 24 hours. IFN-α and IL-6 production in the supernatants were examined by ELISA.

were observed by addition of phosphodiester tailed ODNs (Figure 1(b); sample 1 versus sample 2). We also tested other polynucleotide 40-mer tails such as poly-T(s)40 or poly-C(s)40 instead of poly-A(s)40 and found that the cytokine responses were generally independent of the bases of the polynucleotide tails but were dependent on the presence of the phosphorothioate backbone (Figure 1(b); samples 3–6), although relatively stronger cytokine responses were seen for addition of dAs40 and dCs40 compared to dTs40 tails (Figure 1(b); samples 1, 3, and 5).

3.3. DOTAP Compensates for the Absence of a G-Hexamer Sequence. We also tested the immunostimulatory activities of A-, T-, and C-hexamers containing D35 without phosphorothioate polynucleotide tails on human PBMCs (Figure 2). Consistent with previous reports [5, 22, 23], the immunostimulatory activity of original D35 was dependent on the presence of a G-hexamer (Figure 2). A-, T-, and C-hexamer-containing D35 (D35A, D35T, and D35C) had no effect on human PBMCs (Figure 2; black bar). In contrast, the same ODNs became comparatively active when they were added with DOTAP (Figure 2; white bar), a cationic lipid (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) that has been used for targeting CpG-ODNs to certain endosome compartments where TLR9-mediated signaling starts [24, 25]. This result indicated that the biological activity of A/D type ODNs does not require a G-hexamer sequence if ODNs are targeted to cellular uptake and appropriate intracellular compartments by DOTAP.

Taken together, these results suggested that the immunostimulatory activities of A/D type CpG-ODNs could be regulated by two separate processes: first, efficient cellular uptake through either G-hexamer aggregates or nonspecific binding via phosphorothioate polynucleotide tails and, second, the presence of a CpG motif that induces TLR9-dependent signaling. The fact that DOTAP can convert nonactive G-hexamer-less D35 such as D35A, D35T, and D35C into an immunostimulatory active compound suggests that the



(1) D35-dNs40(A30%, T30%, C30%, G10%)
(2) D35-dNs40(A27%, T27%, C27%, G19%)
(3) D35-dNs40(A24%, T24%, C24%, G28%)
(4) D35-dNs40(A20%, T20%, C20%, G40%)
(5) D35-dNs40(A17%, T17%, C17%, G49%)
(6) D35-dNs40(A14%, T14%, C14%, G58%)
(7) D35-dNs40(A10%, T10%, C10%, G70%)
(8) D35-dNs40(A7%, T7%, C7%, G79%)
(9) D35-dNs40(A3%, T3%, C3%, G91%)
(10) D35-dNs40: GsGTGCATCGATGCAGGGGsGsGs-Ns40
"s" indicates phosphorothioated bond.
"N" indicates any nucleotides.

Figure 7: Nucleotide composition of the tail affects ODN's immunostimulatory activities. Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) for 24 hours. IFN-α and IL-6 production in the supernatants were examined by ELISA. Of note, D35-dNs40 (number 10) did not induce substantial IFN-α production.

presence of a G-hexamer sequence itself is not necessary for CpG motif/TLR9 molecular interactions.

3.4. dAs40-Tailed D35 ODNs Do Not Form Large Aggregates. We also examined several dAs40-tailed D35-related ODNs for their physical properties with DLS (Figure 3). Good manufacturing practice (GMP) grade synthesis of clinically applicable A/D type ODNs has been hampered by G-tail dependent multimerization that results in uncontrollable polymorphisms, aggregation, and precipitation of ODN products [14] (Figures 3 and 8). Original D35 (1 mg/mL) showed variable and heterogenous aggregate formations in PBS resulting in visible turbidity within 24 hours (Figure 3(c)). Of note, this turbidity was not observed in D35 in water. DLS analysis revealed that this turbidity consisted of broadly distributed aggregates (size range from around 50 nm to more than 1 μm in mean diameter; Figures 3(a) and 3(b)). In contrast, the same concentration of D35-dAs40 and D35core-dAs40 in PBS did not form visible aggregations (Figure 3(c)), and the size of the ODNs was less than 20 nm with a sharp peak in DLS (Figures 3(a) and 3(b) and Table 2). TEM analysis confirmed the DLS results and indicated that many globular particle sizes around 50–200 nm were distributed separately or formed stringed clusters of several particles (Figure 3(d)), consistent with a previous report [10, 11]. These pieces of data indicated that the addition of dAs40 tail greatly improved the physical uniformity of A/D type ODNs in PBS, even those containing a G-hexamer sequence such as D35-dAs40 showed virtually no aggregation with the dAs40 tail. We did not observe any meaningful structures in K3, D35-dAs40, D35T-dAs40, D35core-dAs40, and D35coreT-dAs40 by TEM.

3.5. dAs40-Tailed D35-Related ODNs Induce IFN-α in a Dose-Proportional Manner. We further evaluated the immunostimulatory activities of these ODNs by dose titration

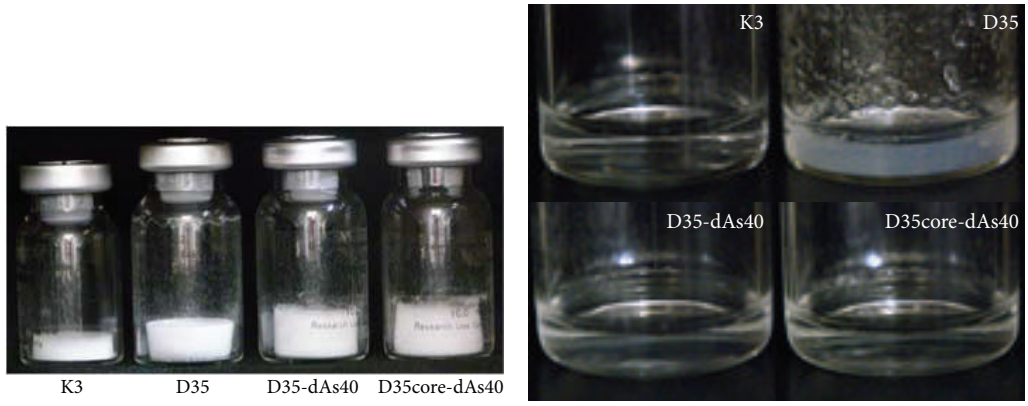


Figure 8: D35-dAs40 and D35core-dAs40 but not original D35 are directly solubilized in saline. (a) GMP grade lyophilized ODN vials (10 mg/vial) of K3, D35, D35-dAs40, and D35core-dAs40. White material is the lyophilized synthetic ODNs. (b) Saline (1 mL) was directly added to each vial. K3, D35-dAs40, and D35core-dAs40 were completely solubilized in saline at 5 min. D35 was not completely dissolved in saline with many visible gelatinous aggregations, and this insolubilized status was unchanged for at least 1 month.

(Figure 4(a)). All ODNs including D35, D35-dAs40 (containing G-hexamer sequence), and D35core-dAs40 (without G-hexamer sequence) induced increased IFN-α and IL-6 responses from human PBMC in a dose-dependent manner (Figure 4(a)). In contrast, a recently reported P-type ODN, 21889, consisting of a phosphorothioate backbone containing two tandem palindromic sequences that promote the formation of dimeric structure or aggregates [4] showed decreased IFN-α and IL-6 responses when higher concentrations were used (Figure 4(a)).

3.6. D35core Plus dAs40-Tail Is Sufficient for IFN-α Production. We also examined the effect of the adjacent sequence from the D35core 12-mer, such as the 5′GG-sequence and 3′T-hexamer on IFN-α production (Figure 4(b)). The core sequence of D35 (12-mer) alone did not induce cytokine responses (Figure 4(b)) whereas the addition of a dAs40 tail to the 12-mer was sufficient to induce comparable amounts of IFN-α. Importantly this result was obtained in the absence of DOTAP. The presence of a T-hexamer seemed to decrease the biological activity. These results suggested that the core adjacent sequences such as the 5′GG-sequence and 3′T-hexamer were not necessary for this type of ODN immunostimulatory activities (Figure 4(b)).

3.7. Longer Phosphorothioated-A-Tailed ODNs Have Increased Immunostimulatory Activity. We also examined the effect of the length of the phosphorothioate A-polymer tail on immunostimulatory activity. When human PBMCs were stimulated with the same amount of the indicated ODNs (1 μM), IFN-α and IL-6 production was positively correlated with the length of the dAs-tail (Figure 4(c)), suggesting that longer-tailed ODN could be more efficiently taken up. When the effect of each ODN concentration was examined, the increased concentration of each ODN (maximum tested 9 μM) ultimately reached similar levels of IFN-α and IL-6 production (Figure 4(d)). Taken together, these data suggested that addition of phosphorothioate polynucleotide tails into a CpG motif containing short ODNs is a useful strategy to produce GMP applicable A/D type ODNs with controlled physical properties.

3.8. More Detailed Requirements and Characterizations of the Phosphorothioate Polynucleotide Tails. We further performed a couple of more detailed experiments to understand the requirement of the phosphorothioate polynucleotide tailing for D35 CpG ODNs, such as the length (Figure 5), the amount of phosphorothioation (Figure 6), and the nucleotides compositions (Figure 7) of the tail. First, D35core with different number of dAs was tested for the induction of IFN-α and IL-6 (Figure 5). No IFN-α induction was observed in D35core with less than dAs6; then the amount of IFN-α was increased by the dA length reaching dAs60. Interestingly, further prolongation of dAs-tail up to 100 did not improve but rather decreased the induction of IFN-α. In contrast, IL-6 production was increased and sustained up to dAs100. Second, by using D35core-dAs40, we changed the amount of phosphorothioation from 100% to 17.5% as indicated in Figure 6. Even with 50% reduction of phosphorothioation in dA40 tail abrogated the IFN-α inducing activities. IL-6 production was less sensitive but also rapidly decreased as the reduction of phosphorothioation amount. We did not observe any IL-6 production with less than 25% phosphorothioation. Third, the requirement of nucleotides compositions was examined by using D35-dNs40. Totally random dNs40 tailed ODNs did not induce substantial IFN-α production (Figure 7; sample 10). We also controlled guanosine amount in the dNs40-tail from 10% to 90%, expecting the increase of the Hoogsteen base pairing formation in the dNs40 tail. As the guanosine amount increased, the IFN-α production increased but D35-dNs40 (91% G) (Figure 7; sample 9) induced relatively small amount of IFN-α. All

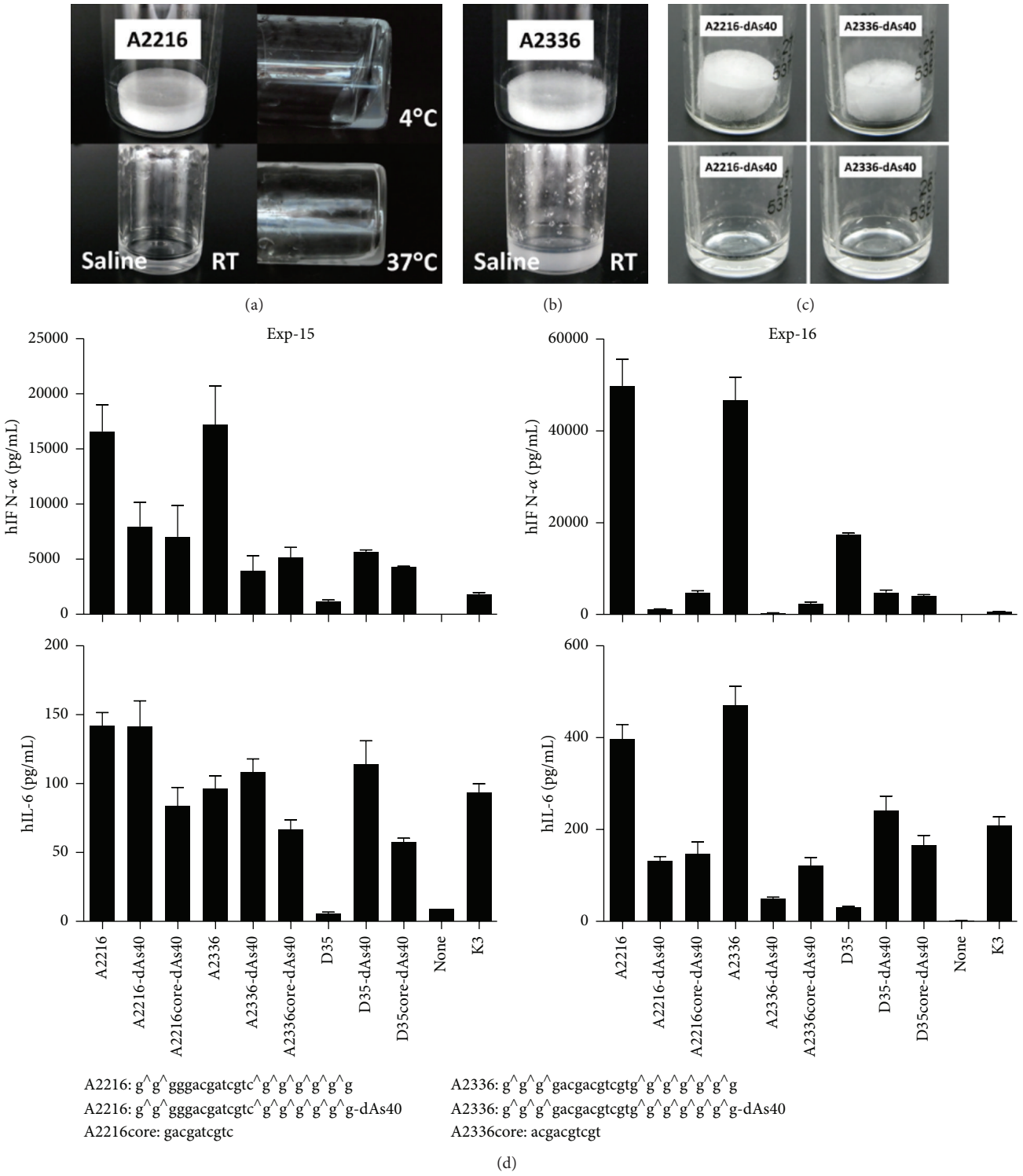


FIGURE 9: 3′ addition of dAs40 sequence to A2216 and A2336 improves direct solubilization in saline. (a) Lyophilized A2216 vial (10 mg/vial) (upper left) was completely solubilized with 1 mL of saline directly at room temperature (RT) (lower left). The solution turned to a gel at 4°C (upper right) and reliquefied at 37°C (lower right). (b) Lyophilized A2336 vial (10 mg/vial) (upper) formed many visible gelatinous aggregation with 1 mL of saline at RT (lower). (c) Lyophilized A2216-dAs40 (0.5 mg/vial) and A2336-dAs40 (0.5 mg/vial) were easily and completely dissolved with 50 μL of saline at RT. (d) Human PBMCs were stimulated with the indicated synthetic ODNs (1 μM) for 24 hours. IFN-α and IL-6 production in the supernatants were examined by ELISA. Bar indicates the mean ± SEM.

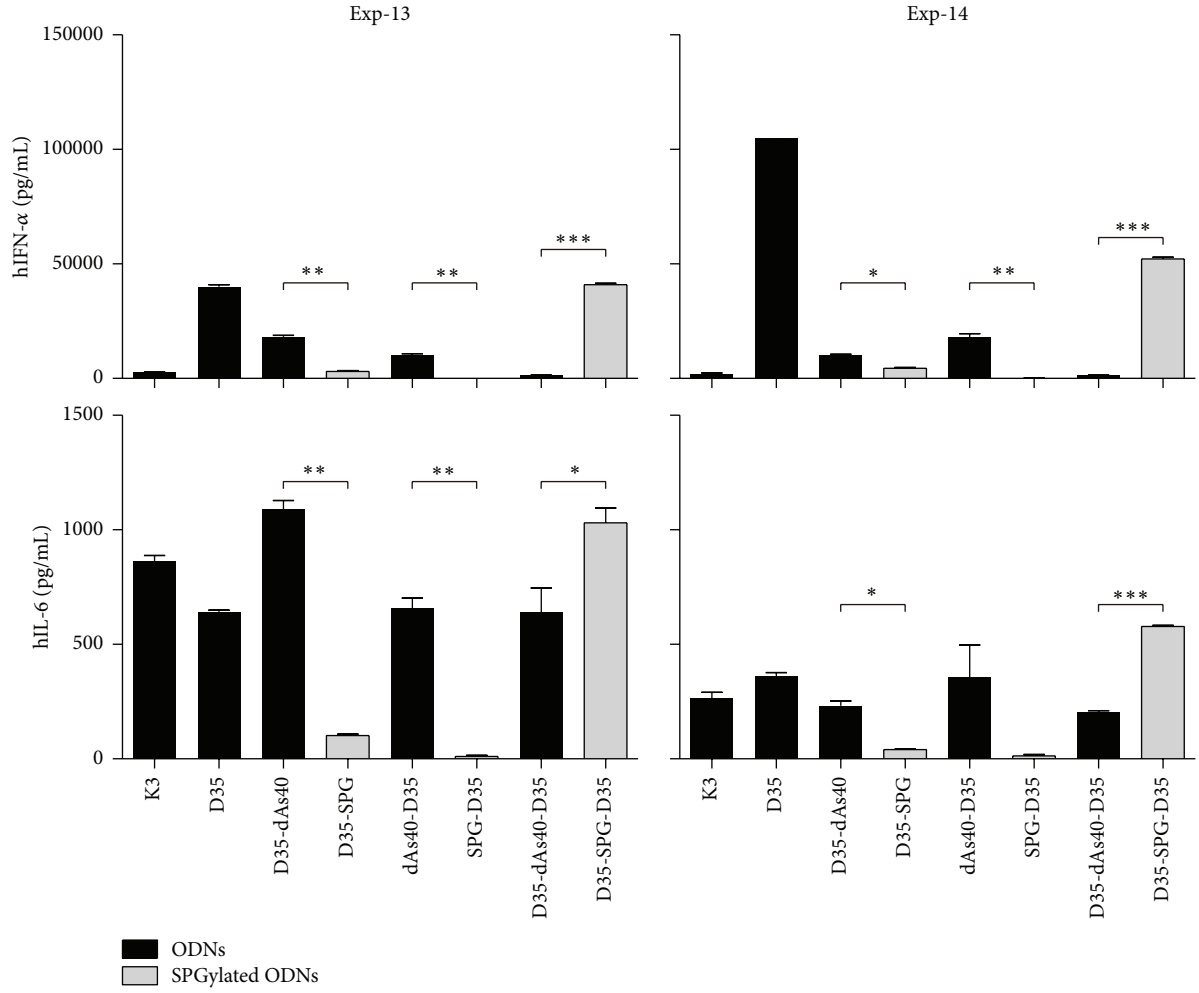


Figure 10: D35 modification with dAs40 at 5′ and/or 3′ ends and their SPGylation effect on cytokine production from human PBMC. Human PBMCs were stimulated with ODNs (D35-dAs40, dAs40-D35, and D35-dAs40-D35; 1 μM each) or their SPGylated ODNs (D35-SPG, SPG-D35, and D35-SPG-D35; 1 μM each ODN amount), and then 24 hours later, IFN-α and IL-6 secretion in supernatants were determined by ELISA. Bar graph shows mean ± SEM in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

together these results suggested that (1) total length, (2) phosphorothioation amount, and (3) the nucleotide composition (A-polymer is better than G-rich randomer) were all important factors affecting the immunostimulating activities of phosphorothioate polynucleotide tailed ODNs. Based on these observations, we selected D35-dAs40 and D35core-dAs40 as prototypes orienting for clinical application.

3.9. Lyophilized D35-dAs40 and D35core-dAs40 in Vials Can Be Directly Formulated with Saline. Direct solubility of immunostimulatory ODNs in a salt-containing solution such as saline is an important requirement to accelerate their clinical applications. Therefore, we tested the direct solubility of lyophilized D35-dAs40 and D35core-dAs40 in saline. Vials containing 10 mg of lyophilized ODN (Figure 8(a)) received 1 mL of directly injected sterile saline solution. K3 and D35core-dAs40 were easily dissolved in saline (Figure 8(b)). D35-dAs40 (containing a G-hexamer sequence) was slowly but completely dissolved within 5 min (Figure 8(b)). Even when the ODN solutions were stored at 4°C for 1 month, no visible precipitation or aggregation was observed. In contrast, original D35 was not readily dissolved in saline and formed heterogeneous gelatinous aggregations (Figure 8(b)). These results demonstrated that D35-dAs40 and D35core-dAs40 could be handled more easily than original D35, especially for clinical applications.

3.10. Addition of dAs40 Tail to Other A/D Type ODNs Improved Their Solubility in Saline. We also examined the direct solubility of other A/D type ODNs such as A2216 and A2336 in saline, with and without the addition of dAs40 tail. Unexpectedly A2216 was gradually but completely dissolved in saline at room temperature. However, the solution turned into a gel at 4°C (Figure 9(a)). This gelation was reliquefied at 37°C incubation (Figure 9(a)). A2336 was not dissolved in saline and formed gelatinous aggregations, similar to D35 (Figure 9(b)). Addition of dAs40 tail to A2216 and A2336 greatly improved their solubility in saline. Both

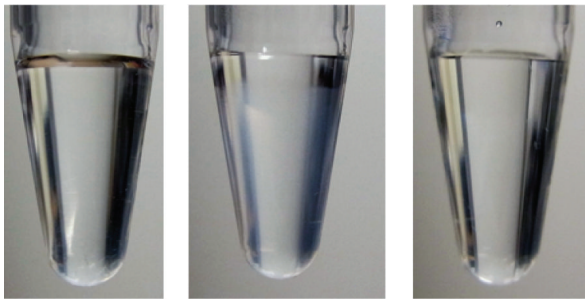


Figure 11: 5′ addition of dAs40 sequence to D35 also prevents visible large aggregate formation in PBS. The indicated ODNs were initially dissolved in distilled water at a concentration of 10 mg/mL (all ODNs were completely solubilized with water and the solutions were clear) and further diluted with PBS at a final concentration of 1.0 mg/mL. Solutions were stored at 4°C for at least 18 hours and then images were captured. D35 developed visible white turbidity during this incubation. In contrast, dAs40-D35 did not develop visible white turbidity.

A2216-dAs40 and A2336-dAs40 readily dissolved in saline (Figure 9(c)) and did not show gelation at the 4°C. A2216-dAs40 and A2336-dAs40 also kept the IFN-α inducing abilities (Figure 9(d)). These results suggested that the addition of dAs40 tail is also useful modification for improving the other A/D type ODNs' manageabilities.

3.11. SPGylation of D35 Does Not Improve IFN-α Secretion from PBMCs. We then attempted to improve the immunostimulatory profile of D35-derived ODNs by complexing them with SPG (SPGylation; see Materials and Methods), performed similarly with previously reported K3-SPG [16]. D35-SPG, SPG-D35, and D35-SPG-D35 were made by complexing SPG with D35-dAs40, dAs40-D35, and dAs40-D35-dAs40, respectively. Complexation efficiency was evaluated by a MultiNA microchip electrophoresis system and the result was as follows: D35-SPG (99.4%), SPG-D35 (96.7%), and D35-SPG-D35 (49.8%). This indicated that ODNs in either D35-SPG or SPG-D35 solution were almost completely complexed, whereas only 50% of ODN was complexed in the D35-SPG-D35 solution. Human PBMCs were stimulated with the SPGylated ODNs, and IFN-α and IL-6 secretion was determined by ELISA (Figure 10). In contrast to K3-SPG [16], 5′ or 3′ SPGylation of D35 did not improve cytokine production but reduced IFN-α and IL-6 secretion compared with the non-SPGylated ODNs (Figure 10). Among them, D35-SPG had a greater immunostimulatory effect than SPG-D35 (Figure 10). Of note, the non-SPGylated ODNs such as D35-dAs40 and dAs40-D35 with a 5′ end addition of dAs40 and 3′ end addition of dAs40 showed comparable immunostimulatory activities (Figure 10). However, we observed slightly better cytokine production with D35-dAs40 compared to with dAs40-D35 in other experiments. Interestingly, dAs40-D35 also did not develop visible large aggregates formation in PBS (Figure 11). D35-SPG-D35 substantially enhanced IFN-α and IL-6 secretion from PBMCs, although the complexation efficiency was only about 50%. Although D35-SPG-D35 showed a cytokine profile improvement similar to that for K3-SPG [16], we did not pursue D35-SPG-D35 development further in this study, because of the ODN size (80 base pairs) and difficulties in achieving full complexation with SPG. Further experiments are required to understand the mechanisms and biological characteristics of these SPGylated D type CpG ODNs.

3.12. D35-dAs40, D35core-dAs40, and D35-SPG Are Better Vaccine Adjuvants for Influenza Split Vaccine Than K3 and Original D35 in Cynomolgus Monkeys. We finally tested the *in vivo* adjuvant potency of D35-dAs40, D35core-dAs40, and D35-SPG in a monkey vaccine model and compared them with K3 and original D35 (Figure 12). Six groups of monkeys ($n = 2$ or 3) were immunized subcutaneously twice (at 2-week intervals) with the indicated SV plus adjuvants, and, 8 weeks after the first immunization, SV-specific IgG responses in sera were examined by ELISA (Figure 12(a)). D35-dAs40 and D35-SPG showed better and more consistent adjuvanticity than K3 and original D35 (Figure 12(b)). We also performed another set of monkey experiments to compare original D35 and D35core-dAs40 and found that D35core-dAs40 also showed better adjuvanticity than original D35 (Figure 12(c)). These results suggested that D35-dAs40, D35core-dAs40, and D35-SPG function as comparable or better adjuvants compared with K3 and original D35 *in vivo* in monkeys, at least for influenza SV vaccination. D35-SPG result in monkey also suggested that IFN-α and IL-6 profiles *in vitro* were not always correlated with the *in vivo* adjuvanticity. We performed more detailed cytokine profiling with a 26-cytokine multiplex using human PBMCs (instead of monkey PBMCs, owing to the limitation of obtaining sufficient amounts of monkey PBMCs for assay) stimulated with D35, D35-dAs40, or D35-SPG (Figure 13) and did not observe apparent correlation between cytokines and *in vivo* adjuvanticity among the 18 detected cytokines (Figure 13).

4. Discussion

In this study, we developed D35-dAs40 and D35core-dAs40, two novel prototypic nonaggregating immunostimulatory A/D type ODNs for clinical use in humans. These ODNs showed similar cytokine profiles to the original D35 with high IFN-α and low IL-6 induction profile from human PBMCs, although the overall balance between these cytokines was slightly shifted toward that of B/K type ODNs (slightly reduced IFN-α and increased IL-6 compared with original D35) (Figure 1). The most important feature of D35-dAs40 and D35core-dAs40 was their excellent solubility in saline. Lyophilized D35-dAs40 and D35core-dAs40 stored in vials can be directly solubilized by injecting saline solution (Figure 8), a requirement for clinical administration, and this feature greatly broadens their application. In addition, both D35-dAs40 and D35core-dAs40 showed better adjuvanticity than original D35 in cynomolgus monkeys when used as an influenza SV adjuvant (Figure 12). This result was not expected, because D35-dAs40 and D35core-dAs40 generally

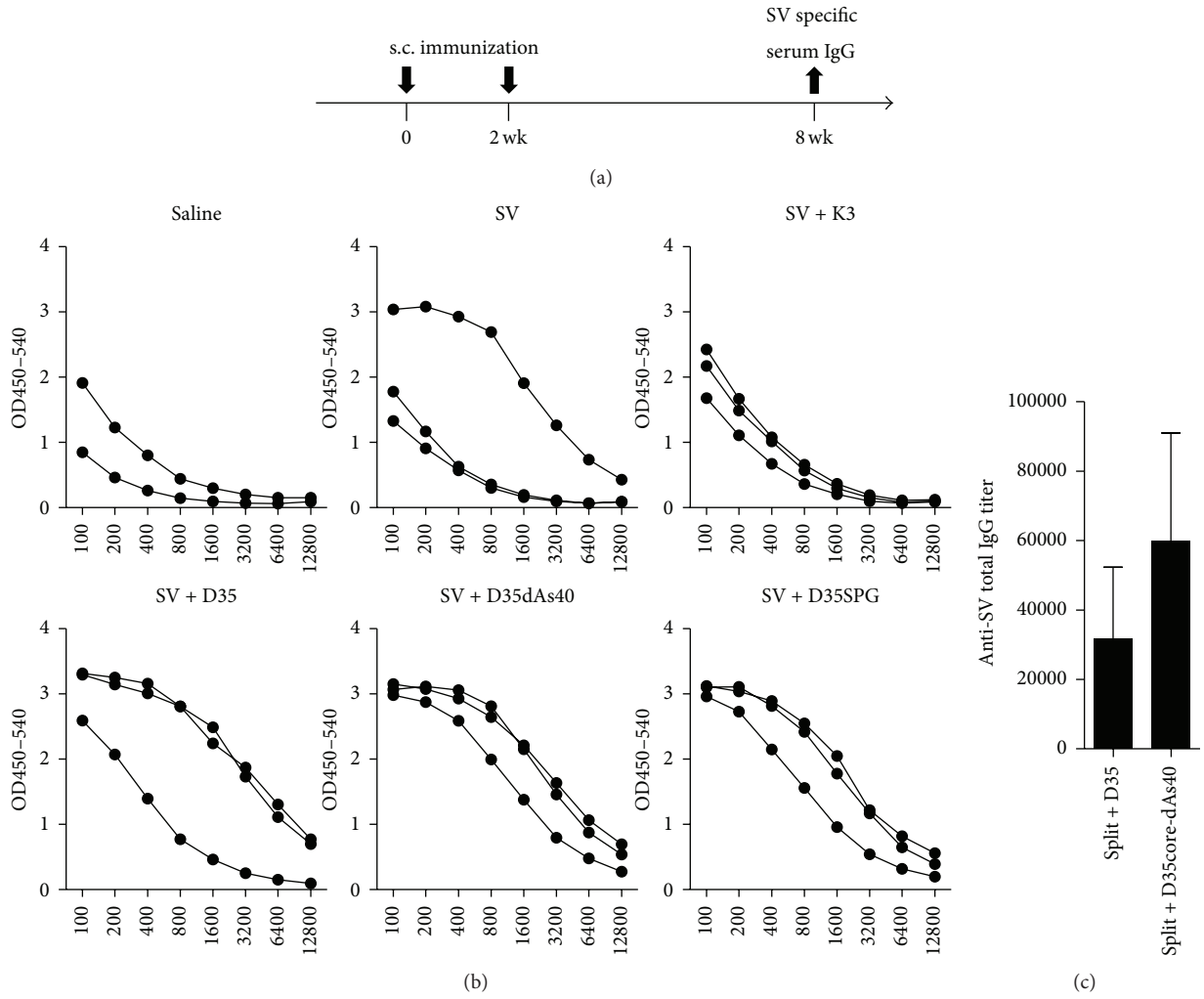
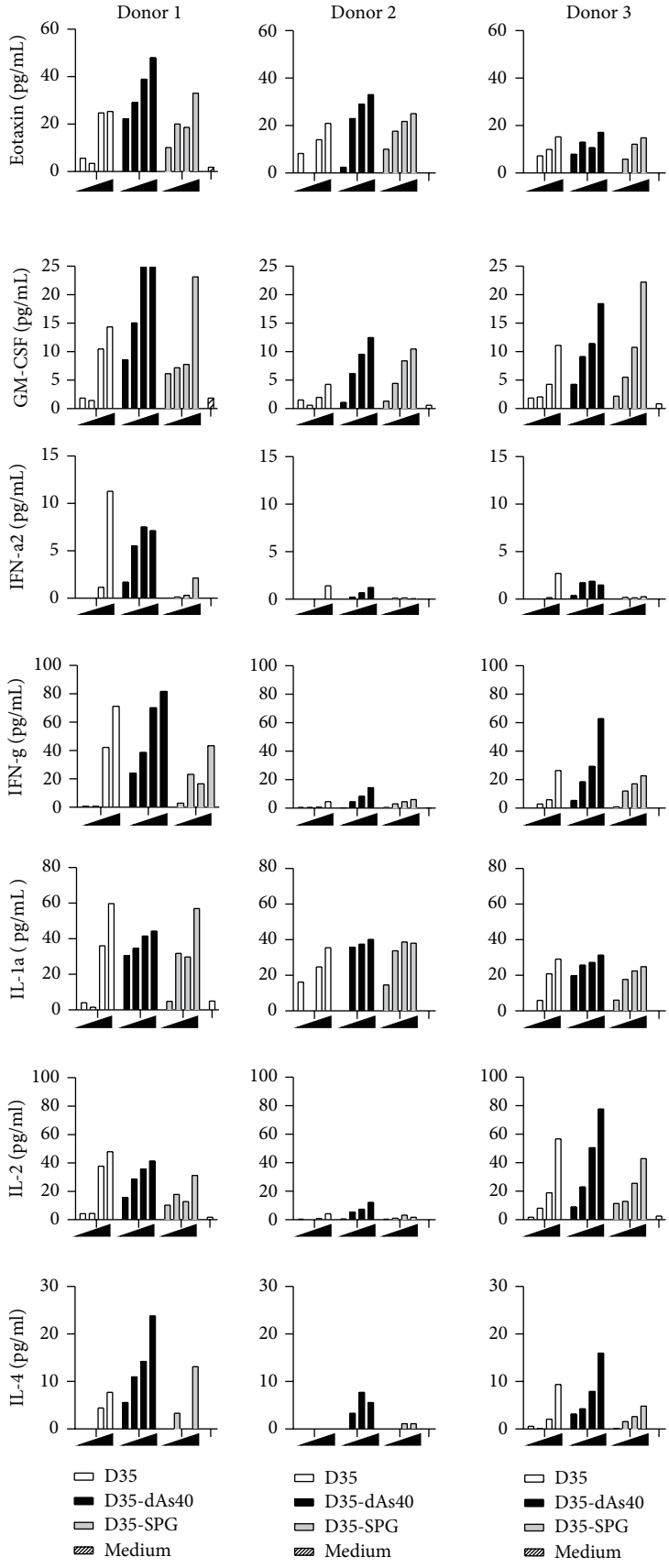


Figure 12: D35-dAs40, D35core-dAs40, and D35-SPG showed better adjuvanticity with influenza SV vaccine in cynomolgus monkeys. (a) Immunization and assay schedule. Six groups of monkeys (n = 2 or 3) were immunized with SV vaccine (A/New Caledonia/20/99, 5 μg/head) s.c. in a total volume of 500 μL with or without the indicated adjuvants (4.7 nmol each: K3; 30 μg, D35; 30 μg, D35-dAs40; 92 μg, D35-SPG; 92 μg as D35-dAs40 amount) twice in 2-week intervals. (b) Anti-SV total IgG in sera was determined by ELISA. X-axis indicates the serum dilutions. Each line indicates an individual monkey. (c) In a separate experiment, two groups of monkeys (n = 3) were immunized with D35 (4.7 nmol = 30 μg) or D35core-dAs40 (4.7 nmol = 80 μg) as in (a). Four weeks after the first immunization, anti-SV total IgG titers were determined by ELISA. Bar indicates the mean ± SEM.

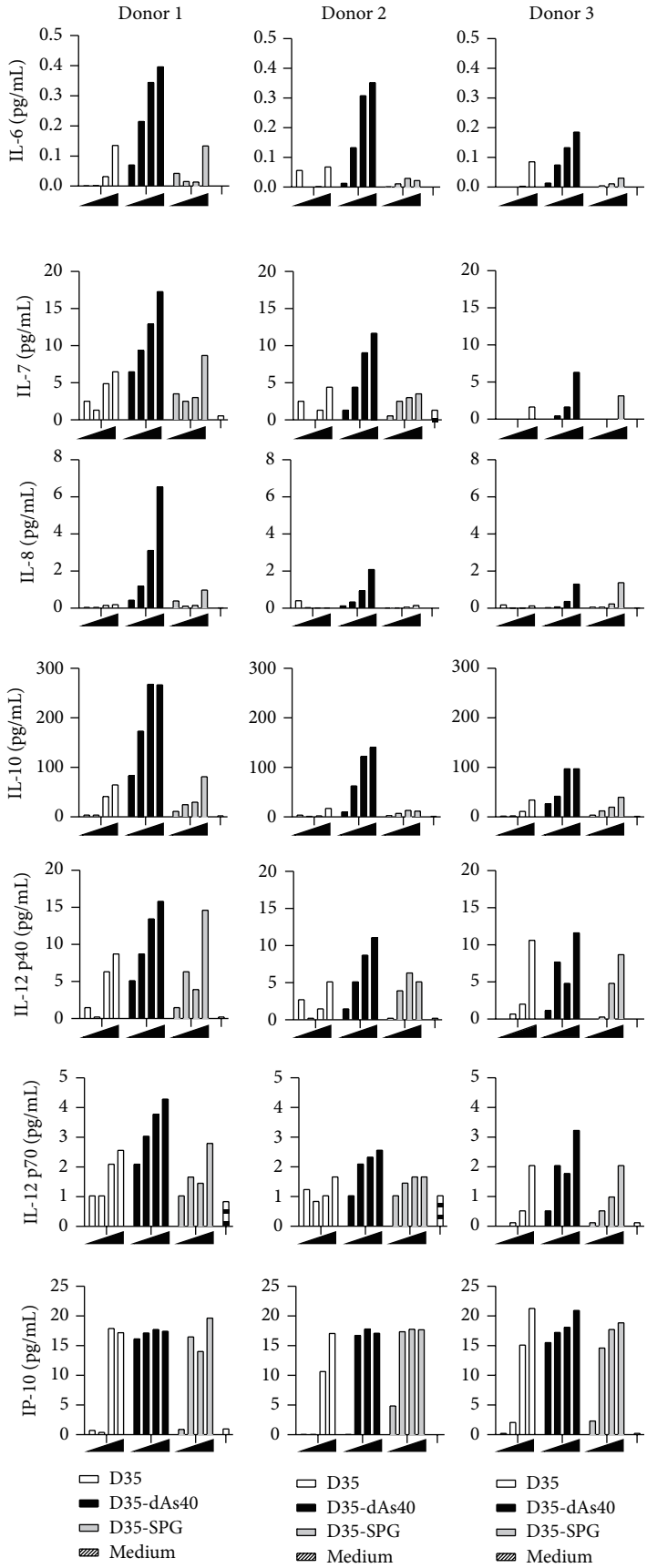
showed relatively reduced IFN-α and more IL-6 compared with original D35 *in vitro* (Figure 4). Even compared with K3, considered to induce better antibody responses owing to the direct activation of B cells and strong IL-6 cytokine induction, D35-dAs40 showed better and more reliable anti-influenza antibody responses in monkeys (Figure 12). This suggested that both D35-dAs40 and D35core-dAs40 are excellent vaccine adjuvants *in vivo*. We also tested D35-SPG (consisting of D35-dAs40 and SPG) in monkeys. Although D35-SPG showed reduced IFN-α and IL-6 levels *in vitro*, D35-SPG had comparable adjuvanticity to D35-dAs40 *in vivo* (Figure 12). These pieces of data indicated that the cytokine amount and quality induced by ODNs *in vitro* do not necessarily correlate with their adjuvanticity *in vivo*. The difference in biodistribution might be another important factor that affects the adjuvanticity of the modified ODNs. In the case of K3-SPG, we observed different biodistribution in the draining LN between K3-SPG and K3 [16]. Further investigation is required in order to understand the relationship between *in vivo* adjuvanticity and the ODN biodistribution.

By dose-response analysis, D35-dAs40 and D35core-dAs40 showed dose-proportional IFN-α and IL-6 responses similar to original D35 (Figure 4), and this is one reason why we consider D35-dAs40 and D35core-dAs40 as "D type" and not C or P type CpG ODNs. C and P type ODNs also induce IFN-α production; however, their "ODN dose-cytokine responses" are usually different from the D type ODN pattern. C and P type ODNs showed reduced IFN-α secretion when a larger amount of ODNs was used for stimulation, and this type of IFN-α response was also observed by us for K3-SPG, which originated from K3 CpG ODN, although it could still induce robust IFN-α



(a)

Figure 13: Continued.



(b)

Figure 13: Continued.

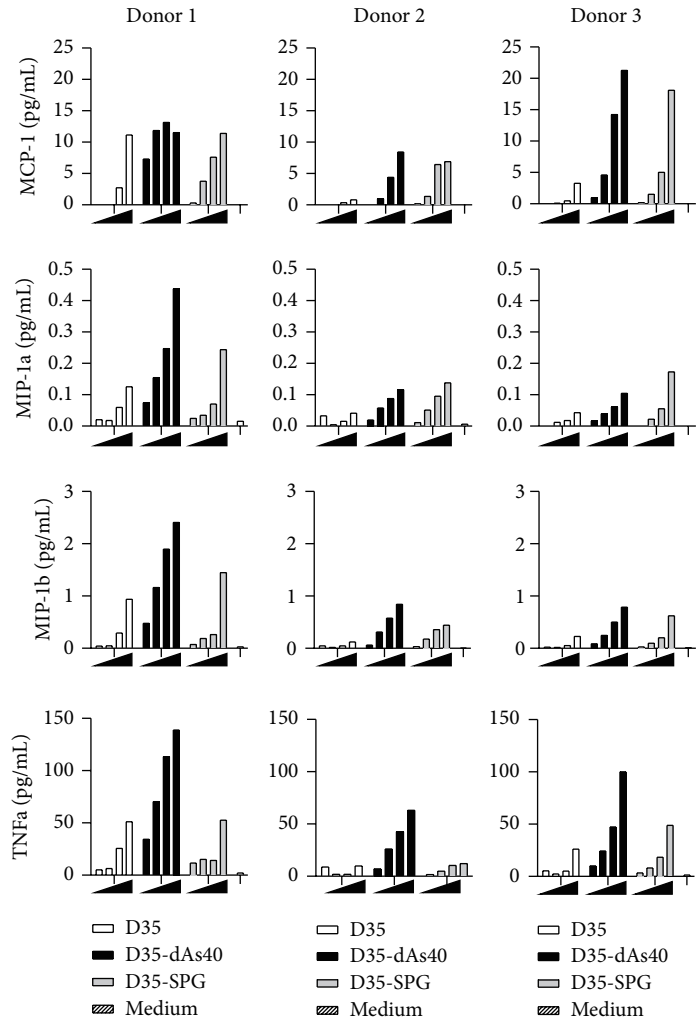


Figure 13: Cytokine profiles of D-type CpG ODNs in human PBMCs. PBMCs from three Japanese adult volunteers were stimulated with D35, D35-dAs40, D35-SPG, or medium. Each ODN was serially diluted (0.74, 2.2, 6.6, or 20 μg/mL). After 24 hours, the cytokine concentrations in the supernatants were determined with Milliplex cytokine assay kit.

production [16]. Because the backbones of C, P, and K3-SPG are all phosphorothioate, this inversely proportional IFN-α response might be caused by the phosphorothioate backbone structures; however, the underlying mechanisms are currently unknown and need to be investigated in the future. Taken together, the present study demonstrated that D35-dAs40 and D35core-dAs40 are promising prototypic D type CpG ODNs with high solubility in saline and high adjuvanticity *in vivo*.

A G-hexamer sequence and the resulting aggregation of ODNs are not necessary for A/D type ODNs to induce IFN-α production from human PBMCs, which was previously believed to be an obligatory requirement. Earlier studies concluded that aggregation was necessary for high IFN-α production by A/D type CpG ODNs [12–14]. However, our data demonstrate that aggregation is not an absolute requirement for high IFN-α production by A/D type CpG ODNs (Figure 1), suggesting that the G-hexamer sequence itself is not directly involved in TLR9-mediated CpG ODN recognition. D35 ODNs not containing G-hexamers (such as D35A, D35T, and D35C) with DOTAP induced strong IFN-α production (Figure 2), thus further supporting this hypothesis, where DOTAP compensated for the aggregation-dependent uptake processes of D-type ODNs. In contrast, IFN-α production was completely dependent on the presence of CpG motifs (Figure 1). These data suggested that the overall immunostimulatory activities of A/D type ODNs could be regulated by two nonoverlapping mechanisms: (1) ODN uptake by cells and (2) CpG motif recognition by TLR9 in the endosome. Taken together, we concluded that the CpG-containing 12-mer palindromic sequence and the phosphorothioated poly-A tail were the two minimal and sufficient components of our newly developed nonaggregating D type ODNs, such as D35-dAs40 and D35core-dAs40.

The presence of a G-hexamer has been reported to contribute to the efficient uptake of phosphodiester backboned

ODNs by cells, possibly through scavenger receptors [19, 23, 26]. It was demonstrated that phosphodiester backboned ODNs require a G-hexamer or related sequences that form aggregations for ODN uptake by cells and the subsequent immunostimulatory functions. However, phosphorothioated single strand CpG ODNs (such as B/K type ODNs) were reported to use DEC-205 for their uptake by cells [27]. In addition, many molecules such as HMGB1, granulin, and LL37 mediate or enhance ODN or DNA uptake and delivery to TLR9 [28]. D35-dAs40 and D35core-dAs40 do not form aggregates but maintain their immunostimulatory activity dependent on the presence of phosphorothioate but not the phosphodiester poly-A tail, suggesting that uptake of D35-dAs40 and D35core-dAs40 is likely mediated by phosphorothioate ODN uptake mechanisms such as DEC-205 and/or possibly other undiscovered accessory molecules. The determination of the precise uptake mechanisms should be addressed in the future.

G-hexamer-mediated aggregation or high order structure formation may also affect subcellular localization and facilitate preferential early endosome localization of ODNs with multimeric form [29]; however, the precise regulation mechanism of this preferential early endosome sorting remains to be determined. It was suggested that IFN-α production signaling starts at CpG/TLR9 interactions residing in the early endosomes [25, 29]. Forced targeting of B/K type ODNs (not a good inducer of IFN-α) to early endosomes with DOTAP also induced IFN-α [25], suggesting the requirement of a backbone chemical feature and high order structures are not strict when CpG ODNs are targeted to the proper compartment for IFN-α production. Similarly, a recent report examined two different higher-order-structured CpG ODNs attached to a nanoparticle surface and found that multimerized-ODNs/nanoparticles induced IFN-α responses while monomeric-ODNs/nanoparticles induced IL-6 responses [30]. These reports suggested that both ODN structures and targeted cellular compartments are important for determining the final preferential cytokine responses, either IFN-α or IL-6. Recently reported AP-3 added another layer to the regulation of IFN-α responses of pDCs [31]. TLR9 localization to lysosome-related organelles that might be derived from late endosomes was required for IFN-α production from pDCs [31], suggesting that IFN-α production by TLR9 signaling was regulated by a more complicated intracellular sorting mechanism than previously thought [32]. D35-dAs40 and D35core-dAs40 are composed of nonaggregated D type sequences and phosphorothioate poly-A tail and can induce both IFN-α and IL-6, which may resemble the intracellular distribution of C type ODNs, although their dose-response reflected D type ODNs. Further study is required to clarify the exact cellular compartment and molecular requirements of D35-dAs40 and D35core-dAs40 induction of IFN-α production.

4.1. Individual Response Differences. Heterogeneity of cytokine responses to B/K type CpG ODNs in human PBMC has been reported [33]. Although overall responses were largely consistent among different human PBMC samples, we also noticed variable responses against nonaggregated derivatives of A/D type ODNs examined in this study. Future investigation of the causes of these individual differences is critical for the clinical application of this type of ODN. However, we believe that nonaggregated forms of A/D type ODNs including D35-dAs40 and D35core-dAs40 are promising starting materials for the further development of clinically applicable A/D type ODNs.

5. Conclusions

In this study, we developed nonaggregating A/D type ODNs and demonstrated that D35-dAs40 and D35core-dAs40 are two promising prototypes of nonaggregating A/D type ODN with advantages of ease of drug preparation for clinical applications as vaccine adjuvants or IFN-α inducing immunomodifiers.

Conflict of Interests

The following authors have conflict of interests to declare. Taiki Aoshi, Hideaki Sato, Ken J. Ishii filed a patent application related to the content of this paper. Hideaki Sato and Hirokazu Nankai are in the employment of GeneDesign, Inc. The other authors have no conflict of interests to declare.

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Research Article

Adsorption of Toll-Like Receptor 4 Agonist to Alum-Based Tetanus Toxoid Vaccine Dampens Pro-T Helper 2 Activities and Enhances Antibody Responses

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Aluminum salts gels (alum) are TLR-independent adjuvants and have been used to boost antibody responses in alum-based vaccines such as diphtheria, pertussis, and tetanus toxoid (DPT) triple vaccine. However, the pro-Th2 activity of alum-based vaccine formulations has not been fully appreciated. Here we found that alum-based tetanus toxoid (TT) vaccine was biased toward a Th-2 profile as shown by TT-induced airway eosinophilic inflammation, type 2 cytokine production, and high levels of IgE anaphylactic antibodies. The adsorption into alum of prototypic TLR4 agonists such as lipopolysaccharides (LPS) derived from *Escherichia coli* consistently dampened TT-induced Th2 activities without inducing IFNγ or Th1-like responses in the lung. Conversely, adsorption of monophosphoryl lipid A (MPLA) extracted from *Salmonella minnesota*, which is a TIR-domain-containing adapter-inducing interferon-β- (TRIF-) biased TLR4 agonist, was less effective in decreasing Th-2 responses. Importantly, in a situation with antigenic competition (OVA plus TT), TT-specific IgG1 or IgG2a was decreased compared with TT sensitization. Notably, LPS increased the production of IgG1 and IgG2a TT-specific antibodies. In conclusion, the addition of LPS induces a more robust IgG1 and IgG2a TT-specific antibody production and concomitantly decreases Th2-cellular and humoral responses, indicating a potential use of alum/TLR-based vaccines.

1. Introduction

Adjuvants fall into two major functional groups based on whether their immune activity is dependent or nor not on Toll-like receptor (TLR) signaling [1–4]. Both groups can be defined as compounds that potentiate humoral and cellular adaptive immune responses to specific antigens. The rationale for using TLR-based adjuvants is supported by their capacity to mimic natural ligands released during infections such as those derived from bacterial walls [5, 6] or from endocytosed nucleic acids. As such, they induce effector Th1/Th17 cells required to protect the host from infections [7, 8]. The molecular mechanisms of TLR-based adjuvants ultimately involve signaling thorough MyD88 or Toll/IL-1R domain-containing adaptor inducing IFN-β-deficient (TRIF) adapter molecules [7, 9]. The augmentation of immune responses by TLR-based vaccine could be due to stimulation of dendritic cells or B cells, which are known to express TLRs [10]. In contrast, aluminum salts (alum) and their gel variations are TLR-independent adjuvants and have also been empirically used to boost antibody responses in alum-based vaccines such as triple vaccine DPT (diphtheria, pertussis, and tetanus), human papillomavirus, and hepatitis vaccines [11, 12]. However, the pro-Th2 activity of alum-based vaccine formulations has not been fully appreciated. In addition, controversy exists regarding the requirement of TLR signaling for the antibody-enhancing effects of adjuvants [13, 14].

It is now apparent that adjuvants act in the early stages of an immune response, activating innate cells that, in turn, release cytokines and chemokines to prime naïve CD4+ T cells towards effector functions [8]. A number of murine studies have demonstrated that immunization with alum provokes strong antigen-induced Th2 responses, characterized by tissue/organ eosinophilic inflammation and elevated

anaphylactic IgE levels [15]. For this reason, alum has been considered a pro-Th2 adjuvant and has been classically used in ovalbumin (OVA) asthma models [16]. However, the pro-Th2 effects of alum, such as promotion of IgE response, might be detrimental in human immunization. Indeed, though being rare, anaphylactic reactions have been reported after DPT vaccine [17].

We have previously shown in a murine OVA model that absorption of bacterial lipopolysaccharide (LPS), a prototypic TLR4 agonist, to alum inhibited the development of OVA-induced Th2 responses in a dose-dependent manner. This inhibition was via MyD88, but not TRIF pathway, and did not induce IFNγ or Th1-like responses in the lung [18, 19]. Therefore, it appears that combining opposing adjuvants blocks the appearance of polarized effector Th2 or Th1 cells. This situation might be particularly important for the development of vaccines aiming to potentiate antibody responses for the elicitation of neutralizing antibodies.

In order to extend these observations and as a proof of concept, we tested the effect of LPS absorption to alum-based vaccine with a deactivated tetanus toxin (TT). Because LPS signals via MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways, we also tested the effect of monophosphoryl lipid A (MPLA), an TLR4 adjuvant associated with TRIF-biased signaling MPLA, which exhibits low toxicity and is currently licensed to human vaccines [20, 21]. In the present study, the parameters used to monitor Th2 activities were serum levels of IgE anaphylactic antibodies and the intensity of airway allergic inflammation is shown in a OVA model of asthma [18, 19].

2. Material and Methods

2.1. Mice. Six- to eight-week-old female C57BL/6 or BALB/c mice were bred at Specific Pathogen-Free Breeding Unit, Institute of Biomedical Sciences (ICB-IV, USP), kept in in ventilated caging system (five animals/cage), and treated according to animal welfare guidelines of ICB (Ethic Protocol 081/09), under National Legislation –11.794 Law 12 h light/dark cycle, food, and water *ad libitum*.

2.2. Sensitization and Challenge. Mice were sensitized on days 0 and 7 subcutaneously with 4 μg of ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO, USA) and/or 0.25 μg of Tetanus Toxoid, kindly provided by Dr. Luciana Cerqueira Cezar Leite (Instituto Butantan, SP Brazil), both adsorbed to 1.6 mg of alum gel in 0.2 mL of PBS. OVA without endotoxin contamination (LPS-free OVA) and alum preparation were performed as previously described [18]. Toll-like receptors (TLRs) agonists PolyI:C (Polyinosine-polycytidylic acid), a TLR3 agonist signaling through TIR-domain-containing adapter-inducing interferon-β (TRIF) and MPLA (monophosphoryl lipid A), a TLR4 agonist TRIF-biased adjuvant [7, 21] extracted from the rough strain *Salmonella minnesota* R595 (Invivogen, San Diego, CA, USA); and LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA), a TLR4 agonist, were adsorbed onto alum gel. The standard dose of all TLR ligands used was 10 μg. On days 14 and 21, mice were intranasally challenged with 10 μg of OVA or TT in 40 μL of PBS. Sensitization and challenge were done under anesthesia with ketamine (50 mg/kg) and xylazine (20 mg/kg). Animals were euthanized by inhaled halothane 24 h after last challenge; samples were collected, unawares numbered, and decoded after analyses.

2.3. Serum Samples and Bronchoalveolar Lavage (BAL). Blood samples were collected by cardiac puncture, centrifuged, and serum stored at −20°C. BAL was acquired after lung washing with 1 mL of cold PBS via trachea. Total and differential cell counts of BAL fluids were determined by haemocytometer and cytospin preparation stained with Instant-Prov Romanowsky-stain (Newprov, Brazil).

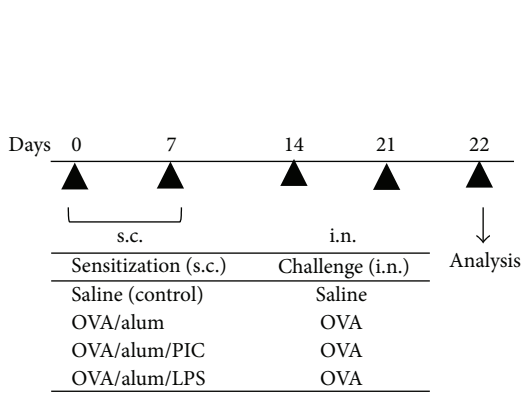
2.4. Enzyme-Linked Immunosorbent Assay (ELISA): Serum and BAL. Total mouse IgE was determined by sandwich-ELISA using kit OptEIA ELISA Set (BD, San Diego, USA). OVA-specific IgE was determined by adding serum at multiple dilutions to plates with anti-IgE (SouthernBiotech, Birmingham, AL, USA). After washing, biotin-labelled OVA was added and revealed with avidin-HRP plus substrate. Internal sample arbitrarily assigned as 1000 U was used as standard [18]. OVA-specific IgG1 and IgG2a were measured by coating the plates with 20 μg/mL of OVA. Serum samples were added at multiple dilutions and anti–mouse HRP-IgG1 or -IgG2a (Invitrogen, San Diego, USA) was revealed. Purified mouse IgG1 or IgG2a (Invitrogen) was used as standard. All ELISAs were performed in 96-well maxisorp plates (Nunc, NY, USA). Levels of cytokines in the BAL fluid were assayed by sandwich kit ELISA (BD, San Diego, USA) [22].

2.5. Measurement of IgE Anaphylactic Antibodies. The anaphylactic activity of IgE to OVA or TT was evaluated by passive cutaneous anaphylactic reaction (PCA) in rat as previously described [18]. All determinations were made in triplicate and the PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. The detection threshold of the technique was established at 1 : 5 dilutions.

2.6. Statistical Analysis. Statistical analyses were performed using Graphpad Prism (V.5; GraphPad Software, USA). One- or two-way ANOVA followed by Bonferroni post-test was performed, as appropriate. Differences were considered statistically significant when P value ≤ 0.05. Data was presented as mean ± standard error (SE).

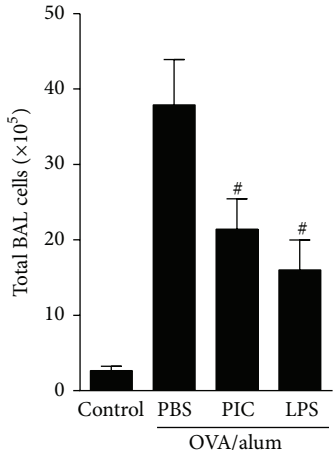
3. Results

3.1. TLR 4 Agonist Is More Effective Than TLR3 Agonist in Dampening OVA-Induced Th2-Mediated Allergic Responses. We have previously shown that TLR4 agonist (LPS) adsorbed to OVA/alum prevented the development of asthma-like responses via MyD88, but not TRIF pathway [18]. In order to ascertain more directly the effect of TRIF signaling, we used the OVA model to compare the effect of Poly I:C, a TLR3 synthetic agonist analog of dsRNA, which signals



Sensitization (s.c.)	Challenge (i.n.)
Saline (control)	Saline
OVA/alum	OVA
OVA/alum/PIC	OVA
OVA/alum/LPS	OVA

(a)



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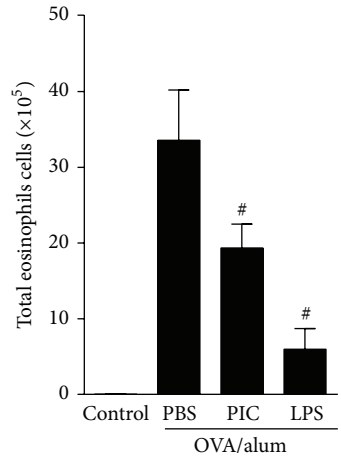

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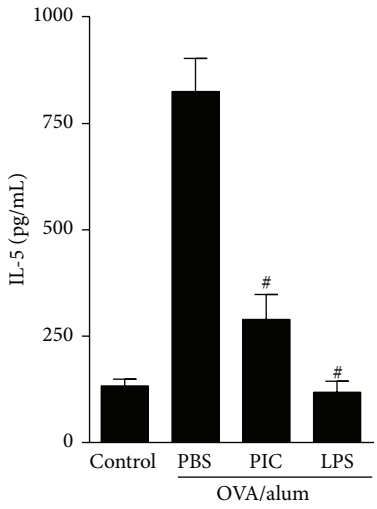

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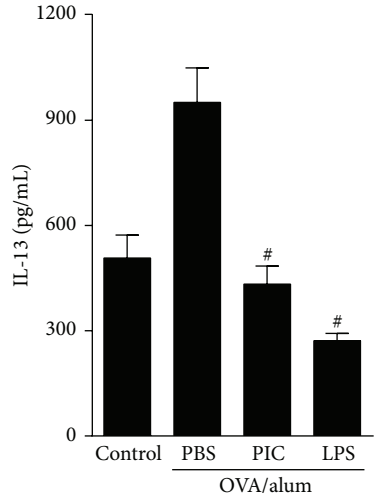

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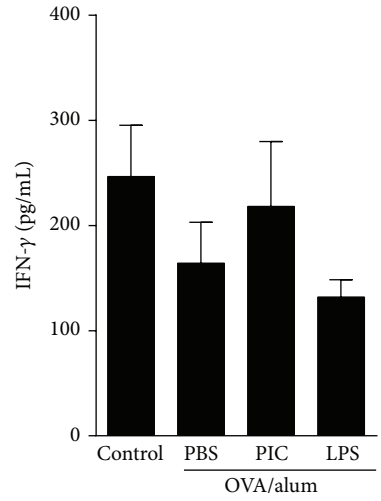
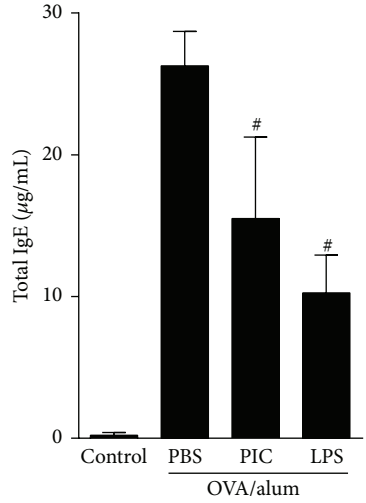
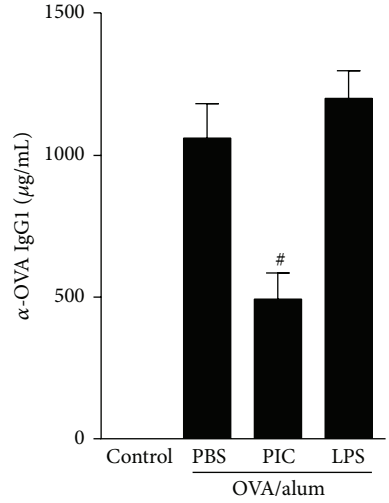


(f)



(g)



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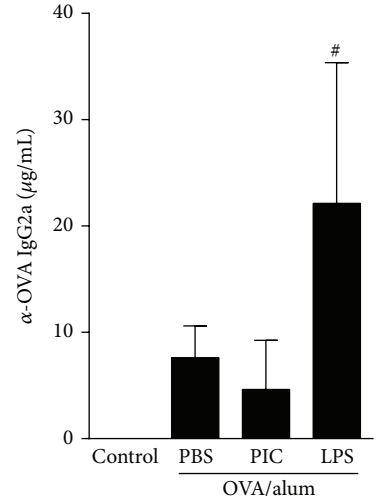

(i)

FIGURE 1: Effects of adsorption of PIC (TLR3) or LPS (TLR4) agonists onto OVA/alum sensitization on OVA-induced cellular and humoral responses. (a) Protocol: C57BL/6 WT mice sensitized with s.c. OVA/alum in the presence or not of PIC (10 μg) or LPS (10 μg) on days 0 and 7 and challenged with OVA i.n. on days 14 and 21. Samples obtained on day 22. (b) Total number of cells and (c) eosinophils in BAL; ((d), (e), and (f)) levels of IL-5, IL-13, and IFN- g in BAL. ((g), (h), and (i)) Levels of serum isotypes: total IgE, OVA-specific IgG1, and OVA-specific IgG2a. Data shown as mean ± SE, one-way ANOVA: $^{\#}P < 0.05$ different from OVA/alum/PBS group ($n = 5$), and experiment was repeated twice.

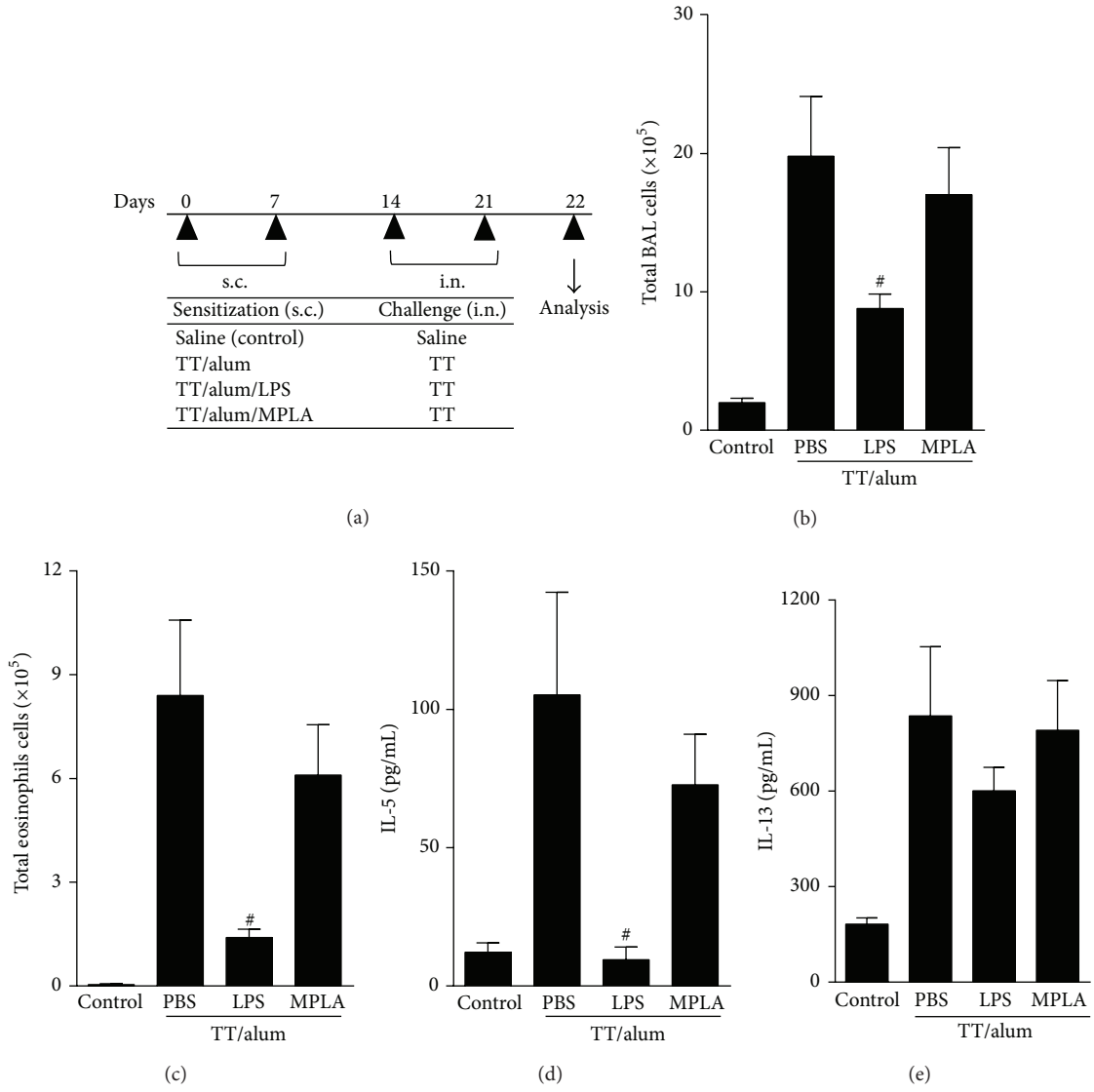

Figure 2: Effects of adsorption of LPS or MPLA onto TT/alum sensitization on TT-mediated airway allergic responses. (a) Protocol: BALB/c mice sensitized with s.c. TT/alum in the presence or not of LPS (10 μg) or MPLA (10 μg) on days 0 and 7, and challenged with TT i.n. on days 14 and 21. Samples obtained on day 22: (b) total number of cells and (c) eosinophils in BAL; ((d) and (e)) levels of IL-5 and IL-13 in BAL. Data shown as mean ± SE, one-way ANOVA: $^{\#}P < 0.05$; different from TT/alum/PBS group ($n = 5$), and experiment was repeated twice.

solely thorough TRIF; with LPS, a TLR4 agonist that signals thorough MyD88 and TRIF pathways. For this, BALB/c mice were sensitized to OVA adsorbed to alum in the absence (allergic group) or presence of agonists of TLR3 (Poly-I:C) or TLR4 (LPS). Overall, both TLRs agonists when adsorbed to OVA/alum dampened Th2 responses when compared to allergic (OVA/alum) group (Figure 1). However, LPS was consistently more effective than PIC in decreasing total cell counts and eosinophil number in BAL fluid (Figures 1(b)-1(c)), IL-5, and IL-13 production (Figures 1(d)-1(e)), and IgE levels (Figure 1(g)). Importantly, the levels of IFNγ in BAL in PIC or LPS groups were not increased and were similar to naive or allergic (PBS) groups (Figure 1(f)). Regarding antibody production, again LPS was more effective than PIC in decreasing IgE (Figure 1(g)). PIC but not LPS decreased OVA-specific IgG1 isotype (Figure 1(h)) while LPS increased IgG2a production (Figure 1(i)). Altogether, these results indicate that LPS was more efficient than PIC in inhibiting Th2-mediated airway allergic response.

3.2. LPS Is More Effective Than MPLA in Dampening Toxoid-Induced Th2-Mediated Allergic Responses. We next adapted the OVA model protocol to tetanus toxoid antigen. As depicted in Figure 2(a), sensitizations of tetanus toxoid adsorbed to alum (TT/alum group) followed by i.n. challenges resulted in airway allergic inflammation, as revealed by increased total cell counts of inflammatory cells in BAL fluid, constituted mainly of eosinophils when compared to control group (Figures 2(b)-2(c)). We also found in TT/alum group an increased level of type 2 cytokines (IL-5 and IL-13) in BAL

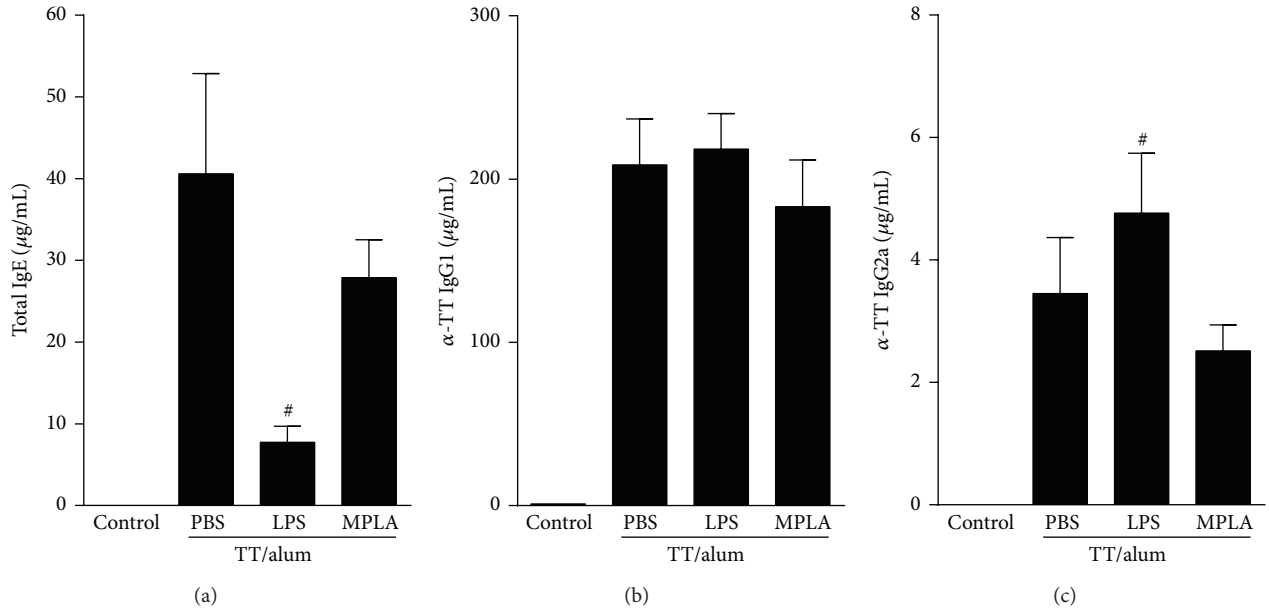

Figure 3: Effects of adsorption of LPS or MPLA onto TT/alum sensitization on serum isotype production. Protocol as shown in Figure 2(a). Concentration of serum isotypes: (a) total IgE, (b) TT-specific IgG1, and (c) TT-specific IgG2a. Data shown as mean ± SE, one-way ANOVA: $^{\#}P < 0.05$; different from TT/alum/PBS group ($n = 5$), and experiment was repeated twice.

when compared to control group (Figures 2(d)-2(e)) thus, confirming the development of airway allergic inflammation. Having established in the OVA model the role of MyD88 and TRIF signaling in the prevention of allergic responses, we now tested the effects of absorbing two different preparations of LPS/lipid A onto tetanus toxoid/alum. One preparation was obtained from *Escherichia coli* 055:B5 that signals thorough TLR4 via MyD88 and TRIF pathways and other designated MPLA, which is a TRIF-biased TLR4 agonist [21]. As shown in Figure 2, the addition of LPS to tetanus toxoid alum preparation inhibited significantly the development allergic airway inflammation, as evidenced by lower number of total cell counts and eosinophils in BAL compared to TT/alum group (Figures 2(b)-2(c)). Also IL-5, but not IL-13, levels in BAL were significantly decreased in LPS group (Figures 2(d)-2(e)). In contrast, although Th2 responses of MPLA group were lower than TT/alum group, these responses did not reach statistical significance (Figure 2). We conclude tetanus toxoid adsorbed to alum behaves like an allergen and that LPS, but not MPLA, efficiently dampens alum pro-Th2 activity. To confirm this, we determined systemic antibody production by measuring serum levels of total IgE. As shown in Figure 3, sensitization and challenge with tetanus toxoid increased IgE levels when compared to control group. The addition of LPS, but not MPLA, to alum decreased significantly IgE levels (Figure 3(a)). Conversely, IgG1 antibodies against tetanus toxoid were similar in PBS, LPS, or MPLA groups (Figure 3(b)) while IgG2a specific antibodies were increased in LPS when compared to PBS group (Figure 3(c)). These results indicate that LPS prevented the production of IgE anaphylactic antibodies, did not interfere significantly in TT-specific IgG1, and augmented TT-specific IgG2a antibody production.

3.3. LPS Potentiate Anti-Toxoid Antibody Production in a Model with Two Antigens. The benefits of combined vaccines such as diphtheria, pertussis, tetanus (DPT) vaccine are multiple including fewer injections and lower cost. However, special attention must be taken with the immunogenicity of individual antigens when combining unrelated antigens since it can be impaired, enhanced, or not affected [23, 24]. To test antigen combination in our model, we used OVA and TT antigens as unrelated antigens, adsorbing them to alum in the presence or absence of LPS, as depicted in Figure 4(a). To monitor Th2 activities we determined OVA-specific responses such as airway allergic inflammation and serum titers of anaphylactic IgE after i.n. OVA challenge. TT-specific responses were determined by serum isotype antibody production after TT s.c challenge. Regarding OVA-specific airway allergic responses, we found that addition of LPS inhibited total cells and eosinophil numbers recruitment to BAL when compared to (PBS) allergic group (Figures 4(b)-4(c)). In addition, IL-5 and, of note, IFNγ levels in BAL were also decreased in LPS group as compared to PBS group (Figures 4(d)-4(e)), indicating that LPS do not shift towards an OVA-induced lung Th1 response. As expected, LPS blocked OVA-specific IgE anaphylactic antibodies as revealed by passive cutaneous anaphylaxis titers (Figure 5(a)), thus confirming the antiallergic effect of LPS in our model. In addition, OVA-specific IgG1 and IgG2a antibodies were increased in LPS group compared to PBS group (Figures 5(b)-5(c)). Regarding TT-specific responses, LPS addition showed a similar effect to that obtained with OVA-specific responses (Figures 5(d)–5(f)). Of note, we found that the concentrations of TT-specific antibodies (IgG1 and IgG2a) were lower (Figures 5(e)-5(f)) than those obtained when sensitization and challenge were done with TT alone (Figures 3(b)-3(c)),

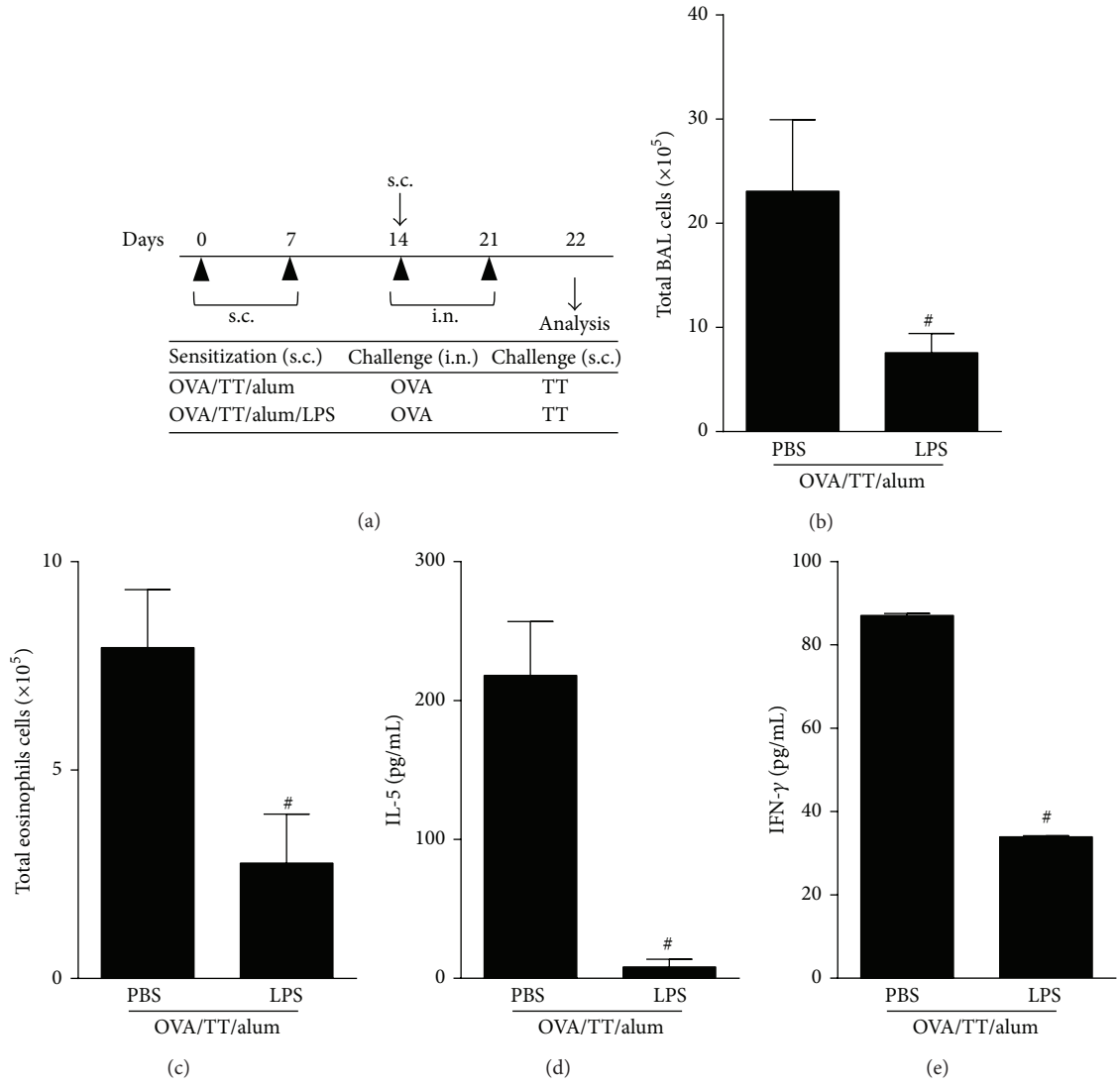

Figure 4: Effects of adsorption of LPS onto OVA/TT/alum sensitization on OVA-induced airway allergic inflammation. (a) Protocol: BALB/c mice sensitized with s.c. OVA/TT/alum in the presence or not of LPS (10 μg) on days 0 and 7 and challenged with i.n. OVA on days 14 and 21. Samples obtained on day 22. (b) Total number of cells and (c) eosinophils in BAL: ((d) and (e)) levels of IL-5 and IFN-γ in BAL. Data shown as mean ± SE, one-way ANOVA: $^{\#}P < 0.05$; different from OVA/TT/alum/PBS group ($n = 5$), and experiment was repeated twice.

indicating that the phenomenon known as antigenic completion has occurred [24]. Nevertheless and, notably, addition of LPS to OVA/TT/alum resulted in a higher production of IgG1 and IgG2a anti-TT antibodies when compared to TT/OVA/alum group. As expected, TT-specific IgE titers were lower in LPS group when compared with PBS group (Figure 5(d)). Therefore, the addition of LPS to alum-based vaccine with two unrelated antigens was able to increase IgG1 and IgG2a antibody production and decrease anaphylactic IgE titers, a more desirable profile for neutralizing antibodies.

4. Discussion

Historically, vaccines have been developed and employed to protect humans or animals against infectious diseases [25]. Alum adjuvant has been empirically used in human vaccination to apparently boost neutralizing antibodies against toxins (DT vaccine) or viruses (hepatitis B) [17]. In contrast, TLRs agonists are viewed as adjuvants that usually favor cellular immunity and the development of Th1 or Th17 cells [26]. Although alum has been used extensively, its pro-Th2 activity, as revealed in animal models of allergies, might limit its efficacy in vaccination against infectious pathogens. We have previously shown that LPS adsorbed onto alum dampened Th2 responses via MyD88 pathway and modulated antibody isotype pattern by increasing IgG2a (Th1) and decreasing IgE (Th2) antibodies [18]. Here we extended our study to tetanus toxoid antigen and tested LPS as well as MPLA, a TLR4 adjuvant associated with a bias toward TRIF signaling [21]. Because TRIF signaling results in type I IFNs production, which are known to exert an inhibitory activity

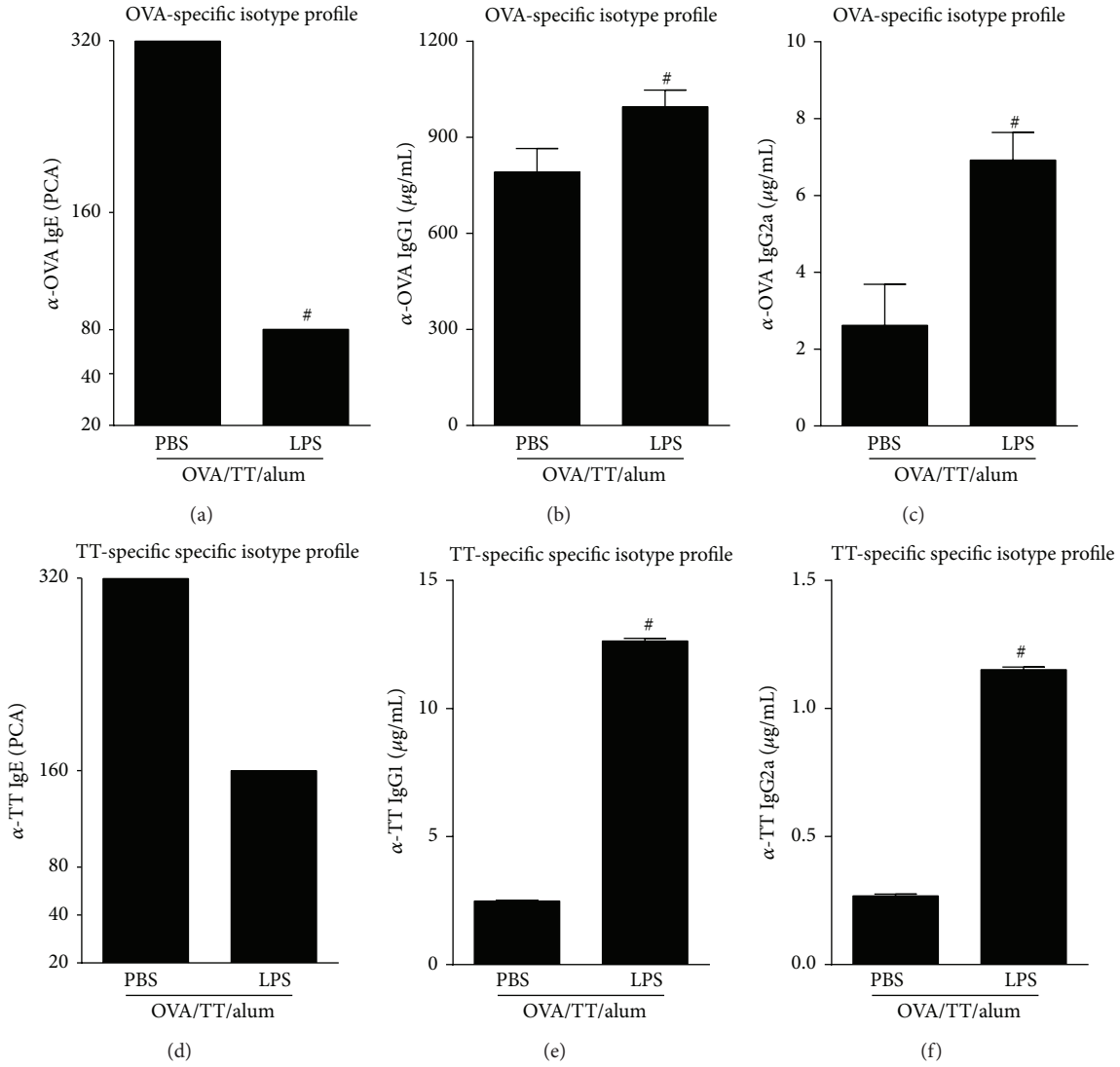

Figure 5: Effects of adsorption of LPS onto OVA/TT/alum sensitization on OVA- or TT-induced isotype production. Protocol shown in Figure 4(a). ((a) and (c)) Concentration of OVA-specific isotypes: (a) PCA titers anaphylactic IgE, (b) IgG1, and (c) IgG2a. ((d) and (f)) Concentration of TT-specific isotypes: (d) PCA levels of anaphylactic IgE, (e) IgG1, and (c) IgG2a. Data shown as mean ± SE, one-way ANOVA: $^{\#}P < 0.05$; different from OVA/TT/alum/PBS group ($n = 5$), and experiment was repeated twice.

on Th2-mediated responses [26–28], we first investigated, in the OVA model, the effect of signaling via TRIF pathway using PIC, a unique TLR3 agonist that signals solely through TRIF pathway. Indeed, we found that signaling through TRIF pathway could inhibit some Th2-mediated responses. However, LPS that signals through MyD88 and TRIF pathways was, by far, more effective than PIC in dampening all tested Th2 associated responses. Even tough, we were still interested in comparing the effect of MPLA, a TRIF-biased adjuvant licensed to human vaccination [21] with LPS using TT, an antigen used in human vaccination. We found that sensitization with TT/alum, as occurred with OVA, induces a strong TT-mediated airway allergic inflammation confirming the pro-Th2 activity of alum and indicating that TT is a good allergen. The Th2-associated airway allergic responses were significantly blocked by LPS, but not by MPLA. More importantly, regarding antibody production, LPS but not MPLA inhibited significantly total IgE production while maintaining the levels of IgG1 or IgG2a anti-TT. Therefore, it is clear that absorption of LPS to alum blocks its pro-Th2 activities that are undesirable because they might reduce anti-infectious immunity. Another fact highlighted in our study is related to antigenic competition. We found that the antibody response to TT was higher when mice were sensitized solely with TT than when sensitized with OVA plus TT. Indeed, when designing vaccine with multiple antigens it is important to test whether the combination does not decrease the immunogenicity of single components. This was the case of a multivalent foot rot vaccine [29]. Also, inactivated poliovirus vaccine combined with diphtheria and TT and whole cell pertussis vaccine diminished antibody response to pertussis vaccine [30]. Conversely, whole cell

pertussis vaccine combined with diphtheria toxoid boosted the immune response to individual components [31, 32]. In our model, although the combination of OVA plus TT decreased TT-specific antibodies indicating antigenic completion, the addition of LPS potentiates IgG1 and IgG2a anti-TT antibodies while decreasing IgE antibodies revealing that LPS boost the production of anti-infectious antibodies. We believe that LPS acts during the sensitization phase impairing Th2 priming. Supporting this assumption it has been shown that TLR ligands can activate dendritic cells to release an uncharacterized MyD88-dependent negative signal that acts on Th cells and impairs Th2 cell development [33]. Also, it is possible that the efficiency of antigen presentation by dendritic cells is dependent on the presence of TLR ligands and that the generation of peptide–MHC class II complexes is controlled by TLRs in the phagosome as shown by Blander and Medzhitov [34].

The high toxicity of LPS precludes its use in humans, but synthetic TLR4 agonists, with low toxicity, have been developed and we attested their antiallergic effect in the OVA model [18]. In addition, other TLRs agonists can be used. For instance, subcutaneous administration of alum vaccine of ragweed-TLR9 agonist was clinically effective in the treatment of allergic rhinitis [35]. In addition, treatment with a novel TLR9 agonist showed clinical efficacy in persistent allergic asthma [36].

5. Conclusions

Our work highlights that the pro-Th2 properties of alum adjuvant can be suppressed by the absorption of LPS indicating the potential use of TLR agonists in alum-based vaccines against a variety of antigens in which antibody production is essential for the immune therapeutic effects and where the alum pro-Th2 activities are detrimental. Our data also show that it is possible to block the development of Th2-mediated responses by TLRs agonists without deviation to a Th1 polarized phenotype.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

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Research Article

Non-CpG Oligonucleotides Exert Adjuvant Effects by Enhancing Cognate B Cell-T Cell Interactions, Leading to B Cell Activation, Differentiation, and Isotype Switching

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Natural and synthetic nucleic acids are known to exert immunomodulatory properties. Notably, nucleic acids are known to modulate immune function via several different pathways and various cell types, necessitating a complex interpretation of their effects. In this study we set out to compare the effects of a CpG motif containing oligodeoxynucleotide (ODN) with those of a control and an inhibitory non-CpG ODN during cognate B cell-T cell interactions. We employed an antigen presentation system using splenocytes from TCR transgenic DO11.10 mice and the ovalbumin peptide recognized by the TCR as model antigen. We followed early activation events by measuring CD69 expression, late activation by MHC class II expression, cell division and antibody production of switched, and nonswitched isotypes. We found that both of the tested non-CpG ODN exerted significant immunomodulatory effects on early T cell and on late B cell activation events. Importantly, a synergism between non-CpG effects and T cell help acting on B cells was observed, resulting in enhanced IgG production following cognate T cell-B cell interactions. We propose that non-CpG ODN may perform as better adjuvants when a strong antigen-independent immune activation, elicited by CpG ODNs, is undesirable.

1. Introduction

Pathogen associated molecular patterns (PAMPs) activate antigen presenting cells (APCs) via pattern recognition receptors (PRRs) and this process is required for the development of an efficient immune response against the pathogens. Bacterial DNA and synthetic oligonucleotides like CpG oligonucleotides [1] are one of the classes of PAMPS that stimulate cells via PRRs. Besides TLR9 several proteins have been described as the candidate receptors for oligodeoxynucleotides (ODNs), such as CD14 [2], membrane bound scavenger receptors like CXCL16 [3] or SR-A and MARCO [4], DEC-205 [5], human CR2 [6], the KIR3DL2 receptor on human NK cells [7], and alpha 2-macroglobulin [8]. It has also been proposed that the uptake of ODN that have a phosphorothioate (PS) backbone differs from that of natural phosphodiester backboned ODN, and PS ODN bind to many proteins due to nonspecific interactions [9, 10], with no consensus about the exact pathways mediating cellular entry, docking, and signalling induced by these ODNs. Recent studies demonstrated that various TLRs, among them TLR9, are also expressed in different murine and human T cell subsets and have costimulating function. In combination with TCR activation, TLR9 ligands have been shown to induce cytokine production and to promote survival [11, 12]. However, TLR9 cannot be solely responsible for this phenomenon, as T cells of TLR9 or MyD88 deficient mice also respond to CpG and interestingly, even to certain non-CpG ODNs, including inhibitory ODNs [13].

Therefore, it would be very important to understand how T cells and B cells costimulated with non-CpG ODN contribute to the development of an adaptive immune response. Our aim was to investigate how non-CpG ODN modulates antibody production following cognate interaction of T cells

with B cells presenting antigen. To this end we examined early and late activation events leading to isotype switching in B cells, a process that enables a more effective host defense against pathogens.

2. Materials and Methods

2.1. Ethics Statement. All the treatments of animals (mice) in this research followed the guidelines of the Institutional Animal Care and Ethics Committee at Eötvös Loránd University that operated in accordance with permissions 22.1/828/003/2007 issued by the Central Agricultural Office, Hungary, and all animal work was approved by the appropriate committee.

2.2. Animals and Cell Culturing. BALB/c mice were purchased from Charles River Laboratories; DO11.10 mice (on the BALB/c background) were derived from The Jackson Laboratory. Both strains were bred and maintained under specific pathogen free conditions in the animal unit of the Eötvös Loránd University. Mice were used at 6–18 wk of age. Spleen or lymph node cell suspensions were cultured in RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA, US) supplemented with 5% heat-inactivated FCS (GIBCO), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, US), 100 U/mL penicillin (Sigma-Aldrich), 100 μg/mL streptomycin (Sigma-Aldrich), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 1 mM sodium pyruvate (Reanal, Budapest, HU).

2.3. Oligodeoxynucleotides and Ovalbumin Derived Peptide. All oligodeoxynucleotides (ODN) had phosphorothioate (PS) linkages between the nucleobases (marked with capital letters), except the one preceding the last two 3′ base (marked with lowercase letters). CpG (ODN 1668; TCCATGACGTTCCTGATGCt), Control (ODN 1720; TCCATGAGCTTCCTGATGCt), and Inhibitor (ODN 2088; TCCTGGCGGGGAAGt) were purchased from Sigma-Aldrich. The ovalbumin derived peptide (OVA) with the following sequence Biotin-KISQAVHAAHAEINEAGR was synthesized by the CASLO Laboratory ApS (Lyngby, Denmark).

2.4. In Vitro Cell Activation. Freshly isolated spleen or lymph node cells (pooled from the subiliac, popliteal, proper and accessory axillary, superficial parotid, mandibular, and sciatic lymph nodes) were plated onto 96-well plates in 2×10^5 cells/well density. Inhibitor, Control, and CpG ODNs were added in low (0.25 μM) or high (2.5 μM) concentrations with or without a suboptimal activating dose (25 nM) of OVA peptide. Cells were incubated for 1 day at 37°C in 5% CO_2 humidified atmosphere and CD69 expression was measured or cultured for 2 days with addition of 5-ethynyl-2′-deoxyuridine (EdU) on the first day, and the percent of divided cells was measured on the second day using a "click" reaction assay, according to the manufacturer's protocol (Click-iT EdU flow cytometry assay kit, Invitrogen). MHCII expression or number of antibody secreting cells was determined with flow cytometry or fluorescent ELISPOT, respectively, from 4-day cultures.

2.5. Flow Cytometry. Fluorescently labeled mAbs obtained from eBioscience (San Diego, CA, US) were the following: anti-mouse CD45R-PerCP-Cy5.5 (clone: RA3-6B2), rat IgG2a-PerCP-Cy5.5 isotype control (clone: eBR2a); BD Biosciences (Franklin Lakes, New Jersey, US): anti-mouse CD4-PE (clone: (L3T4) (RM4-5)), rat anti-mouse I-A/I-E-PE (clone: M5/114.15.2), rat IgG_{2b}, κ-PE isotype control; BioLegend (San Diego, CA, US): anti-mouse CD69-A647 (clone: H1.2F3). For cell surface staining, cell suspensions were incubated on ice for 20 min with different combinations of mAb, diluted in FACS buffer (PBS supplemented with 1% heat-inactivated FCS and 0.1% sodium azide). Nonspecific binding was blocked using heat inactivated mouse serum in 4-time dilution. After staining with fluorescently labeled mAb, cells were washed and acquired by a FACSCalibur (BD Biosciences) flow cytometer and results were analyzed using FCSExpress (De Novo Software). In $CD45R^+CD4^-$ lymphocyte-sized cells were considered B cells and $CD45R^-CD4^+$ lymphocyte-sized cells were considered T cells. Dead cells were excluded on the basis of their light scattering properties.

2.6. Fluorescent ELISPOT. 16-pad nitrocellulose-covered glass slides (UniSart, Sartorius Stedim Biotech, Goettingen, Germany) were put into slide modules (ProPlate, Grace Bio-Labs, Bend, OR, USA) and rinsed with PBS for 5 min before use and coated overnight with 2.5 μg/mL anti-mouse kappa capture antibody (Southern Biotech, Birmingham, AL, USA) in PBS. Slides were then washed with PBS three times and blocked for 1 hour at 37°C with RPMI-1640 medium supplemented with 10% FCS. For the measurement of IgG and IgM antibody secreting cells (ASCs) 2/3 volume for the measurement of IgG1 and IgG2a isotype ASCs 1/15 volume of the original 4-day spleen or lymph node cultures was added to the slide. After 12 hours of incubation, slides were washed with PBS and then with PBS-Tween. Labeling antibodies (anti-mouse IgG1-A488 (Invitrogen), anti-mouse IgG2a-Cy5 (SouthernBiotech), anti-mouse IgM-A647 (Invitrogen), and anti-mouse IgG-A488 (Invitrogen)) were diluted 5000-fold in PBS containing 5% BSA (Sigma-Aldrich) and 0.05% Tween 20 (BSA-PBS-Tween). After incubation with the labeling antibodies for 1 h at RT, slides were washed with PBS-Tween, arrays were dried and scanned with an Axon GenePix 4300A scanner, and data were analyzed with ImageJ 1.43 M software or with visual inspection.

2.7. Statistical Analysis. Statistical difference was calculated using two-tailed Wilcoxon signed rank test in case of flow cytometric data and two-tailed permutation test was applied for fluorescent ELISPOT results. The permutation test was performed as follows: Values from the two groups to be compared were randomly reassigned to two groups and the difference between the group means was calculated. Distribution of 5000 randomizations was drawn and the two-tailed P value corresponding to the real sample assignments was determined. The arithmetic mean of 50 such P values was accepted as the probability of alpha error. For simplicity, only

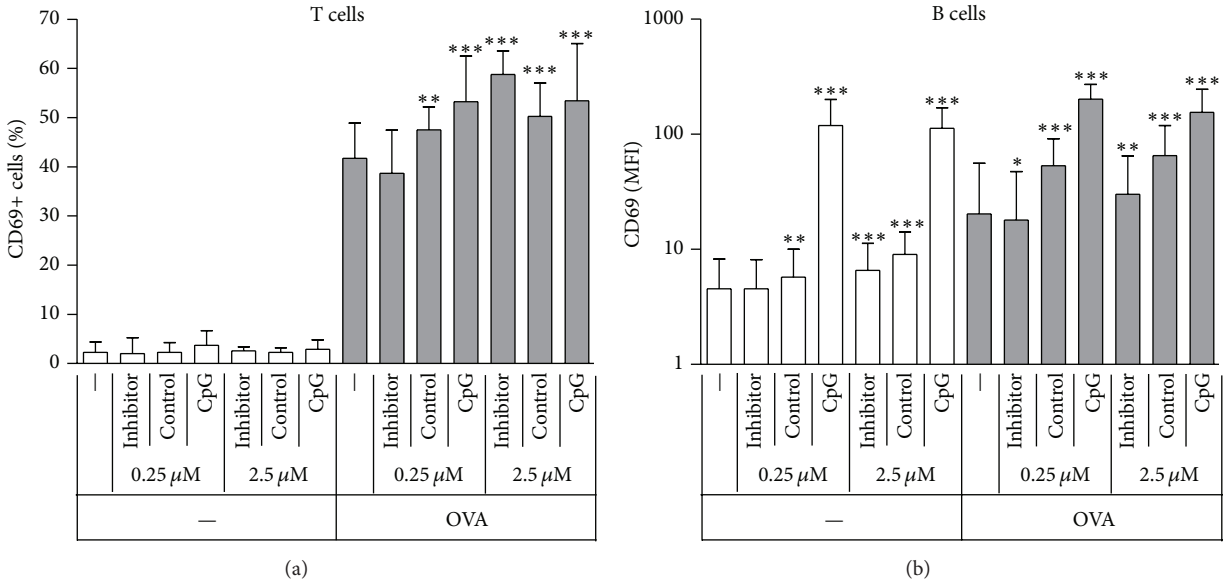

Figure 1: Non-CpG ODN enhances CD69 expression in T and B cells engaging in cognate interaction. DO11.10 splenocytes were incubated with different combinations of ODNs and OVA; after 24 hours CD69 expression was measured on T and B cell populations by flow cytometry ((a)-(b)). Medians and interquartile ranges of 13 independent experiments are shown. Asterisks indicate significant difference from the group receiving no ODN at all, within the respective OVA treatment group. MFI: mean fluorescence intensity; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the results of the comparison of OVA versus OVA + ODN treatments are indicated.

3. Results

3.1. Non-CpG ODNs Enhance Early Activation Events upon Antigen Presentation. In order to extend earlier observations showing costimulation of T cells by non-CpG ODN [13], we utilized an antigen presentation system based on the transgenic expression of ovalbumin specific T cell receptor. Helper CD4 positive T cells from the DO11.10 mouse strain recognize an ovalbumin peptide sequence (referred to as OVA from here on). This peptide, displayed on MHCII of APCs served as TCR stimulus in our experiments. We used suspensions of splenocytes, which due to their abundance B cells (around 50% of all splenic white blood cells) strongly contribute as APC, especially when peptides that are taken up by pinocytosis and require no further processing are used as antigen. The concentration of OVA was set to 25 nM, which confers suboptimal T cell activation (Figure 1(a)) and thus enabled us to observe modulation of the outcome of antigen presentation. Along the same lines of thought, modulating ODNs were used at 0.25 μM and 2.5 μM concentrations, at which non-CpG ODNs (Inhibitor and Control) were found to only modestly activate B cells, in contrast to CpG ODN (Figure 1(b)).

After 24 hours of incubation about 40% of T cells expressed the early activation marker CD69 in the presence of OVA (Figure 1(a)). This basic activation was enhanced not only by coincubation with CpG ODN but also by Control and at higher concentration, Inhibitor ODN, resulting in 50–60% of the T cells being activated (Figure 1(a)). In a similar fashion, the presence of OVA resulted in increased CD69 expression on B cells, due to cognate interactions with T cells during antigen presentation (Figure 1(b)). While CpG strongly activated B cells without OVA, inducing more than tenfold increase in CD69 expression, this effect was not further intensified by the addition of antigen. In contrast, non-CpG Control ODN showed improved enhancing effects in the presence of OVA, implying the need for T cell help for the effect to take place. Thus, non-CpG ODNs enhance the activation of both T and B lymphocytes when these cells engage in cognate interaction.

3.2. Non-CpG ODN Effects on Late Activation Events. After receiving appropriate activation stimuli lymphocytes express various surface markers and start to differentiate and divide. To better understand the enhancing effects of non-CpG ODN exerted on T and B cells upon cognate interaction, we looked at two late activation events: expression of MHC class II on B cells and the synthesis of novel DNA as a preparation for cell division in both B and T cells.

Interestingly, the expression of MHCII did not reflect our observations with CD69. Non-CpG ODN triggered the increase of MHCII in the absence of OVA (both low and high concentrations of Control and high concentrations of Inhibitor) but had no effects in the presence of OVA (Figure 2(a)). As expected, proliferation of T cells, expressed as the percentage of cells that incorporated the nucleotide analogue EdU, was only observed in the presence of OVA (Figure 2(b)). Proliferation of both T and B cells reflected the pattern of early activation marker expression (Figure 2(c)). Thus, non-CpG ODN alone induced moderate increase of B cell MHCII expression and synergized with OVA to increase the number of dividing B cells.

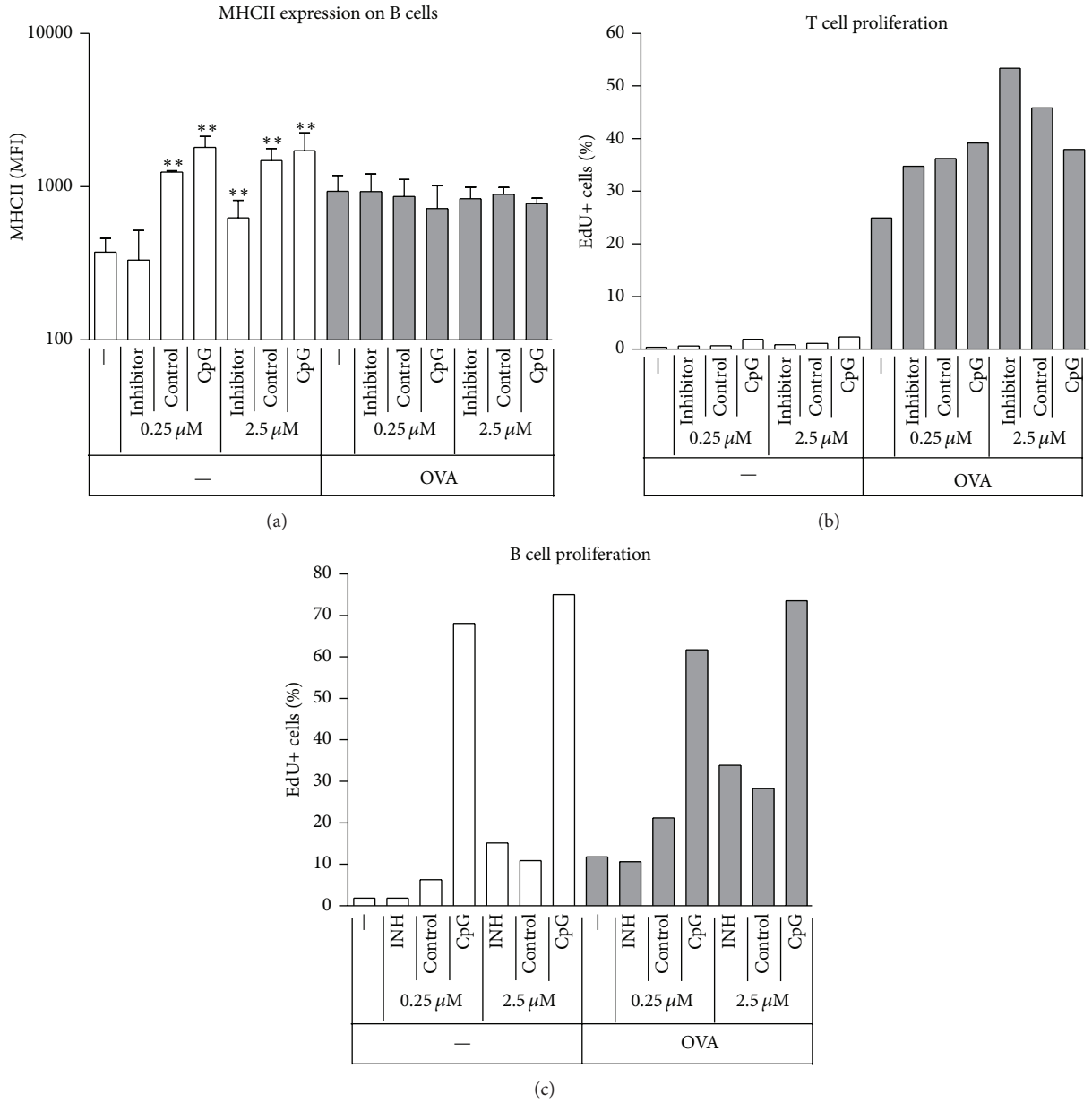

Figure 2: Effects of non-CpG ODN on late activation events. Spleen cells were incubated with the different combinations of ODNs and OVA as indicated. For MHCII expression measurements cells were harvested and stained for flow cytometry after 4 days (a). Medians and interquartile ranges of 9 independent experiments are shown. Asterisks indicate significant difference from the group receiving no ODN at all, within the respective OVA treatment group. MFI: mean fluorescence intensity; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For proliferation studies EdU was added on day 1 and left for incorporation for another day. The percentage of divided cells was then measured by flow cytometric analysis of EdU incorporation ((b), (c)). Results shown are from a single experiment; identical results were observed when EdU was added after 2 days.

3.3. Modulation of Differentiation into Antibody Secreting Cells (ASC) by Non-CpG ODN. B cells differentiate into antibody secreting plasmablasts and plasma cells when they receive proper stimuli. This stimulus may include T cell help, involving MHCII-TCR interactions along with the engagement of coreceptors but may also take place without T cell help. We examined the effects of non-CpG ODN on B cell differentiation into ASCs by measuring the number of cells producing antibodies of various isotypes. Using the very same experimental setup of coincubation of splenocytes with or without OVA and with the addition of ODN, after 4 days of culture the cells were transferred into wells coated with antibodies capturing light chains.

The number of both IgM and IgG-producing ASCs was higher in the presence of Inhibitor or Control ODNs, as well as in the presence of CpG ODN (Figures 3(a) and 3(b)). This was true in both absence and presence of OVA; that is when T cell help was provided for B cells, implying that not only

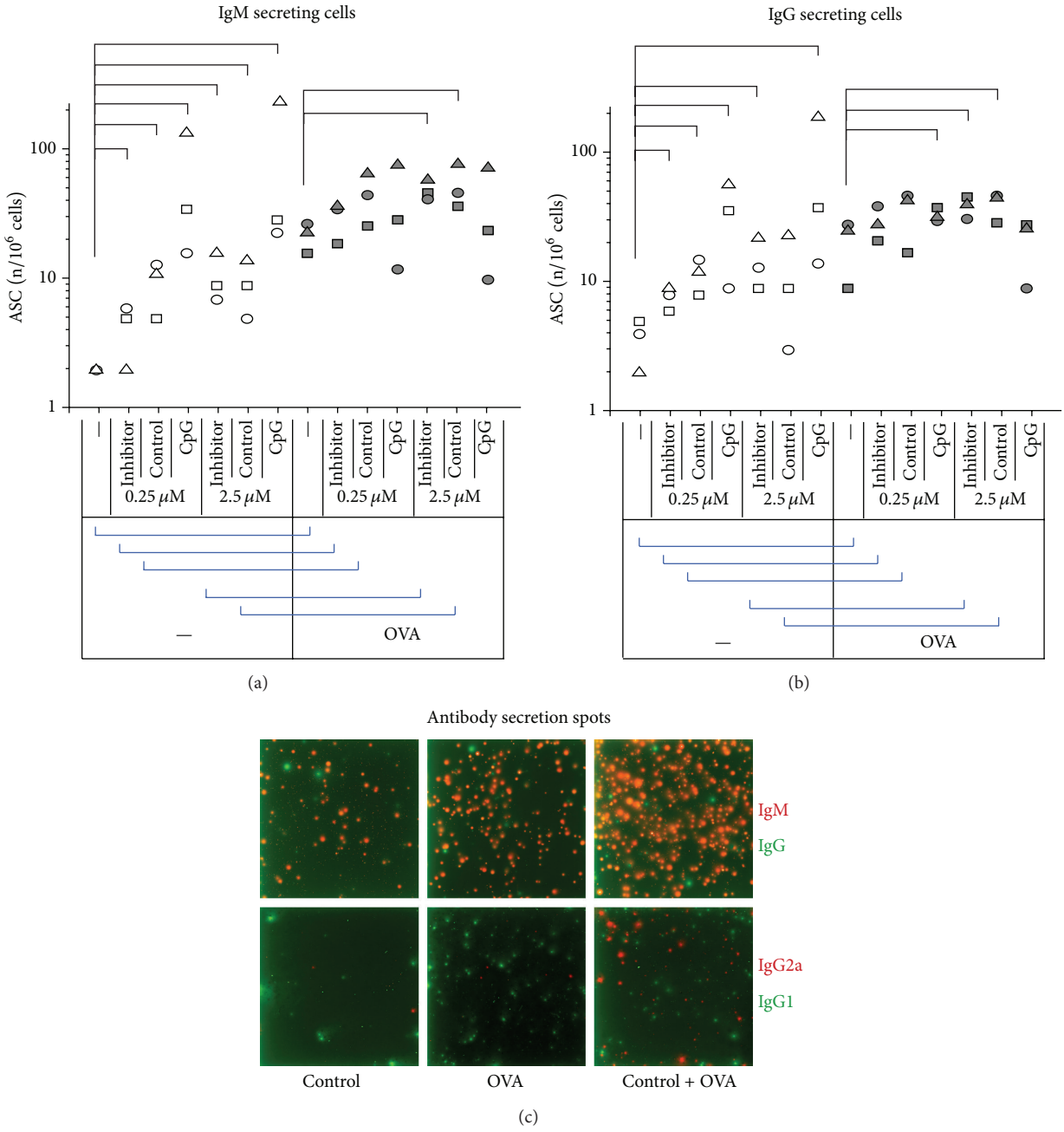

FIGURE 3: Enhancement of ASC formation by non-CpG ODN. Cells were incubated with the different combinations of ODNs and OVA as indicated; then after 4 days the cultures were transferred onto nitrocellulose-covered slides coated with light chain capture antibodies. After 10 hours of incubation, slides were washed and IgM (a) and IgG (b) spots produced by ASCs were detected using labeled antibodies and a fluorescent scanner. A representative fluorescent spot experiment is shown in (c). Statistical significance was calculated using two-tailed permutation test. Treatment pairs with significant differences ($P < 0.05$) are indicated by connecting brackets; thin lines for within OVA treatment group comparisons and thick lines for OVA treatment effects. ASC: antibody secreting cell.

CpG but also non-CpG ODN promoted differentiation of B cells into ASC. Importantly, pairwise comparisons of non-CpG but not CpG treatments with and without OVA showed additive effects (Figures 3(a) and 3(b)).

The same trends were observed in the case of IgG1- and IgG2a-producing cells, but differences were not statistically significant (a representative measurement is shown in Figure 3(c)). Non-CpG ODNs thus enhance antibody production and isotype switching during cognate B cell-T cell interactions.

3.4. Cognate Interaction Is Required for Non-CpG Modulation of T Cell Induced Antibody Production. To further confirm

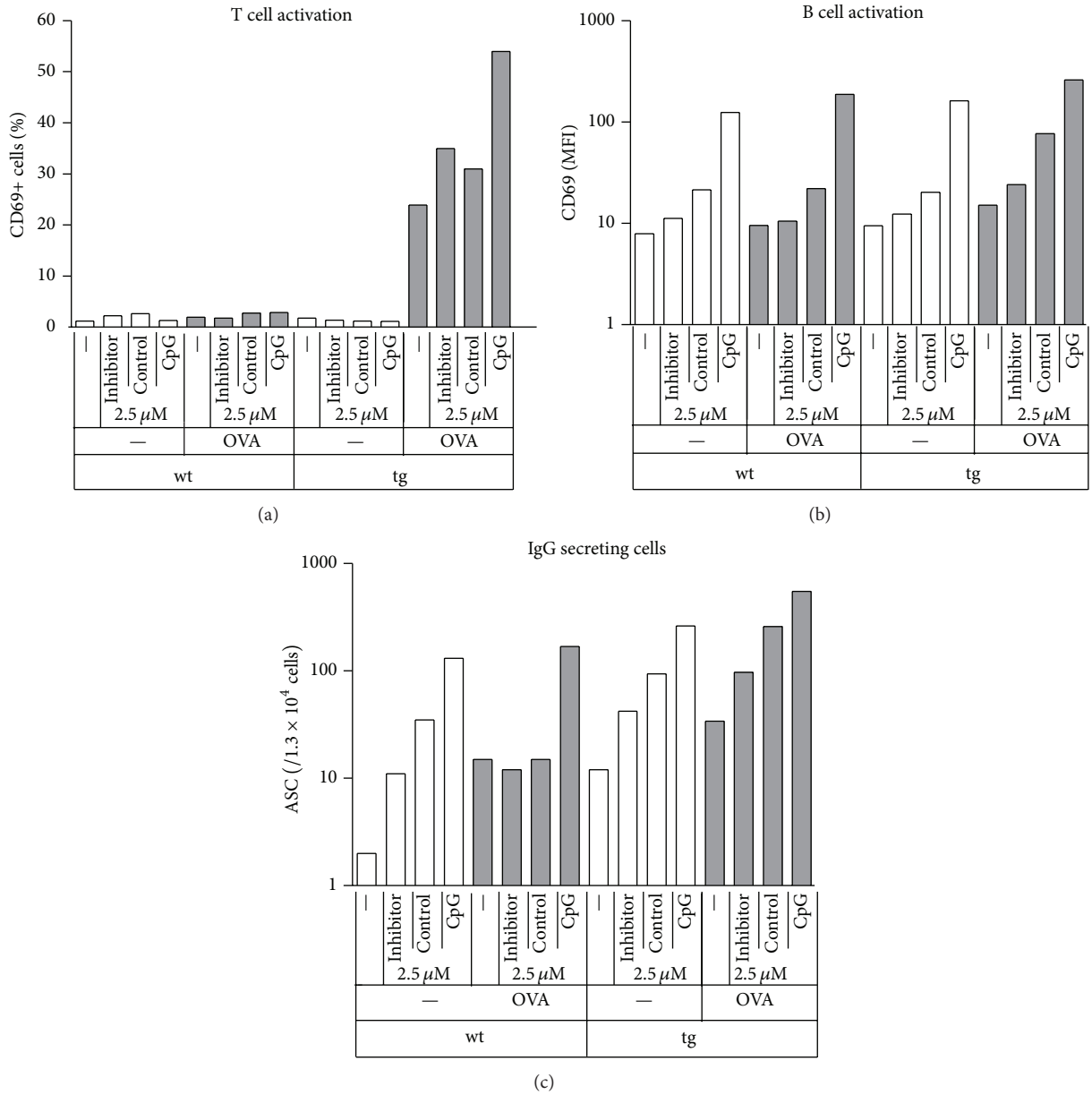

Figure 4: No synergism between OVA and ODN treatments when cognate T cells are not present. Wild type (wt) or TCR transgenic (tg) splenic cells were incubated with different combinations of ODN and OVA for 1 day ((a), (b)) or 4 days (c). After 1 day CD69 expression was measured on B and T cell populations by flow cytometry ((a), (b)). Cells from the 4-day cultures were washed and then transferred onto nitrocellulose-covered slides that were previously coated with light chain capture antibodies. After 10 h of incubation, slides were washed and spots indicating IgG producing ASCs were detected using fluorescently labeled antibodies and a fluorescent scanner (c). Results represent a single experiment.

that non-CpG ODN synergize with T cell derived stimuli in enhancing antibody production and isotype switching in B cells, we repeated experiments using wild type (wt) mice. While some OVA specific T cells may be present in naïve wt mice, their numbers are expected to be so low as to be negligible (1 : 10 000 or lower). Indeed, the percentage of CD69 positive T cells was unaffected by the addition of OVA in wt mice (Figure 4(a)). In the absence of OVA, the pattern of CD69 expression in B cells was similar in wt and DO11.10 mice (Figure 4(b)). The higher concentration of non-CpG ODN used here induced modest elevation of CD69 in both strains. The presence of OVA induced further increase only in DO11.10 mice. In a similar manner, the presence of OVA enhanced IgG production only in transgenic mice (Figure 4(c)). Therefore, it is not OVA itself but the interaction of B cells presenting the peptide to T cells in its presence that further increases mutual activation of the cells and enhances antibody production and isotype switching.

4. Discussion

Efficient vaccination presents two challenges to the immunologist: one is finding the appropriate antigen that will stimulate the ideal B and T lymphocyte clones for achieving pathogen recognition, neutralization, and removal; the other is the formulation and administration of the antigen, in a way that the optimal costimulation is provided for those specific lymphocyte clones. Adjuvants promote these latter events, where a delicate balance should be reached between overstimulation, causing unacceptable side effects, and understimulation, leading to poor vaccination response. Immunostimulatory oligodeoxynucleotides are potent adjuvants [14], acting at least partly via the TLR9 pathway on a number of different cell types [15–17]. Depending on the dosage and administration route, unwanted systemic effects may be triggered, which call for caution regarding their human usage.

The exact mechanism by which different ODNs exert their costimulatory effect on T cells is not fully elucidated yet, as there is incongruity between reports regarding the dependence of ODN effects on the presence of TLR9 and MyD88 [13, 18]. Therefore, it is not clear if there are other receptors and mechanisms that could be responsible for the costimulatory potential of non-CpG ODN.

Our approach of examining non-CpG ODN with moderate adjuvant effects [19, 20] was an extension of observations on the T cell stimulatory properties of such ODN [13]. Landrigan et al. used an artificial T cell stimulation system with CD3 and CD28 triggered activation. In contrast, we set up an experimental system where APCs (predominantly B cells) in the spleen and lymph node suspensions present to transgenic T cells their cognate antigen (OVA). Activated T cells then provide help for B cells for activation and maturation. This approach has the advantages of being closer to T cell activation upon antigen presentation *in vivo* and of being suitable for studying the effects on B cells, as well. The disadvantage is that ODN effects exerted on B or T cells cannot be distinguished.

As expected based on previous observations, T cells were not activated when treated by different ODN alone, even when B cells were activated by CpG ODN treatment (Figure 1). When antigen and ODN treatments were combined, synergism was observed in every aspect studied, ranging from early activation events (Figure 1) through late activation events (Figure 2) to promotion of B cell differentiation into ASCs (Figure 3). This effect was not due to separated APC- and T cell activating effects of ODNs, as non-CpG ODNs further increased APC activation only when given in combination with T cell antigen. The synergism was not observed when wt cells were used, ruling out the possibility that the OVA peptide itself or contamination in the peptide preparation would be responsible for the enhancement (Figure 4).

ODNs that do not or only moderately activate B cells could be used in combination with antigen to achieve B cell activation, differentiation and isotype switching via cognate interaction with antigen specific T cells. From our experiments we cannot categorically conclude whether non-CpG ODN showed antigen presentation enhancing effects via B cells, T cells, or both. The non-CpG ODN dosage we utilized showed modest effects on early B cell activation in itself and enhanced MHCII expression and ASC formation. The contribution of this effect on B cells, therefore, should be taken into account. The experiments of Landrigan et al. clearly showed a costimulatory effect of non-CpG ODN on T cells [13]. Our proposition is that non-CpG ODNs act on both B and T cells, and this effect becomes prominent when these two cell types engage in cognate interaction during antigen presentation. As we used a small peptide antigen, we assume that antigen was taken up potentially by all B cells and BCR-mediated uptake was not needed. Therefore, presumably all B cell clones presented antigen to T cells and were all partners of helper functions of costimulated tg T cells. It remains to be examined whether antigen uptake via the BCR would restrict the B cell clones affected by non-CpG treatment in our experimental system to those that specifically recognize antigen.

Class switching in murine B cells follows a pattern that is the function of the number of cell divisions [21]. Our experiments tracking dividing B cells (Figure 2(c)) showed nice correlation with the number of IgG producing cells (Figures 3(b) and 3(c)), suggesting that non-CpG ODN synergism with T cell help enhanced isotype switching via the augmentation of proliferation.

Although there is evidence concerning the requirement of CpG DNA-mediated PI-3 kinase activation via T cell-expressed MyD88 in a $CD4^+$ T cell-dependent response *in vivo* [18], the mechanism of action and possible role of other types of ODN is not elucidated. Our study focused on the B cell response, following T cell costimulation with different CpG and non-CpG ODN, and verifies their impact on T cell mediated B cell responses *in vitro*. Further studies are needed to assess the effect of ODN-costimulation of T cells *in vivo*, preferentially using ODNs that do not stimulate APC. Studying human samples would also be important, as murine and human TLR expression profile and certain TLR signaling pathways show species-specific differences.

5. Concluding Remarks

Taking into account that CpG ODN induce cell activation irrespective of the coadministration of antigen, we propose that nonstimulatory ODNs are promising candidates as possible vaccine adjuvants, because they costimulate only T cells responding to the administered antigen and in turn provide help for appropriate B cell clones presenting the antigen, a feature that could provide a more restricted and focused B cell activation and differentiation profile.

Abbreviations

APC: Antigen-presenting cell
ASC: Antibody-secreting cell
CpG: Cytosine guanine dinucleotide-containing unmethylated DNA motif (in this study: ODN 1668)
Control: Control oligonucleotide (in this study: ODN 1720)

IFA: Incomplete Freund's adjuvant
Inhibitor: TLR9 antagonist oligonucleotide (in this study: ODN 2088)
LN: Lymph node
MFI: Mean fluorescence intensity
ODN: Oligodeoxynucleotide
OVA: Ovalbumin peptide (in this study: Biotin-KISQAVHAAHAEINEAGR)
PS: Phosphorothioate.

Conflict of Interests

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

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Review Article

Innate Immune Memory: The Latest Frontier of Adjuvanticity

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Recent findings in the field of immune memory have demonstrated that B and T cell mediated immunity following infections are enhanced by the so-called *trained immunity*. This effect has been most extensively investigated for the tuberculosis vaccine strain Bacillus Calmette-Guérin (BCG). Epidemiological studies suggest that this vaccine is associated with a substantial reduction in overall child mortality that cannot be solely explained by prevention of the target disease but that it seems to rely on inducing resistance to other infections. Upon infection or vaccination, monocytes/macrophages can be functionally reprogrammed so as to display an enhanced defensive response against unrelated infections. Epigenetic modifications seem to play a key role in the induction of this "innate memory." These findings are revolutionising our knowledge of the immune system, introducing the concept of memory also for mammalian innate immunity. Thus, vaccines are likely to nonspecifically affect the overall immunological status of individuals in a clinically relevant manner. As a consequence, future vaccine strategies ought to take into account the contribution of innate memory through appropriate design of formulations and administration scheduling.

1. Introduction

Vaccination is the most effective medical intervention introduced within the last 300 years. Its effectiveness results in a reduction of mortality and an increase of life expectancy by the prevention of contagious diseases. A recent report shows that vaccines prevented more than 100 million cases of disease over the last century in the United States alone [1], and every year immunisation programs save 2.5 millions of lives worldwide [2]. Vaccination started as an empirical approach until the emergence of more sophisticated technologies (from recombinant DNA to reverse and structural vaccinology) that allowed more specific and safer formulation of vaccines [3]. One of the challenges of vaccinology has been and still is the development of vaccines that improve and support immature, failing, and compromised immune system in immunologically frail population groups such as newborns, elderly, and chronically ill patients, respectively. Adjuvants have been crucial for vaccine success. Adjuvants are immunostimulatory molecules, such as aluminium phosphate or hydroxide salts (known as Alum), Toll-like receptors agonists (TLRa), such as monophosphoryl lipid A (MPLA) and CpG oligonucleotides, emulsions (e.g., oil-in-water emulsions such as MF59 and AS03), combinations of TLRa with Alum (e.g., AS04), and liposomes/nanoparticles [4, 5]. The name adjuvant (from Latin *adiuvans* = the one who helps) underlines the ability of these agents to help the development of an adaptive immune response against a vaccine antigen by inducing a mild innate inflammatory response [6]. Over 50% of vaccines either licensed or in clinical trials are formulated with adjuvants. The role of adjuvants in inducing effective immunisation has recently been discussed in several extensive reviews [7–9].

In the last years, important discoveries changed the way of looking at the innate immune system. Features as specificity and memory, the main traits of the adaptive immune system, are now also considered to some extent for innate immunity.

The discovery of Patter Recognition Receptors (PRRs) has introduced the concept of specificity in innate recognition, although not in the highly specific fashion characterising adaptive immune recognition. The existence of different classes of innate receptors (such as TLR, C-type lectin receptors (CLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I) helicases) allows innate immune cells to identify different pathogenic microorganisms based on the recognition of pathogen-associated molecular patterns (PAMPs). The

Table 1: Innate memory versus adaptive memory.

	Innate memory	Adaptive memory
Organisms	Plants, invertebrates, vertebrates	Higher vertebrates
Cell types	NK cells, monocytes, macrophages	B and T lymphocytes
Mechanisms	Functional re-programming (e.g., epigenetic modification)	Antigen-specific antibodies and receptors after gene rearrangement
Duration	Medium- to long-term (?)	Long-term
Specificity	No (?)	Yes
Protection	Broad	Limited, highly specific

discovery of TLR and elucidation of their functions has led to the selection of a new class of adjuvants, that is, the TLR agonists [10–12].

Revisited old knowledge on the repeated stimulation of the innate immune responses has reintroduced the old concept of innate immune memory [13, 14], redubbed "trained immunity," as proposed by Netea et al. [15].

Evidence in both plants and invertebrates (that do not possess adaptive immunity and classical memory) indicates that phagocytes can respond much better to a challenge if they have been prestimulated with the same or with another agent [16]. Thus, innate immunity can have a memory, although different from acquired immune memory. Recently, "memory" of innate immune cells has been observed in vertebrates [17]. Table 1 summarises the main differences between innate and adaptive memory.

The concept of innate memory might help to develop new strategies of adjuvanticity in the near future.

Generally, vaccines have antigen-specific protective effects, but they can also improve the resistance to other infectious diseases. This phenomenon of nonspecific memory induction may go both ways, as we will better describe later; that is, it can also result in decreased reactivity to an unrelated subsequent challenge. Accordingly, a vaccine is not only a preventive strategy that improves the immune response against a specific infection, but a "biological preparation that alters the resistance towards unrelated pathogens" [18]. Interestingly, recent data reveal that trained immunity/innate memory accounts for nonspecific effects of vaccines along with the well-known role of T and B cell mediated adaptive immunity [18, 19]. Actually, innate immune memory is not a recent discovery in vaccinology, although only recently it has gained a wide interest in the context of the mechanisms underlying the activation of protective immunity.

This review summarises the current knowledge and hypotheses on innate immune memory and its role on vaccine efficiency, focusing on mononuclear phagocytes as the main innate immune cells involved and on the role of innate immune memory on nonspecific immunity. We will also highlight which questions are still unanswered. Table 2 defines some properties of the immune system, which are mentioned throughout the review.

Table 2: Definitions.

Adaptive Memory Adaptive memory is long-term, antigen-specific ability of T and B lymphocytes to respond more rapidly and more efficiently to a specific antigen upon second encounter.
Innate Memory Innate memory is the ability of an organism to adapt its immune response depending on a previous infections or vaccination, mediated by NK cells and monocytes/macrophages. This immunological re-programming can result in non-specific suppression (tolerance) or increased innate immune response (training) against reinfection by the same or different pathogens.
Trained Immunity/Memory Trained immunity/memory is the enhanced nonspecific protection against infections after previous exposure to certain microbial components (e.g., β-glucans), possibly involving epigenetic and metabolic re-programming in the cell.
Tolerance Tolerance is the refractory state of monocytes/macrophages, involving epigenetic remodelling, induced by microbial components (e.g., LPS). Upon subsequent challenge, even with a high dose of LPS, a less robust induction of pro-inflammatory cytokines ensues.
Nonspecific Effects "Nonspecific" immune effects are induced by a vaccination or infection, against unrelated and antigenically diverse infectious agents. Nonspecific effects are mediated by cross-reactive lymphocytes and innate memory cells, and might be either beneficial or detrimental, depending on the type of memory of the cells involved.

2. Role of Innate Immune Memory in Nonspecific Vaccination Effects

Some vaccines have been associated with a high decrease in mortality that not only is accounted for by their specific effects against a certain pathogen, but also depends on the induction of a nonspecific protection against unrelated infections and pathogens [18]. This nonspecific effect, most likely mediated by both T cell cross-reactivity and innate memory induction, has been extensively investigated for the tuberculosis vaccine strain Bacillus Calmette-Guérin (BCG) [20–23]. The BCG vaccine has been associated with an overall reduction in mortality [18, 24]. In developed countries, in which mortality rates are low, BCG vaccination is related to decreased morbidity outcomes, such as sepsis-related hospitalisation or melanoma risk [23, 25]. Positive nonspecific effects on mortality and morbidity in high and low income countries have been reported also for other live vaccines, for example, against measles [26–28] and smallpox [29]. Conversely, negative effects were observed for inactivated vaccines such as the diphtheria-tetanus-pertussis (DTP) vaccine [30]. In a nutshell, live vaccines are accompanied by positive nonspecific effects, while inactivated vaccines may in some circumstances induce negative outcomes. Time and sequence

of vaccine administration and sex of the vaccinees apparently influence the possibility of negative nonspecific effects, at least in less-developed countries [31]. These observations underlie the need of designing appropriate immunisation schedules, aiming at using vaccination to its greatest benefit by optimising efficacy and reducing the possibility of nonspecific deleterious effects [32].

As already mentioned, the favourable nonspecific effects of vaccines are presumably mediated by both adaptive and innate immunity. A study on SCID mice (which are devoid of T and B cells) clearly shows BCG induced nonspecific protection against an unrelated pathogen, thereby underlining the crucial role of innate immune mechanisms in the BCG induced protection [17]. Moreover, the same study reports BCG-dependent trained memory induction in human circulating monocytes, assessed as increased inflammatory cytokine release upon stimulation with unrelated pathogens, and shows that this effect is associated with epigenetic modifications. This trained memory state persisted for at least 3 months [17]. Likewise, NK cells from BCG-vaccinated individuals show an increased inflammatory cytokine release upon *ex vivo* stimulation up to 3 months after immunisation [33]. Interestingly, a study on nonspecific effects of BCG vaccination on subsequent endotoxemia did not show any immunomodulatory capacity of the vaccine [34]. It should be noted that the BCG vaccine used in the study was an inactivated γ-irradiated BCG vaccine. Considering that live BCG is detectable for up to 4 weeks at the challenge site [35], it is conceivable that the different immunomodulatory properties of the two vaccines depend on the bacterial persistence (prolonged for the live bacteria, reduced for the inactivated vaccine). In line with this, the capacity of live BCG to induce trained memory in mononuclear phagocytes might vary depending on variations during the production of the vaccine, as a very recent study found elevated memory induction in monocytes from slow growth rate BCG compared to BCG batches with normal growth rates [36].

3. Innate Immune Memory: Cells and Mechanisms Involved

A very interesting notion is that the innate memory is at least in part nonspecific, which implies that an improved defensive response can be obtained by prechallenging the host with (almost) any kind of agents. This concept breaks the current dogma that innate immunity is a stable and nonvariable type of response, always the same at every challenge, as opposed to acquired immunity that "learns" after the first encounter and generates more rapid and more efficient responses upon subsequent challenges due to the presence of memory cells.

The mechanisms underlying trained innate immunity have not been fully elucidated. Among the innate immune cells, the most active innate memory cells are monocytes/macrophages and NK cells. Both are cells with low turnover rates and thus more easily trainable compared, for instance, to terminally differentiated and short-lived neutrophils. In mice, memory NK cells mediate protection against viral infections in a T and B cell-independent manner, and memory properties apparently depend on a differential expression of the virus-specific LY49H receptor [37]. Moreover, hepatic $CXCR6^+$ NK cells of T and B cell-deficient mice develop nonspecific memory upon vaccination with structurally diverse antigens [38]. In humans, NK cell memory has been observed after cytomegalovirus infection [39]. Recently, it also has been demonstrated that cytokine combinations including IL-12, IL-15, and IL-18 can induce memory-like properties in human [40] and murine [41] NK cells.

It is very interesting that monocytes/macrophages are able to develop different kinds of memory depending on the type of priming. Thus, monocytes/macrophages can develop a memory that leads them to be less reactive to some challenges (tolerance, to avoid extensive tissue damage) or to an enhanced response (training, to improve tissue surveillance, e.g., against tumours). These different ways depend on the nature of the first challenge. Both mild and severe stimulations with LPS trigger a strong reaction but, upon a second challenge, macrophages react much less because they aim at avoiding an excessive reaction to a minor challenge and the consequent risk of unwanted tissue damage [42]. On the other hand, challenge with fungal components and ultralow LPS stimulation (implying a long-term slow infection with tissue debilitation) induces an innate memory that results in enhanced reactivity to subsequent stimuli, necessary for the adequate defense of a weakened tissue [43, 44]. One mechanism that has been identified as possibly underlying this trained memory is the epigenetic reprogramming of monocytes during their differentiation into macrophages, or during LPS tolerance and trained memory effects [44–46]. Some epigenetic markers have been identified that are associated with the acquisition of a trained or a tolerant phenotype, such as trimethylation of the histone 3 (H3) lysine at position 4 (H3K4me3) and acetylation of the H3 lysine at position 27 (H3K27ac) [17, 44]. Epigenetic reprogramming may be induced after infection and vaccination, and innate memory leading to enhanced reactivity can explain at least in part the BCG-induced nonspecific protective properties. H3K4me3 is associated with the trained memory-inducing effect of BGC vaccination in monocytes, an effect that involves the intracellular PRR NOD2 [17]. Moreover, the trained memory induced by BGC on human monocytes persists for at least 3 months after vaccination, with some of the protective effects lasting up to 1 year [33].

The question that arises from these observations is how can monocytes, which possess a relatively short half-life in circulation, be responsible for this long-term protection? A possible explanation is that a reservoir of epigenetically modified monocytes (memory monocytes) persists in the body, possibly located in the spleen, as hypothesized for NK cells. Alternatively, monocyte precursors could be "trained" directly in the bone marrow by the local microenvironment. The latter hypothesis is supported by a recent work that demonstrates how TLR2 stimulation of myeloid progenitor cells can influence the functional phenotype of the macrophages that develop from them [47]. Thus,

maintenance of epigenetic modifications can occur during myelopoiesis in the bone marrow, thereby having the potential to influence myeloid cell functions for longer periods.

Other interesting aspects of trained memory are changes in metabolic processes, as already observed in macrophage polarisation [48]. Recent evidence underlines the importance of metabolism in shaping the functional phenotype of macrophages in response to distinct polarising stimuli in the tissue microenvironment, under normal conditions, and in pathological settings [48–51]. Whereas different metabolic pathways are apparently involved, the glucose metabolism seems to play a major role in both polarised and memory macrophages. In response to inflammatory stimuli, macrophages display a metabolic shift towards an aerobic glycolytic pathway (with the transformation of pyruvate to lactate and the rapid energy production, similarly to anaerobic glycolysis), as opposed to the classical aerobic glycolysis (oxidative phosphorylation of pyruvate in mitochondria, with lower rates of energy production) that occurs in alternatively activated macrophages. Likewise, induction of monocyte trained memory by β-glucan requires a metabolic shift towards the high energy-producing type of aerobic glycolysis, which is referred to as the "Warburg effect" [52]. The switch to the Warburg type of glycolysis seems to depend on the activation of mTOR through the Dectin 1-AKT-HIF1α-dependent pathway [52].

In addition to epigenetic and metabolic reprogramming, other putative mechanisms involved in establishing monocyte memory include the involvement of different monocyte subpopulations (e.g., $CD14^{dim}CD16^{+}$, $CD14^{+}CD16^{-}$), a topic that has not yet been fully investigated [15]; an increased expression of PRRs on the cell membrane following BCG vaccination [17, 53]; and the role of soluble mediators, such as inflammatory cytokines. The latter mechanism is supported by the fact that peripheral inflammation can modulate immune response in the central nervous system despite the inability of microbial components (such as LPS) to pass the blood-brain barrier [54]. Moreover, plants possess the ability to develop SAR, "systemic acquired resistance" [16, 55], mediated by soluble factors. It is tempting to speculate that similar principles apply also to the innate memory of mammals.

The main mechanisms of trained memory are summarised in Figure 1. Whether all these mechanisms are concomitantly involved or which one is mainly responsible for shifting innate immune cells toward a memory-like phenotype is still a matter of investigation.

4. Improving Adjuvanted Vaccine Formulations by Exploiting the Concept of Innate Immune Memory

Nonspecific side effects of vaccines are a highly debated topic, as an increasing number of parents refuse to immunise their children, fearing side effects and unforeseeable long-term problems [56, 57]. This worrisome trend compromises herd immunity and can lead to serious disease outbreaks, which would not occur in the case of vaccination compliance. In Europe alone, more than 30,000 measles cases have been registered in 2013 [58]. As already pointed out, the nonspecific effects of vaccination are a fact. In most cases such effects increase and broaden protection, while only in some instances have they caused problems. The nonspecific effects of vaccination should be thoroughly investigated, in order to avoid the adverse consequences and optimise the beneficial effects of vaccines.

Thus, the development of future vaccines should take into account not only pathogen-specific immunity but also the nonspecific effects mediated by innate memory. Several issues should be considered on the contribution of innate immune memory to vaccine formulations:

(1) Adjuvants that are already in use and act *via* PRR signalling (e.g., TLRa) possibly hold the potential of inducing innate memory and could thereby mediate long-term changes in host defense. Particular attention should be paid to potential variability of reaction depending on sex, ethnicity, and age.

(2) Boosting innate defense mechanisms through trained memory induction seems particularly appealing for vulnerable populations that show impaired resistance to pathogens in general. However, boosted nonspecific immunity might also have beneficial outcomes on herd immunity in an average population against widespread diseases, such as the common cold.

(3) PAMPs that are able to robustly induce trained memory might also feature potential adjuvant capacity.

(4) Enhancing nonspecific effects induced by vaccination can affect the immune response to other routine immunisations, modulating the antibody titre and improving overall protective response, as seen for BCG vaccination [22].

(5) Sequence/timing and combination of vaccines against different pathogens are very important aspects of vaccination programmes. Importantly, detrimental nonspecific effects have been noted only when an inactivated vaccine was the most recent one [31]. Thus, changing the current vaccine policies with an improved schedule of vaccinations could be advantageous to avoid negative side effects of vaccines and fully exploit their potential benefits [32, 59].

(6) Induction of trained immune memory might improve the induction of specific protection by low-efficiency vaccines.

(7) Nonspecific effects of established vaccines have to be further investigated in order to determine their potential in long-term innate immune memory.

(8) The memory-inducing capacity of a vaccine might depend on various factors (e.g., the microorganism growth rate) during the vaccine production process.

(9) Well-known vaccines with beneficial nonspecific effects could be (re)introduced in countries where they are not part of the immunisation schedule.

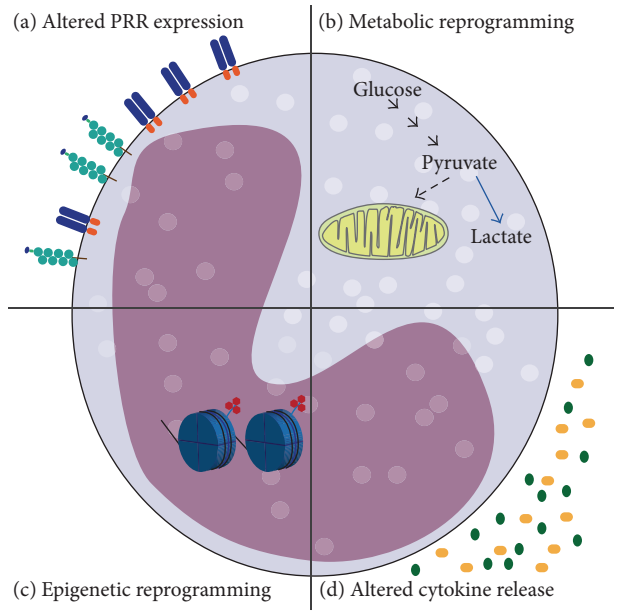

Figure 1: Main mechanisms involved in trained immune memory. In the picture the main mechanisms believed to underlie innate memory are shown. (a) *Altered PRR expression*. Phenotypic changes of innate immune cells with memory properties involve increased expression of PRRs on the cell surface and improved pathogen recognition. (b) *Metabolic reprogramming*. Innate immune memory requires a metabolic shift, which involves Warburg metabolism. The metabolism of glucose is shifted toward increased glycolysis with production of lactate and decreased oxidative phosphorylation. (c) *Epigenetic reprogramming*. Trimethylation of H3 at lysine 4 (H3K4me3) is a marker of promoter activation for proinflammatory genes specifically induced by β-glucan-dependent memory. (d) *Altered cytokines release*. Trained memory responses are characterised by an enhanced protective inflammatory reaction. The different patterns of cytokine release may be involved in the systemic establishment of a memory phenotype, reaching far/secluded anatomical sites (as suggested for brain responses and demonstrated in plants).

5. Concluding Remarks

The increased awareness of the properties of innate memory is changing our understanding of host defense and immunological memory and could lead to defining new classes of vaccines and adjuvants. Two major aspects have to be fully addressed, the in-depth identification of the molecular and cellular mechanisms involved and the duration of protection provided by innate memory, which is lifelong in plants and insects but not well evaluated in mammalian systems. Both epigenetic and metabolic reprogramming can be induced during establishment of innate memory. No information is however available on the possible cross-talk and cross-regulation between these events.

Several questions are still open, concerning the epigenetic memory upon infection or vaccination. Does an epigenetic inheritance during myeloid cell linage division exist? Can epigenetic reprogramming be maintained during cell differentiation or upon reinfection? How long lasting are the memory reprogramming effects? Future studies will shed light on these open questions.

A better understanding of innate memory mechanisms in general, and of those induced by licensed and candidate adjuvants and vaccines in particular, will help us to exploit in full the beneficial potential of vaccination and reduce all possible side effects.

Conflict of Interests

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

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Research Article

Possible Triggering Effect of Influenza Vaccination on Psoriasis

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Psoriasis is a chronic, recurrent, immune-mediated inflammatory disease and it can be provoked or exacerbated by a variety of different environmental factors, particularly infections and drugs. In addition, a possible association between vaccination and the new onset and/or exacerbation of psoriasis has been reported by a number of different authors. The aim of this study is to investigate the effects of influenza vaccination on patients with psoriasis. Here, we report the findings from 43 patients suffering from psoriasis (clinical phenotypes as mixed guttate/plaque lesions, palmoplantar or scalp psoriasis) whose diseases had been triggered after influenza vaccination applied in the 2009-2010 season. The short time intervals between vaccination and psoriasis flares in our patients and the lack of other possible triggers suggest that influenza vaccinations may have provocative effects on psoriasis. However, further large and controlled studies need to be carried out to confirm this relationship.

1. Introduction

Vaccination is a proven and well-established strategy for the prevention of infectious diseases in the general population and in patients with immune-mediated chronic inflammatory diseases. This group of patients has an increased risk of contracting complications of some vaccine-preventable infections due to the nature of the disease and immunomodulatory treatments [1]. However, concerns have emerged regarding the safety of vaccinations in immune-mediated inflammatory diseases following recent publications that highlight a stimulating effect of vaccination at the onset of inflammatory disease or during its course. Although a direct and causal relationship between vaccination and the flare of the disease has not been detected by any substantial research studies, these publications have given rise to a belief among some clinicians that vaccination may have a triggering effect [1–4].

Psoriasisis one of the world's most frequent chronic, recurrent, and inflammatory diseases, affecting around 2% of the population and is characterized by erythematous scaly plaques on the skin [5]. It is now recognized as an immune-mediated inflammatory disease [6]. Furthermore, the principle pathogenetic factor is defined as a T-cell mediated autoimmunity which is directed against poorly defined antigens [7]. On a genetic basis, various endogenous and exogenous triggering factors move the patient from a state of latency to clinical disease. Physical or chemical factors, infections, and various types of medications are the most important among these and they may affect the course of the psoriasis by many different mechanisms [8, 9]. However, induction or worsening of the psoriasis followed by some vaccinations has only rarely been reported in literature. Most of the available publications refer to case reports and a few observational studies [10–17]. Here, we report on the findings from 43 psoriasis patients whose diseases were triggered after influenza vaccination with no other detected possible provoking factors.

2. Materials and Methods

We collected cases that were on the onset of contracting psoriasis or where the disease had worsened, within 3 months following immunization with commercial influenza vaccines that were used in the 2009-2010 season. Trade names and the date of the applications were noted for all patients together with the historical and clinical features of the eruption. The diagnosis of psoriasis and its clinical phenotype classification were established for all patients after

clinical and histopathological evaluations by an experienced dermatologist. Patients were asked whether they had used any other drug and/or vaccination prior to their eruption. Also, various factors including focal infections that may have a triggering effect on their psoriasis were investigated. Routine hematological and biochemical analysis, urinalysis, HIV and VDRL tests, throat and urine cultures, Water's and thorax radiographs, and tooth examinations were performed. Patients were enrolled into our study group if no other trigger of psoriasis had been identified such as infection or intake of drugs.

3. Results and Discussion

Our observational clinical studyevaluated 43 psoriasis patients whose diseases had been triggered after receiving vaccinations for influenza. Patients had a history of using one of the two types of inactivated influenza vaccine trivalent types A and B (split virion). Among these, Vaxigrip Sanofi Pasteur, which contained three different strains of the influenza virus A/Brisbane/59/2007- (H1N1-) like strain, A/Brisbane/10/2007- (H3N2-) like strain, and B/Brisbane/60/2008-like strain with some adjuvants (e.g., ovalbumin, thimerosal, formaldehyde, and neomycin), was used in 34 of the patients (79.1% of the total sample); Fluarix GlaxoSmithKline Biologicals, which contained three different strains of the influenza virus A/Brisbane/59/2007, IVR-148 (H1N1), an A/Brisbane/10/2007-like virus A (H3N2), and B/Brisbane/60/2008 with some adjuvants (e.g., ovalbumin, formaldehyde, and gentamicin sulfate), were used in 9 of the patients (20.9% of the total sample).

Of the 43 patients (26 female, 17 male), 37 (86%) had mixed plaque type and guttate psoriasis, three of the patients (7%) suffered from palmoplantar psoriasis, and another three (7%) suffered from psoriasis on the scalp. There was an exacerbation of preexisting psoriasis after vaccination in 36 (83.7%) of them while it was the first induction of psoriasis in the remaining 7 (16.3%) patients. The latent period for the induction or exacerbation of psoriasis after vaccination was between 2 weeks and 2 months, but most patients contracted it within a period of 2 to 3 weeks. While 38 (88.4%) patients had a history of vaccination prior to their psoriasis without any other drug intake, the remaining five (11.6% of the sample) patients had a history of using some drugs but these were not identified as being responsible for the induction or exacerbation of psoriasis. The patients in our study group had no other vaccination experience in the past except the influenza vaccine and had no other triggering factors such as infections at the time they were enrolled into the study.

Although increased susceptibility to infection in patients with psoriasis remains a matter of debate, it should be emphasized that some special consideration should be given to vaccination strategies in psoriasis patients, especially in the current era of biological therapies [18]. Despite this, it was reported that the rates of vaccination among patients with psoriasis remain low [19, 20]. Recently, in the largest study conducted by Sbidian et al. to assess the coverage of 2009 monovalent H1N1 influenza vaccination, the overall influenza vaccine coverage was found to be 19% among 1308 French patients with psoriasis. In this study, the patients who had not received vaccination for the H1N1 influenza expressed the risk of vaccine-related adverse effects (54%), uncertainty about the vaccination efficacy (50%), and fear concerning the psoriasis flare triggering effect (17%) as major concerns [19].

Substantial advances have been achieved in understanding the genetics and pathomechanisms of psoriasis in recent years. It is considered to be a primarily Th1-type disease characterized by Th1 cytokines and a predominance of CD8+ cells in the epidermis and CD4+ cells in the dermis [5, 6]. Currently, it is recognized as one of the immune-mediated inflammatory diseases [5]. Increasingly, psoriasis is being understood as an autoimmune disease although no definitive autoantigen or immunogen has been identified responsible for the inflammation [6, 7]. Autoimmunity results from complex interactions between genetic predisposition and environmental factors and can be triggered by a number of stimuli, including local inflammation as well as viral, bacterial, and parasitic infections [3, 4]. Vaccinations may trigger autoimmunity by two mechanisms which are antigen specific or antigen nonspecific as in natural infections [1–4]. Because the vaccines also contain adjuvant materials (e.g., aluminum salts, thiomersal, squalene, sorbitol, albumin, neomycin, and gentamycin), we cannot identify, with certainty, the responsible agent for the autoimmune phenomena either the infectious component of the vaccine or the adjuvant [2, 3, 21]. Most of the reported psoriasis exacerbations in the literature were related with influenza vaccines with adjuvants, although only one patient with guttate psoriasis described an exacerbation after 2009 monovalent H1N1 vaccine infusion without adjuvant [17].

Over the years numerous reports have raised the suspicion of the safety of vaccines in autoimmunity and in persons already diagnosed with autoimmune conditions. Because of the popularity and the widespread use of influenza vaccine, its effects have been examined in many autoimmune conditions [2]. Influenza vaccines contain formol-inactivated, purified influenza virus antigens, with or without adjuvants. The vaccine strain composition is reconsidered each year by the WHO and the European Union. Although each vaccine can have a unique potential to provoke immune-mediated problems, trials comparing the adverse effects of the two types of influenza vaccines (split virion and surface antigen) did not find any significant difference [22]. Although H1N1 vaccines and other seasonal vaccines are generally safe and effective, many serious and nonserious vaccine-related adverse events have been reported [20–23]. The vaccination against influenza has been associated with several autoimmune adverse events including Guillain-Barre syndrome, microscopic polyangiitis, rheumatoid vasculitis, thrombotic thrombocytopenic purpura, and increased antiphospholipid antibodies [3, 24]. Occasionally, some cutaneous adverse effects can also occur at the site of injection or at a distant area as local or generalized reactions. Local reactions including transient redness, induration, edema, pain, and ecchymoses are not rare and are usually related to a nonspecific stimulation of host immune system [22]. Additionally, there have been many reports displaying various cutaneous side effects

including a wide range of dysimmune reactions and autoimmune phenomena as well as full-blown autoimmune diseases such as Stevens-Johnson syndrome, urticaria, eczematoid lesions, pityriasis rosea, pemphigus, bullous pemphigoid, papular acrodermatitis, erythromelalgia, and vasculitis following influenza vaccinations [24]. However, there are still only a few reports (one case report and one observational clinical study) in the literature describing the new onset and/or exacerbation of psoriasis following influenza vaccination [16, 17]. Shin et al. recently described a 26-year-old woman with multiple erythematous scaly macules scattered on the extremities and trunk compatible with psoriasis, who had been injected with an inactivated split-virus influenza A/H1N1 vaccine without adjuvant (Green Flu-S, Green Corp.) [16]. So far, only one available clinical study has been carried out into the triggering effect of influenza vaccination on psoriasis. In that study, Sbidian et al. evaluated psoriasis patients who had been vaccinated against influenza and had exhibited induction or exacerbation of psoriasis. They sent a declaration request questionnaire once by e-mail to nearly 3,000 French dermatologists through the institutional channels to request reporting any case who describes psoriasis onset or flare after H1N1/seasonal vaccination. They received feedback from 6 dermatologists concerning 10 patients presenting with a psoriasis of new onset ($n = 7$) or with a worsening of previously diagnosed psoriasis ($n = 3$) within 3 months following the 2009 monovalent H1N1/seasonal vaccination. Among them, six patients with the first episode of psoriasis had exhibited the guttate/plaque mixed clinical phenotype, while the remaining patient had displayed a plaque type with a median onset of 8 days after vaccination (range 6–74 days). Even though data for these patients is available, the date could not provide a reliable estimation of the rate of vaccination-related reaction because of the uncertainties concerning the actual number of psoriatic patients undergoing vaccination, an underestimation of the incidence due to underreporting, and underdiagnosis. Consequently, the authors claim that even if it is not a very strong effect, influenza vaccination is associated with psoriasis flare [17].

Our study has a number of limitations including the lack of a control group and follow-up evaluations which could prove causal correlation between vaccine and clinical manifestation. Despite these potential limitations, our observations may partially support the apparent association between influenza vaccination and the development of psoriasis. The fact that no other provoking factors were found in our patients promotes this relationship.

Although staphylococcal, streptococcal, measles, and varicella vaccines have also been applied previously in the treatment of psoriasis, an early report described two cases of psoriasis induced by BCG vaccination and influenza vaccination under the name of "psoriasis vaccinalis" [10, 20]. Subsequently, extremely rare cases of psoriasis, psoriasis-like guttate eruptions, and psoriatic arthropathy have been reported following BCG vaccination and a case of psoriasis was reported, as triggered by the tetanus-diphtheria vaccination [11–14]. Additionally, a case-control study reported rubella vaccination as a risk factor for psoriatic arthropathy [15]. The etiological relationship between psoriasis and the vaccination remains uncertain. It was claimed that the vaccination may trigger an exacerbation of psoriatic skin lesions itself; however, these views also concur with the well-known Koebner phenomenon that occurs in psoriasis, that is, the development of new plaques at the locations of skin injury [1]. Researchers also indicated that the BCG and tetanus-diphtheria vaccines, due to the mycobacterial heat shock proteins in the case of the BCG and diphtheria toxoid in the case of the tetanus-diphtheria vaccines, induce IL-6 production, which, in turn, promotes the development of Th17 cells [12–14]. Previous studies had also demonstrated a significant increase in IL-6 values after the administration of the influenza vaccination [25, 26]. In the study of the influenza vaccine in a murine model, the cytokine profile demonstrated a robust cellular immune response with enhanced Th1 and Th17 immunity that provided balanced immunity against both intracellular and extracellular forms of the virus [27]. Consistent with these findings, it could be speculated that an immunological reaction to the influenza vaccination may mediate the IL-6 production and generation of IL-22-producing Th17 cells which are a key player in the development of characteristic epidermal changes of psoriasis [9, 16, 17]. These mechanisms are also probably responsible for the activation of the immune system and provocation of psoriasis in our cases. However, positive rechallenges may provide some support for a causal relationship between a vaccine and an adverse event and represent a reasonable basis for further assessment [17].

4. Conclusion

Consequently, our data suggests that the H1N1 influenza vaccines, which were used in the 2009-2010 season, have the potential to trigger development of psoriasis. Therefore, it is important not only to know the protective potential of influenza vaccines but also to understand their potential to provoke psoriasis.

However, as previously suggested, although the administration of the influenza vaccine has been associated with psoriasis in some patients, their very low incidence and mild clinical course, combined with the general lack of high level of evidence, do not warrant an abandonment of the immunization practice considering the favorable cost-effectiveness ratio of the vaccine use. Therefore, we recommend the follow-up of such individuals and suggest further large-sized, controlled, and well-constructed clinical research studies going forward, which may confirm this relationship.

Conflict of Interests

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

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Research Article

Differential Immune Response against Recombinant *Leishmania donovani* Peroxidoxin 1 and Peroxidoxin 2 Proteins in BALB/c Mice

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We assessed the immune response against recombinant proteins of two related, albeit functionally different, peroxidoxins from *Leishmania donovani*: peroxidoxin 1 (LdPxn1) and peroxidoxin 2 (LdPxn2) in BALB/c mice. We also evaluated the effect of coadministration of TLR agonists (CpG ODN and GLA-SE) on the antigen-specific immune response. Immunization with recombinant LdPxn1 alone induced a predominantly Th2 type immune response that is associated with the production of high level of IgG1 and no IgG2a isotype while rLdPxn2 resulted in a mixed Th1/Th2 response characterized by the production of antigen-specific IgG2a in addition to IgG1 isotype. Antigen-stimulated spleen cells from mice that were immunized with rLdPxn1 produced low level of IL-10 and IL-4 and no IFN-γ, whereas cells from mice immunized with rLdPxn2 secreted high level of IFN-γ, low IL-4, and no IL-10. Coadministration of CpG ODN or GLA-SE with rLdPxn1 skewed the immune response towards a Th 1 type as indicated by robust production of IgG2a isotype. Furthermore, the presence of TLR agonists together with rLdPxn1 antigen enhanced the production of IFN-γ and to a lesser extent of IL-10. TLR agonists also enhanced a more polarized Th 1 type immune response against rLdPxn2.

1. Introduction

Infection by parasites of the genus *Leishmania* results in a chronic disease known as leishmaniasis. It is transmitted when an infected female phlebotomine sandfly injects the metacyclic promastigotes into the host during a blood meal. The flagellated promastigotes are taken by macrophages where they transform into aflagellated amastigotes that multiply and disseminate the infection [1]. The outcome of *Leishmania* infection depends on the species of *Leishmania* as well as the host immune response. Clinical manifestation of leishmaniasis ranges from self-healing cutaneous form to fatal visceral disease [2]. The disease is prevalent worldwide infecting millions of people in more than 90 countries in the tropics, subtropics, and southern Europe (Center for Disease Control and Prevention, http://www.cdc.gov/parasites/leishmaniasis/). About 1.3 million new cases and about 30000 deaths are recorded each year with the majority of these cases occurring in poor regions of the world (World Health Organization, http://www.who.int/mediacentre/factsheets/fs375/en/, [3]) where the afflicted populations have low accessibility to health care. Chemotherapy is available but its usefulness is compromised by toxicity of some drugs and drug resistance by the parasite [4]. In addition, the emergence of *Leishmania*/HIV coinfection compounded the problem. Concomitant infection with HIV increases the cases of active VL in otherwise asymptomatic individuals by 100 to 1000 times and it increases the likelihood of drug toxicity as well as relapse of the disease [5].

Experimental studies have shown that protection against leishmaniasis is mediated by T helper 1 (Th1) type $CD4^+$ cells that produce a high level of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) whereas progression of the disease is associated with Th2 type $CD4^+$ cells which produce IL-4, IL-5, IL-10, and IL-13 [6–9]. The Th1 and Th2 cells

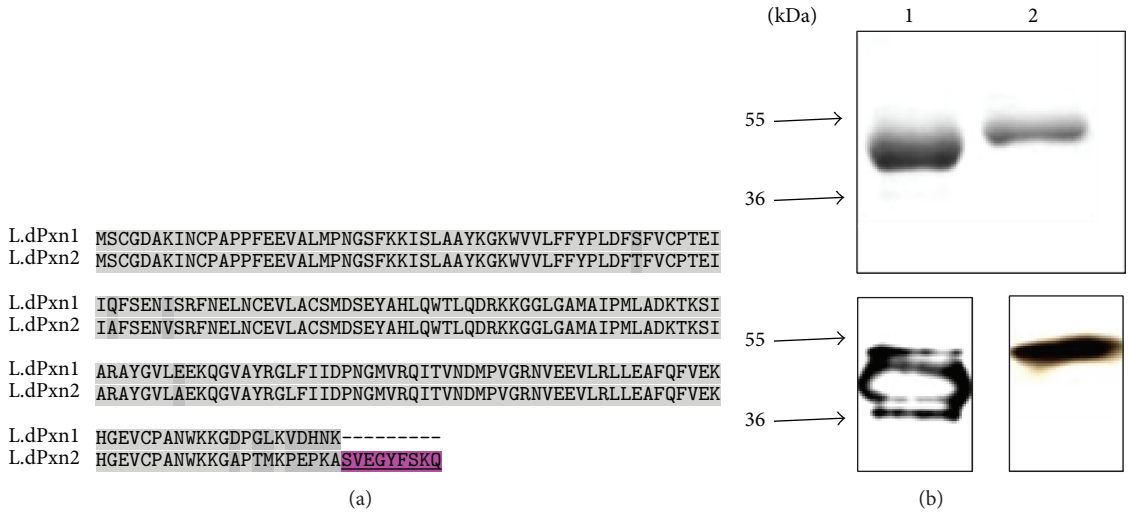

Figure 1: (a) Sequence comparison of *Leishmania donovani* Pxn1 and Pxn2. Alignment of amino acid sequence depicts the high homology between LdPxn1 and LdPxn2. Highlighted areas show positions of mismatch. LdPxn2 possesses extra 9 amino acids at the carboxy terminus (underlined) that are missing from LdPxn1. (b) SDS-PAGE and western blot of rLdPxn1 and rLdPxn2 proteins. One microgram per lane of rLdPxn1 (lane 1) and rLdPxn2 (lane 2) was separated on a 12% SDS-PAGE and stained with Coomassie blue, top. The separated samples were transferred to Hybond-P membrane and were probed with pooled sera from mice immunized with the respective recombinant protein, bottom. Molecular weight in kDa is shown on the left.

have differential capabilities in stimulating B cells to secrete different antibody isotypes where Th1 type cells elicit IgG2a antibody production and Th2 type cells induce IgG1 antibody secretion [10]. This differential effect is brought about by the regulatory effect of cytokines on the immunoglobulin isotype switching. *In vitro* studies have shown that IL-4 and IFN-γ stimulate the production of IgG1 and IgG2a, respectively [11, 12].

Leishmania parasites are highly successful in parasitizing macrophage cells which are otherwise hostile to pathogens. Generally, uptake of pathogenic organisms by macrophages results in oxidative burst which is associated with the production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH^-) and reactive nitrogen species (RNS) including nitric oxide (NO). These reactive species are highly destructive to the infecting pathogen and they can interact with each other forming more potent oxidants such as peroxynitrite ($ONOO^-$) [13].

One of the evasive mechanisms used by *Leishmania* parasites to bypass the microbicidal effect of free radicals produced by macrophages is the expression of antioxidant enzymes known as peroxidoxins. These enzymes are conserved and highly abundant proteins in almost all living organisms which suggest essential function in oxidative homeostasis. It has been shown that peroxidoxins from different organisms including *Leishmania* are important in the protection of these organisms against oxidative stress [14–16]. We isolated and characterized three peroxidoxins as part of a multigene family from *L. donovani* complex: Pxn1, Pxn2, and Pxn3 [14, 17]. Both Pxn1 and Pxn2 are cytosolic whereas Pxn3 is predicted to be glycosomal. A fourth mitochondrial peroxidoxin, Pxn4, has also been identified in *L. donovani* [18]. In addition to the common localization of Pxn1 and Pxn2 in the cytoplasm, the two proteins have 89.4% homology. The difference between these two proteins is brought about by an extra 9 amino acids at the carboxy terminus of Pxn2 plus few nucleotide mismatches along the entire sequence [14, 17] (Figure 1(a)). Despite the high similarity between LdPxn1 and LdPxn2 at the amino acid level, there are striking differences between the proteins encoded by the two genes. Unlike LdPxn1, which is upregulated during the amastigote stage, LdPxn2 is expressed at high levels during the promastigote stage and the expression declines towards the amastigote stage. In addition, while recombinant LdPxn1 protein has been shown to detoxify various free radicals including ROS and RNS, LdPxn2 can only detoxify H_2O_2 [14].

In this study, we assessed the immune responses against LdPxn1 and LdPxn2 as recombinant GST-fusion proteins in BALB/c mice to test if the differences observed in gene expression and functionality between these two antigens are paralleled by different immune response profile. In addition, we evaluated the immune response against these proteins in the presence of two Th1 adjuvants: bacterial CpG oligodeoxynucleotide (CpG ODN) and glucopyranosyl lipid A in a stable emulsion (GLA-SE), which are Toll-like receptor 9 (TLR-9) and TLR-4 agonists, respectively. Our results indicate that mice immunization with LdPxn1 induces a predominant Th2 type response, whereas immunization with LdPxn2 stimulates a mixed Th1/Th2 response. Our data also show that repeated injections with coadministration of Th1-adjuvants enhanced the immune response against LdPxn1 and LdPxn2 which is more biased towards Th1 type.

2. Materials and Methods

2.1. Mice. Female BALB/c mice (4–6 weeks old) were purchased from Charles River Laboratories (QC, Canada) and

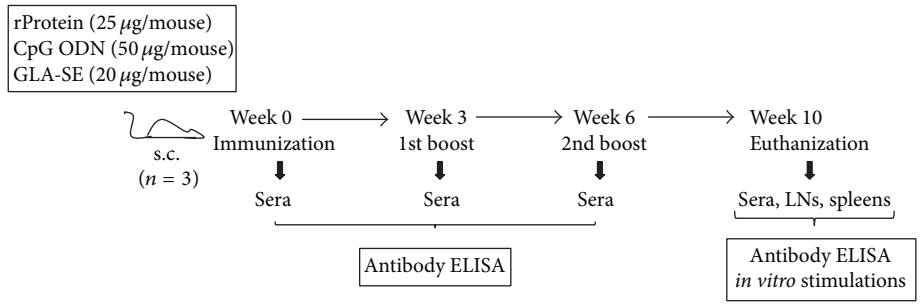

Figure 2: Schematic representation of the immunization protocol. Six- to 8-week-old female BALB/c mice were immunized s.c. in the hind foot pad with recombinant LdPxn1 or LdPxn2 protein (25 ug/mouse) with or without CpG ODN (50 ug/mouse) or GLA-SE (20 ug/mouse) adjuvants. Mice were boosted twice in 3 weeks interval. Sera were collected at each time of injection. Four weeks after the last boost mice were euthanized and sera, lymph nodes, and spleens were collected. Samples were used for antibody and cytokine analysis using standard protocols.

were housed in a specific pathogen-free facility at the University of Calgary and provided water and food *ad libitum*. Mice were acclimatized for one week and randomly distributed into experimental groups and controls. Animal protocols were approved by the Life and Environmental Sciences Animal Care Committee (LESACC) of the University of Calgary, Alberta, Canada.

2.2. Cloning, Expression, and Purification of Recombinant LdPxn1 and LdPxn2 in E. coli. Cloning of LdPxn1 and LdPxn2 as GST-fusion proteins was performed by using the prokaryotic expression vector, pGEX-2T (Amersham Pharmacia Biotech) following the procedure described previously [17]. Briefly, the coding regions of LdPxn1 and LdPxn2 were amplified by PCR using specific primers. The amplified fragments were then cloned into pGEX-2T vector. To express the recombinant proteins, transformed *E. coli* BL21 (DE3) cells were grown in a 37°C shaker overnight in Luria-Bertani (LB) broth in the presence of 100 μg/mL ampicillin. The cultures were induced with 0.2 mM isopropyl beta-D-thiogalactoside (IPTG) and continued to grow for 3–6 hours. Fusion proteins, GST-LdPxn1 and GST-LdPxn2, were harvested by sonication and passing over a glutathione-agarose resin column (Sigma) as described by Smith and Johnson [19]. Endotoxins were removed using Detoxi-Gel Affinity Pak prepacked columns following the manufacture's instruction (Pierce Biotechnology, USA). Endotoxin level of protein samples was measured at the Infectious Disease Research Institute (Seattle, USA) using Limulus Amebocyte Lysate (LAL) assay. Samples of endotoxin levels <10 EU/mg protein were used.

2.3. Immunization. Immunization protocol is schematically represented in Figure 2. Mice were randomly divided into groups of three and were immunized subcutaneously (s.c.) with recombinant LdPxn1, rLdPxn1 plus CpG ODN, rLdPxn1 plus GLA-SE, rLdPxn2, rLdPxn2 plus CpG ODN, and rLdPxn2 plus GLA-SE. Recombinant proteins, CpG ODN 1826 (Coley Pharmaceutical Group, Canada) and GLA-SE (Infectious Disease Research Institute, Seattle, USA), were given at 25, 50, and 20 μg/mouse, respectively. Two booster injections were given in three-week interval. Sera were isolated from blood (collected every three weeks starting from the time of first immunization (week 0) until the time of euthanization) and stored at −20°C. Mice were euthanized four weeks after the last boost, and lymph node and spleens were aseptically harvested and processed for the isolation of single cell suspensions. The isolated lymph node and spleen cells were used for *in vitro* antigen stimulation experiments.

2.4. Western Blotting. For western blotting, 1 μg of each of the recombinant proteins was separated by SDS-PAGE and transferred to Hybond-P membrane (GE Healthcare, QC, Canada). The membrane was blocked with 5% skim milk dissolved in phosphate buffered saline (PBS) containing 0.05% tween-20 (PBS-T) for 2 hr at room temperature. Then, it was incubated overnight at 4°C with mice serum that was collected four weeks after the last immunization with the respective antigen. After washing three times with PBS-T, the membrane was incubated with a horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, QC, Canada) for 45 min at room temperature (RT) followed by three washing steps. Immunoreactivity was detected by chemiluminescence using ECL reagents following the manufacturer's instructions (GE Healthcare, QC, Canada).

2.5. Antibody Measurement. The presence of antibody specific to LdPxn1 and LdPxn2 in serum samples was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Sarstedt, USA) were coated overnight at 4°C with 1 μg/mL recombinant protein in bicarbonate buffer, pH 9.6. The plates were blocked with 5% (w/v) skim milk in PBS-T for 1 hr at RT. After three washes with PBS-T, 100 μl/well of sera diluted 1 : 100 in blocking buffer was added to the plates and incubated for 1 hr at RT. After washing, 100 μl/well of biotinylated goat anti-mouse IgG1 or IgG2a antibody was added to the wells and incubated for 1 hr at RT followed by 1 hr incubation with streptavidin-HRP. The reaction was then developed by adding 100 μl/well TMB (3,3′,5,5′-tetramethylbenzidine) substrate (BD Biosciences, ON, Canada). After the reaction was stopped by adding

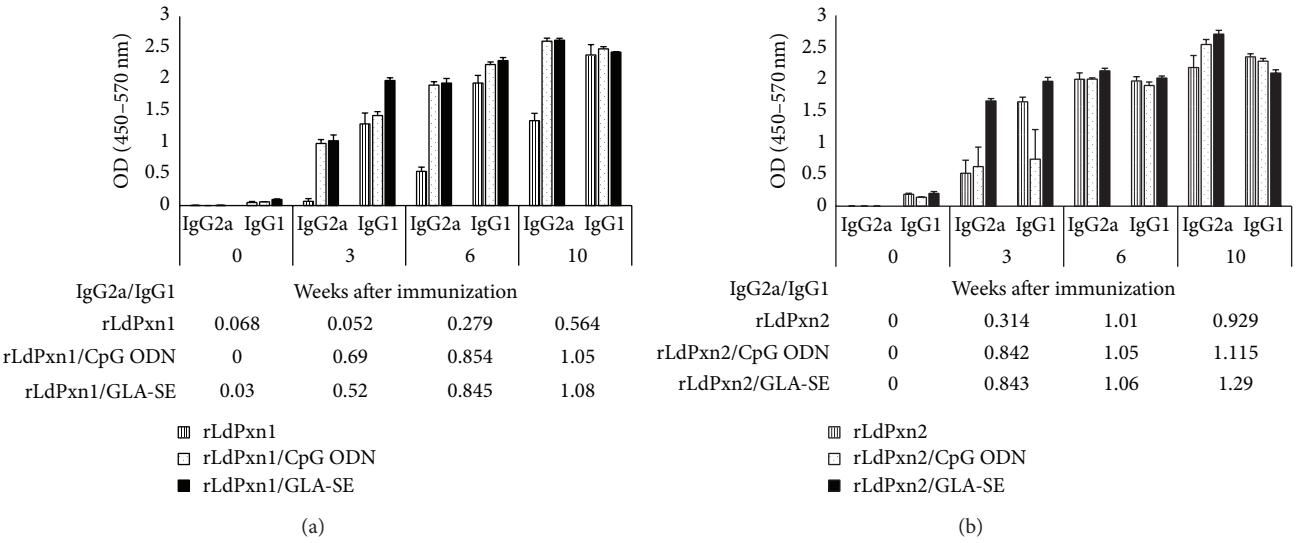

Figure 3: Anti-LdPxn1 and -LdPxn2 antibodies in immunized mice. Mice were immunized subcutaneously with recombinant LdPxn1 or LdPxn2 proteins with or without CpG ODN or GLA-SE. Mice were boosted twice in 3-week intervals. The levels of IgG1 and IgG2a isotypes were measured on sera collected at different time points using ELISA. Data are presented as the mean OD ± S.E.M. of IgG1 and IgG2a of sera from mice immunized with rLdPxn1 (a) and rLdPxn2 (b). The IgG2a/IgG1 ratios are shown in tables below each figure.

50 μl/well 1 N H_2SO_4, the plates were read at 450 nm in a microplate reader (Molecular Devices, USA).

2.6. In Vitro Antigen Stimulation and Cytokine Measurement. Mice were euthanized 4 weeks after the last immunization and lymph node and spleen cells were isolated as described previously [20]. Cells from lymph nodes of mice from the same group were pooled before *in vitro* stimulation. For stimulation assays, cells from individual spleens or from pooled lymph nodes were dispensed at 2×10^5 cell/100 μL media/well in 96-well flat bottomed tissue culture plates (Sarstedt, USA) and incubated with 2 or 10 μg/mL of recombinant protein in complete medium (RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, penicillin (100 U/ml) plus streptomycin (100 lg/mL), and 50 μM β-Mercaptoethanol) for 72 hr at 37°C in a humidified incubator with 5% CO_2. Cells were also incubated with 5 μg/ml concanavalin A (ConA) or with medium alone as positive and negative control, respectively. Culture supernatants were collected and cytokine production was measured using cytokine ELISA kits as per the manufacturer's instructions (BD Bioscience, ON, Canada) as described previously [20]. The amount of IFN-γ and IL-10 produced by lymph node or spleen cells was expressed as ng/ml. In addition, the production of IL-4 was measured in spleen cells and was expressed as pg/mL.

2.7. Statistics. Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student's *t*-test. *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Recognition of LdPxn1 and LdPxn2 by Immune Sera. To demonstrate the immunoreactivity of recombinant LdPxn1 and LdPxn2 in BALB/c mice, we tested the interaction between sera collected from immunized mice and the respective recombinant protein by western blot analysis. As depicted in Figure 1(b), mice immune sera bound to the respective recombinant protein immobilized onto the membranes as indicated by the prominent bands of the expected molecular size of the GST-fused proteins. This indicates that both proteins are immunogenic in BALB/c mice.

3.2. Comparative Analysis of Humoral Immune Response to Recombinant LdPxn1 and LdPxn2 Proteins. To analyze the isotype profile of antibody response in mice immunized with rLdPxn1 or rLdPxn2, we measured antigen specific IgG1 and IgG2a isotypes in sera collected at different time points after immunization. In addition, we calculated the ratio of IgG2a to IgG1 as a surrogate marker for Th1 type immune response.

As shown in Figure 3(a), immunization of mice with rLdPxn1 by itself stimulated a high level of IgG1 isotype and barely detectable amount of IgG2a at 3 weeks after the first immunization. The amount of specific IgG2a stimulated in this group increased upon booster immunization; however it remains significantly lower than the amount of IgG1 ($P < 0.05$). Concomitant injection of CpG ODN or GLA-SE with rLdPxn1 triggered a high level of IgG1 and more importantly a high level of IgG2a as well (Figure 3(a)). Similar to immunization with rLdPxn1 alone, the production of anti-rLdPxn1 antibodies in mice immunized with rLdPxn1 plus adjuvants was augmented by booster immunization (Figure 3(a)). The augmentation effect of booster injections together with

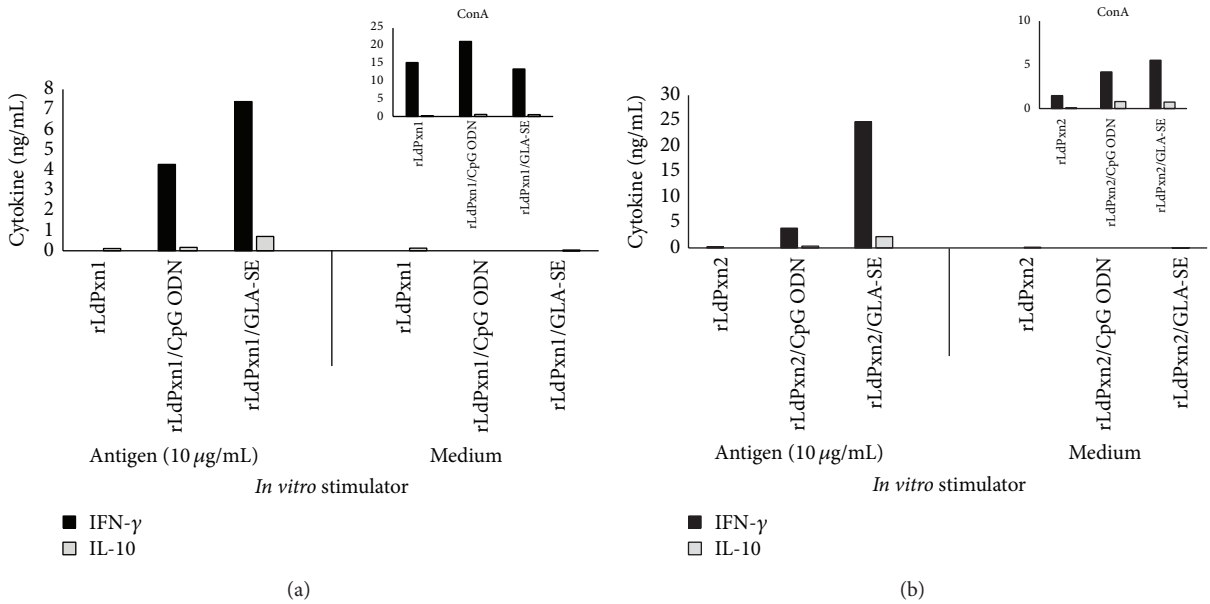

Figure 4: Cytokine responses in lymph node cells of rLdPxn1 and rLdPxn2 immunized mice. Mice were immunized subcutaneously three times at three-week intervals with rLdPxn1 or rLdPxn2 with or without CpG ODN or GLA-SE. Four weeks after the last immunization, cells from pooled lymph nodes were stimulated *in vitro* with the respective antigen (10 μg/mL) or ConA (5 μg/mL). The release of IFN-γ and IL-10 in mice immunized with rLdPxn1 or rLdPxn2 was measured in supernatants after 72 hr of *in vitro* stimulation at 37°C. Results are presented as the amount of IFN-γ (ng/mL) and IL-10 (ng/mL) for rLdPxn1 (a) and rLdPxn2 (b).

the presence of TLR agonists in the immunization protocol resulted in the induction of IgG2a level as high as IgG1 four weeks after the last boost.

In contrast to rLdPxn1, rLdPxn2 alone was able to induce specific IgG2a production, in addition to IgG1 isotype, as early as 3 weeks after the first immunization (Figure 3(b)). The level of both isotypes was enhanced by booster immunizations in this group and the level of IgG2a was comparable to the level of IgG1 after the second injection, that is, the first boost (Figure 3(b)). Coadministration of CpG ODN or GLA-SE adjuvants with rLdPxn2 resulted in the production of high and comparable levels of both IgG2a and IgG1 isotypes as early as 3 weeks after the first boost (week 6) (Figure 3(b)). Coadministration of CpG ODN with rLdpxn2 induced lower antibody response as compared to GLA-SE after the first injection. However, the level of both isotypes in all groups receiving rLdPxn2 was comparable after the second booster immunization. At this time point, the level of IgG2a was slightly less or slightly more than IgG1 in the group immunized with rLdPxn2 alone or rLdPxn2 plus adjuvant, respectively.

These results indicate that, in BALB/c mice, priming with recombinant LdPxn1 induces a predominantly Th2 response (IgG2a/IgG1 ratio of 0.052) whereas priming with recombinant LdPxn2 stimulates a mixed Th1/Th2 response (IgG2a/IgG1 ratio of 0.314). The data also reveal that, four weeks after the second boost, the ratio of IgG2a/IgG1 increased to 0.564 and 0.929 for rLdPxn1 and rLdPxn2, respectively. These results suggest that booster immunization can enhance the immune response against rLdPxn1 and rLdPxn2. Our findings also show that CpG ODN and GLA-SE adjuvants have the capacity to skew the immune response against rLdPxn1 and rLdPxn2 toward a more Th1 type (IgG2a/IgG1 ratio > 1.0 after the last boost).

3.3. Antigen Specific Cellular Immune Response. To understand the type of cell-mediated immune response (CMI) against rLdPxn1 and rLdPxn2, we measured the level of IFN-γ and IL-10 in antigen-stimulated lymph node cells and the level of IFN-γ, IL-10, and IL-4 in the spleen cells of immunized mice. Lymph node cells from mice in each group were pooled and stimulated *in vitro* with 10 μg/mL of recombinant proteins whereas spleen cells from individual mouse were stimulated with 2 or 10 μg/mL of recombinant proteins. No stimulation or stimulation with 5 μg/mL Con A was added as negative and positive controls, respectively. Culture supernatants were collected 72 hr later and the amount of cytokines was determined by ELISA.

There was no spontaneous release of cytokines by unstimulated lymph node cells in any of the groups (Figures 4(a) and 4(b)). No detectable cytokine was released by lymph node cells from mice immunized with the recombinant proteins alone (Figures 4(a) and 4(b)). However, immunization of mice with the recombinant proteins in the presence of TLR agonists resulted in the production of a high level of IFN-γ and a low level of IL-10 (Figures 4(a) and 4(b)). While coadministration of CpG ODN triggered the production of similar amounts of IFN-γ in mice immunized with rLdPxn1 or rLdPxn2, GLA-SE stimulated the release of more IFN-γ in the group receiving rLdPxn2. The amount of IFN-γ produced by lymph node cells of mice from this group

Table 1: The ratio of IFN-γ/IL-10 in lymph node cells of immunized mice.

	Antigen (10 μg/mL)			Medium			ConA		
	Antigen	+CpG ODN	+GLA-SE	Antigen	+CpG ODN	+GLA-SE	Antigen	+CpG ODN	+GLA-SE
rLdPxn1	0	24.74	10.42	0	0	0	75.35	36.59	24.6
rLdPxn2	0	10.83	11.11	0	0	0	19.42	5.19	7.38

(Figure 4(b)) was more than 3-fold higher than the level produced by lymph node cells from mice immunized with rLdPxn1 alone (Figure 4(a)). Interestingly, CpG ODN and GLA-SE triggered the production of lower amount of IL-10 in mice receiving rLdPxn1 as compared to rLdPxn2 (Figures 4(a) and 4(b)). Lymph node cells stimulated with ConA mitogen produced comparable levels of cytokines (Figures 4(a) and 4(b) in sets) with the exception of the group immunized with rLdPxn2 alone which produces lower level of cytokines (Figure 4(b) in set). The results of cytokine analyses in lymph node cells demonstrate that rLdPxn1 and rLdPxn2 can stimulate lymph node cells of immunized mice to produce cytokines only in the presence of adjuvants. The results also show that CpG ODN and GLA-SE adjuvants favor a Th1 type response against the two antigens as indicated by the high IFN-γ/IL-10 ratios (Table 1). Moreover, the results show that while coadministration of GLA-SE exerts comparable effect on both antigens as indicated by comparable ratios of IFN-γ/IL-10 (Table 1), CpG ODN induces stronger Th1 in mice receiving rLdPxn1 as compared to rLdPxn2 (IFN-γ/IL-10 ratio of 24.74 and 10.83 for rLdPxn1 and rLdPxn2, resp.) (Table 1).

Production of IFN-γ, IL-10, and IL-4 by spleen cells of immunized mice is depicted in Figure 5.

Spleen cells from mice immunized with rLdPxn1 by itself did not produce any detectable level of IFN-γ and very low level of IL-10 upon i*n vitro* stimulation with 2 or 10 μg/ml rLdPxn1 (Figure 5(a)). In contrast, immunization with rLdPxn2 alone was able to stimulate mice spleen cells to produce considerable amount of IFN-γ but low IL-10 in *in vitro* recall experiments with 2 and 10 ug/ml rLdPxn2 (Figure 5(b)). Both rLdPxn1 and rLdPxn2 stimulated the production of a low level of IL-4 in spleen cells of immunized mice when stimulated *in vitro* with 2 μg/mL of the respective protein. However, IL-4 production was only detected in spleen cells from the group receiving rLdPxn2 upon stimulation with 10 μg/mL (Figure 5(c)).

Administration of rLdPxn1 in the presence of CpG ODN results in the production of a low level of IFN-γ and almost no IL-10 when the spleen cells were stimulated with 2 μg/ml of the antigen (Figure 5(a)). At this concentration, stimulated spleen cells from mice receiving rLdPxn1 plus GLA-SE produced low but comparable levels of IFN-γ and IL-10. The level of IFN-γ and IL-10 produced by spleen cells from mice that received rLdPxn1 plus the adjuvants was dose-dependent with the production of higher levels of each cytokine upon stimulation with 10 μg/mL of the recombinant protein (Figure 5(a)).

In vitro stimulation of spleen cells from mice immunized with rLdPxn2 plus CpG ODN or GLA-SE with 2 and 10 μg/ml produced a high level of IFN-γ and a low IL-10 (Figure 4(b)). Interestingly, as shown in Figure 5(c), the presence of the TLR agonists in the immunization protocol induced the production of a higher level of IL-4 by the spleen cells from mice immunized with rLdPxn2 as compared to low or none from those immunized with rLdPxn1. Spontaneous release of a low level of IL-10 by spleen cells from mice receiving rLdPxn1 by itself or with CpG ODN was observed (Figure 5(a)) as well as a low level of IL-4 by spleen cells from mice immunized with rLdPxn2 alone or plus CpG ODN (Figure 5(c)).

These results show that rLdPxn1 alone stimulates a weak cell-mediated immunity in the spleens of immunized mice as indicated by the low level or absence of detectable cytokines in *in vitro* recall experiments. Administration of rLdPxn1 in the presence of CpG ODN or GLA-SE increased the immune response with higher IFN-γ/IL-10 ratio (3.78 and 2.03 for rLdPxn1-CPG ODN and rLdPxn1 GLA-SE, resp.) (Table 2) and low IL-4 (Figure 5(c)). On the other hand, immunization of mice with rLdPxn2, in the presence or absence of adjuvants, results in a mixed Th1/Th2 type response in spleen cells of immunized mice associated with high IFN-γ/IL-10 ratio (12.82, 10.88, and 7.55 for rLdPxn2, rLdPxn2 CpG ODN, and rLdPxn2 GLA-SE, resp.) (Table 2) and high level of IL-4 (Figure 5(c)). This observation indicates that, independently of the adjuvant use, rLdPxn2 is capable of inducing a mixed Th1/Th2 response biased toward a Th1 type.

4. Discussion

In this study, we report differential immune responses against two cytosolic *Leishmania donovani* peroxidoxins: LdPxn1 and LdPxn2. These two antioxidants are highly homologous, yet they are differentially expressed. The expression of LdPxn1 is upregulated during the mammalian amastigote stage whereas LdPxn2 is highly abundant in the promastigote stage [17]. In addition, LdPxn1 and LdPxn2 are functionally different; LdPxn1 has been found to detoxify a wide range of reactive species (ROS and RNS) while LdPxn2 can only neutralize H_2O_2 [14]. The main focus of this work was to examine and compare the humoral and cellular immune responses against recombinant LdPxn1 and LdPxn2 GST-fusion proteins in BALB/c mice and to investigate the potential of two TLR agonists as adjuvants that can be used with these recombinant proteins.

Our findings show that recombinant LdPxn1 protein induces a predominant Th2 type immune response in mice, whereas rLdPxn2 stimulates a mixed Th1/Th2 response biased toward a Th1 type. Our data also demonstrate that coadministration of CpG ODN and GLA-SE favors the stimulation of a polarized Th1 type response with increased ratios of IgG2a/IgG1 and IFN-γ/IL-10. This finding is not

Table 2: The ratio of IFN-γ/IL-10 in spleen cells of immunized mice.

	Antigen ($2\,\mu$g/mL)			Antigen ($10\,\mu$g/mL)			Medium			ConA		
	Antigen	+CpG ODN	+GLA-SE	Antigen	+CpG ODN	+GLA-SE	Antigen	+CpG ODN	+GLA-SE	Antigen	+CpG ODN	+GLA-SE
rLdPxn1	0	2.88	0.53	0	3.78	2.03	0	0	0	22.04	34.62	28.91
rLdPxn2	3.37	18.3	7.75	12.82	10.88	7.55	0	0	0	60.24	80.84	34.76

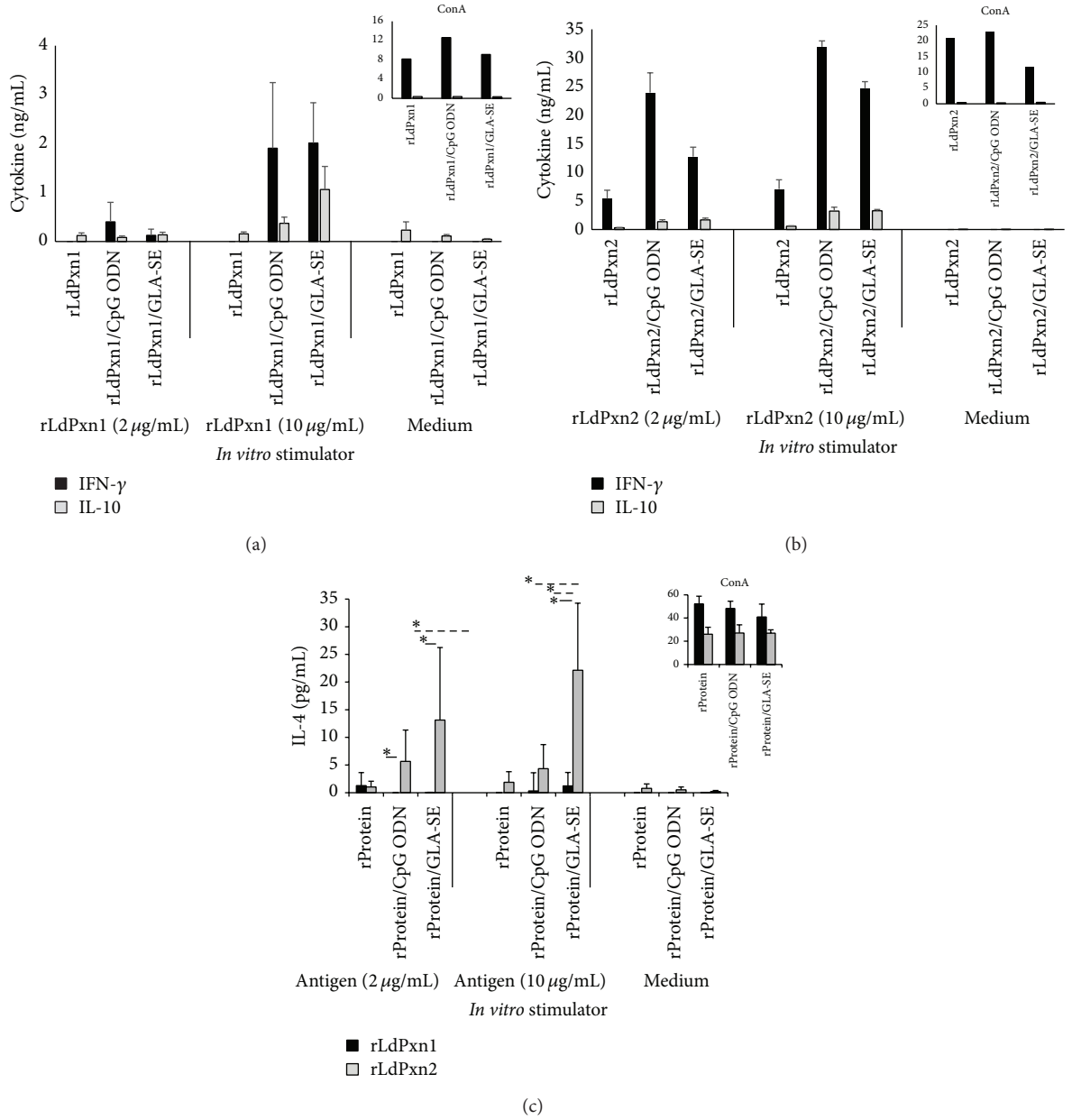

Figure 5: Cytokine responses in spleen cells of rLdPxn1 and rLdPxn2 immunized mice. Mice were immunized s.c. three times at three-week intervals with rLdPxn1 or rLdPxn2 with or without CpG ODN or GLA-SE. Four weeks after the last immunization, spleen cells were prepared and *in vitro* stimulated with the respective antigen (2 and 10 μg/mL) or ConA (5 μg/mL). The release of IFN-γ (ng/mL), IL-10 (ng/mL), and IL-4 (pg/mL) in immunized mice was measured in supernatants after 72 hr of *in vitro* stimulation at 37°C. Results are presented as the amount of IFN-γ (ng/mL) and IL-10 (ng/mL) for rLdPxn1 (a) and rLdPxn2 (b) or the amount of IL-4 (pg/mL) of rLdPxn1 and rLdPxn2 (c).

unprecedented since several previous studies have also shown the ability of these TLR-based adjuvants to stimulate a protective Th1 response against *Leishmania* antigens [21–27].

The mechanism by which recombinant LdPxn1 and LdPxn2 stimulate different immune responses in BALB/c mice was not investigated in this study and it remains to be defined; however, possible explanations are discussed below.

One possibility is that rLdPxn1 and rLdPxn2 are recognized by different TLRs which may result in the stimulation of different effector mechanisms. It has been reported that a mycobacterial early secreted antigenic target protein 6 (ESAT-6) can directly bind to Toll-like receptor 2 and modulate the host immune response [28, 29]. Studies have suggested that lymphocyte-derived cytokines released following TLR ligation can regulate T helper cell differentiation and the type of induced immunity (reviewed in [30]). Additional explanation is that the two antigens may differ in their intracellular trafficking such that they undergo different processing and presentation with major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs). This

possibility has been proposed as a possible cause for the differences in immune responses triggered against *L. major* TSA and LmsTI1 antigens [31]. Dendritic cells (DCs) are professional antigen presenting cells capable of stimulating T-cell activation [32]. Studies have shown the participation of these cells in Th1/Th2 polarization through differential production of IL-12 and IL-10 [33], as well as IFN-γ [34]. Thus, interaction of antigens with DCs is central to the priming and differentiation of T cells.

Also requiring further study is the relationship between rLdPxn1 and rLdPxn2 structures and the immune response as these two antigens may have structural differences, in particular differences in their antigenic epitopes that might affect the humoral immune responses generated against them. *Leishmania* possesses the typical 2-Cys peroxidoxins which have two conserved cysteine (Cys) residues the peroxidatic cysteine Cys47 located at the N-terminus and the resolving cysteine Cys170 placed near the carboxyl terminus [35, 36]. In general, active peroxidoxins exist as homodimers arranged in a head-to-tail orientation such that the N-terminus cysteine of one monomer is juxtaposed with the C-terminus cysteine on the opposing subunit. The transition of peroxidoxins from the reduced to the oxidized state is commonly associated with a conformational change involving the C-terminus tail. Although the structure of LdPxn1 and LdPxn2 is not available, we anticipate that, following conformational changes (resulting from changes in redox state or from antigen processing), the LdPxn2 C-terminus amino acid extension, composed of the terminal 9 amino acids plus few up-stream amino acids which also exhibit differences from LdPxn1, might present different epitopes and consequently stimulates immune response distinct from LdPxn1. A simple experiment to examine the contribution of LdPxn2 C-terminus extension in shaping the immune response can be done by testing its immunoreactivity in mice. Alternatively, mice immune response to a mutated LdPxn2 molecule depleted of the C-terminus extension or of LdPxn1 molecule to which the LdPxn2 C-terminus extension is introduced can be tested and compared to the immune response against the original molecules.

It is important to note that LdPxn1 and LdPxn2 were examined in the form of recombinant GST-fusion proteins. It has been documented that the immune responses generated against GST-fusion proteins are greatly affected by the carrier portion of the protein as well as the adjuvant used [37, 38]. Moreover, it has been suggested that GST fusion may cause conformational changes of proteins permitted by the flexible linker region [39]. Despite the fact that the rLdPxn1 and rLdPxn2 used in this study were generated in the same way, we believe that it is important to analyze the possible effects that GST fusion may have on the immune response against these proteins.

Our future studies will focus on elucidating the possible mechanisms that regulate mice immune responses against LdPxn1 and LdPxn2. We believe that it is important to understand the potential mechanisms by which these antigens interact with the host immune system to shed light on the factors behind the difference in the immune response to seemingly similar antigens.

5. Conclusions

In conclusion, we observed distinct immune response against rLdPxn1 and rLdPxn2 in BALB/c mice. Recombinant LdPxn1 induced a predominant Th2 type whereas rLdPxn2 triggered a mixed Th1/Th2 with predominant Th1 type response. We also found that CpG ODN and GLA-SE enhance the production of a polarized Th1 type regardless of the initial response. In a recent study, we showed that priming with LdPxn1 DNA in the presence of murine granulocyte macrophage colony-stimulating factor (mGMCSF) and boosting with recombinant LdPxn1 protein stimulates multifunctional $CD4^+$ T cells and protects mice against *L. major* infection [40]. It will be interesting to examine the protective effect of recombinant LdPxn1 and LdPxn2 proteins individually or combined with or without adjuvants against *Leishmania* infection.

Conflict of Interests

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

Acknowledgments

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