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Cell-Mediated Immunity and Vaccines

Guest Editors: Jaya Kumari, Senthamil R. Selvan, Stephane Becart, Subhasis Chattopadhyay, and Roy Ambli Dalmo

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Editorial Board

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Editorial

Cell-Mediated Immunity and Vaccines

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Cell-mediated immunity (CMI) is one of the important effector arms during immunological responses to infection and vaccine development. Therefore, a key challenge in developing new vaccines that are effective involves the induction of optimal memory T cell responses [1]. This requires a better understanding of the mechanisms and signals involved in the generation and maintenance of the host CMI response. Thus, a rational strategy should be designed based on crucial factors such as the level and duration of antigen exposure, the use of adjuvants that can enhance CMI and antibody response, and also establishing accurate correlates of protection from diseases. The papers presented in this special issue focus on the leveraging knowledge of CMI and its probable role in novel vaccine strategies.

C. E. Jäkel et al. reviewed twenty recent clinical studies regarding the application of cytokine-induced killer (CIK) cells for the treatment of gastric, pancreatic, hepatocellular, and colorectal cancers. They showed that in all studies, CIK cell therapy was well tolerated and safe as well as superior to conventional therapies alone, since CIK cells have a favorable therapeutic effect with non-MHC-restricted tumor targeting and uncomplicated isolation and cultivation. The clinical study reviewed here thus provides a promising approach in cancer therapy.

Since the last decades of cancer research, numerous approaches have been initiated aiming at activating cytotoxic immune reactions against tumors. Besides targeting the adaptive immune system, stimulators of the innate immune system gained much attention. In this context and resulting from their strong immune stimulatory capacity, ligands for Toll-like receptors (TLRs) were extensively studied. In line with this, two of the research papers deal with those aspects of TLR ligands that have hitherto not been explored in great depth. The paper by Naumann et al. explores the physiochemical and immunostimulatory properties of RGC100, a TLR3 agonist, which may thus represent a promising adjuvant for prophylactic and therapeutic vaccination strategies. They showed that RGC100 has a defined chemical structure and length and has good solubility and stability as well as induces CD1c+ DC driven T cell proliferation. Furthermore, S. Stier et al. clarified the two-sided roles of TLRs expressed by tumor cells. Utilizing the potential of various TLR ligands, alone or in combinations, they demonstrated control of tumor growth and activation of immune cells but also unraveled the requirement of choosing the right combinations as certain combinations accelerated the tumor growth. The findings underscore the rationale for using TLR ligands in cancer immunotherapy, preferably together with conventional chemotherapy, as strongest oncolytic effects were observed in the presence of a functional immune system. Further, their studies offer insights for elucidating the exact balance between pro- and antitumor activities of TLR agonists as single agents but especially of combinations.

S. L. Yingst et al. raise new questions about the importance of CD8+ T cells in defense against *Brucella* infection. They showed a limited role of CD8+ T cells, as IFN-γ

producers or cytotoxic cells, in secondary immunity to *B. melitensis* in contrast to previous studies, focused on *B. abortus*-induced CD8+ T cell responses. Therefore, the authors suggest that a vaccine strategy aimed at sensitizing CD8+ T cells may have limited value, although future investigation is necessary.

New insights on the interaction between *Haemophilus parasuis* and cytokine expression in pigs for better understanding Glasser's disease pathogenesis and effective vaccine development are described in the paper by R. Frandoloso et al. In this context, they describe the capability of subunit vaccines (NPAPTim and NPAPTit: native proteins with affinity to porcine transferrin) to dampen the transcription of several chemokines and cytokines (CCL-2, CXCL-8, IL-1α, IL-1β, IL-6, IL-10, and TNF-α) directly related to severe inflammation in systemic and target infection organs of nonimmunized animals. This highlights that NPAPT antigens might be a suitable candidate to control Glasser's disease caused by *H. parasuis* Nagasaki strain.

We hope the collection of papers in this special issue will enrich our readers and researchers with latest information with respect to wide but important field of cell-mediated immunity and its relationship with novel vaccine development.

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Review Article

Clinical Studies Applying Cytokine-Induced Killer Cells for the Treatment of Gastrointestinal Tumors

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Tumors of the gastrointestinal system represent a significant share of solid tumors worldwide. Despite the advances in diagnosis and treatment, the prognosis of gastrointestinal tumors is still very poor and improved therapies are indispensable. Cytokine-induced killer (CIK) cells are feasible for an immunotherapeutic approach as they are easily available and have an advantageous biologic profile; they are rapidly proliferating and their high cytotoxicity is non-MHC-restricted. We summarize and discuss twenty recent clinical studies applying CIK cells for the treatment of gastric, pancreatic, hepatocellular, and colorectal cancer. Autologous CIK cells were transfused intravenously, intraperitoneally, or via the common hepatic artery. In all studies side effects and toxicity of CIK cell therapy were mild and easily controllable. The combination of CIK cell therapy with conventional adjuvant or palliative therapies was superior to the standard therapy alone, indicating the benefit of CIK cell therapy for cancer patients. Thus, CIK cells represent a promising immunotherapy for the treatment of gastrointestinal tumors. The optimal treatment schedule and ideal combination with conventional therapies should be evaluated in further clinical studies.

1. Introduction

Tumors of the gastrointestinal (GI) system constitute a major part of the cancer incidence and mortality statistics. Worldwide, colorectal cancer is the most frequent type of GI cancer: it is the third most common cancer in men and the second most common in women. Moreover, colorectal cancer accounts for the largest share of GI cancer-related deaths in women, while liver cancer is the most common cause of death among GI tumors of men [1].

Despite the recent advances in diagnosis and therapy, outcomes for patients with GI tumors remain very poor. Often, GI tumors are diagnosed only at advanced stages due to the lack of specific symptoms and screening methods. As a result, 5-year survival rates are low [2–5].

Adoptive cell immunotherapy might be used in combination with standard therapies—as adjuvant postsurgical treatment and as palliative treatment—to improve survival and quality of life of GI cancer patients. Cytokine-induced killer (CIK) cells have the best credentials to be effective in this therapeutic approach. Compared to lymphokine-activated killer (LAK) cells, CIK cells can be obtained more easily and reveal a higher tumor-specific cytotoxic activity [6–10]. Similarly, there are several factors hampering the adoptive cell therapy with tumor-infiltrating lymphocytes (TILs), for example, the difficulty to recover sufficient quantities of these cells and their poor migration to the tumor side [11, 12].

CIK cells can be easily developed from peripheral blood lymphocytes (PBLs) and stimulated with interferon-γ (IFN-γ), monoclonal antibody against CD3 and interleukin (IL)-2. The heterogeneous cell population gains its potent, nonmajor histocompatibility complex (MHC)-restricted cytotoxicity mainly through expansion of $CD3^+CD8^+CD56^-$ cells to CD56-positive natural killer (NK) T cells [10, 13, 14]. CIK cell cytotoxicity is mediated by perforin release and dependant on NKG2D recognition and signaling [15, 16]. The addition of high doses of IL-2 during generation of CIK cells is critical for the expression of the NKG2D adapter protein DAP10 which in turn is essential for cytolysis [16]. NKG2D ligands (e.g., MICA, MICB, and ULBP 1–4) are expressed on both solid and hematologic tumors [17–19].

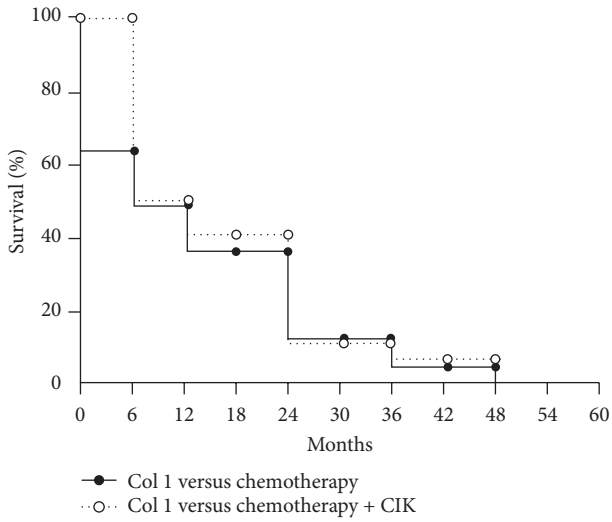

Figure 1: Cumulative survival rate of the patients analyzed by the Kaplan-Meier method. No patient was alive after five years (modified from [21]).

In the following sections, clinical studies applying CIK cells for the treatment of GI tumors are reviewed and discussed.

2. Gastric Cancer

Gastric cancer is the third leading cancer-related cause of death among men and the fifth most common among women. It is therefore a major health issue worldwide. Apart from dietary aspects, *Helicobacter pylori*, a bacterium colonizing the stomach, is the most prominent known risk factor for gastric cancer [1, 20]. Today, combinations of surgical resection, different platin, fluoropyrimidine, and taxane-derived chemotherapies and radiotherapy are the standard treatment options for patients with stomach cancer. At the time of diagnosis these patients generally present with advanced or even metastatic disease. Thus, the cure rates remain poor and novel treatment strategies are required [1].

Jiang et al. provide a study applying CIK cells in combination with chemotherapy in patients with advanced gastric cancer who had all undergone palliative gastrectomy [21]. After gastrectomy, twenty-five patients were treated with three cycles of folinic acid, 5-fluorouracil (5-FU), and oxaliplatin (FOLFOX) chemotherapy. Thirty-two patients were treated with FOLFOX chemotherapy plus autologous CIK cell therapy consisting of five transfusions of CIK cells (1×10^9) after each chemotherapy. In both treatment groups, serum levels of tumor markers were significantly ($P < 0.05$) lower after therapy. The decrease was more pronounced in the patient group receiving additional CIK cell therapy. In the beginning, there was an increase in the cumulative survival rate of the patients treated with CIK cell transfer but after two years, there was no difference in survival between the two groups (Figure 1). Still, the authors conclude that there is a benefit of combined chemo- and CIK cell therapy for patients with advanced gastric cancer.

A similar study was conducted by Shi et al. a few years later [22]. The final analysis included 151 patients with gastric cancer in locally advanced stage. All patients had undergone gastrectomy. During the generation of CIK cells *in vitro*, the number of $CD3^+CD56^+$ cells increased severely (on average 700-fold) and all cell populations used for immunotherapy had no less than 30% $CD3^+CD56^+$ cells. Patients received six cycles of multidrug adjuvant chemotherapy based on 5-FU. Seventy-four patients additionally received at least three cycles of autologous CIK cell therapy (immunotherapy group). One cycle consisted of five CIK transfusions.

One week after immunotherapy the mean percentages of $CD3^+$ and $CD4^+$ cells and the $CD4^+/CD8^+$ ratio increased in the patients' blood and remained at a higher level for up to two months (after three cycles of CIK cell therapy). All side effects occurring during or after transfusion could easily be treated with symptomatic therapy in case they did not resolve on their own within 24 hours. The most common side-effects were fever, chills, headache, rash, nausea, and vomiting (ranging from 5.0 to 20.8%).

The median followup was 50.5 months; in the end 137 patients (90.7%) had died and 143 (94.7%) had relapsed (mostly hematogenous recurrence). The disease-free survival (DFS) rate was significantly better ($P = 0.044$) in the immunotherapy group than in the control group. A trend towards an improved overall survival (OS; $P = 0.071$) could be observed in the immunotherapy group as well. Moreover, by retrospective subgroup analysis, patients with intestinal-type tumors could be found to benefit most from CIK cell therapy (OS: $P = 0.045$; DFS: $P = 0.023$; diffuse or mixed-type tumors: OS: $P = 0.970$; DFS: $P = 0.962$).

On the whole, CIK cell immunotherapy prolonged the DFS in patients with locally advanced stomach cancer and also the OS in patients with intestinal-type tumors. Therefore, the intestinal-type tumor might be a prospective inclusion criterion for CIK cell immunotherapy.

Wang et al. published a study combining capecitabine and oxaliplatin chemotherapy with intraperitoneal (i.p.) perfusion of CIK cells [23]. Forty-two advanced gastric cancer patients with ascites were enrolled in two groups: the chemotherapy group (22 patients) and the combination group (chemotherapy plus CIK perfusion; 20 patients).

The combination of chemotherapy and CIK cells was well tolerated, and there were no serious adverse reactions after CIK perfusions. Compared to chemotherapy alone, the combined therapy was able to reduce the volume of 2-cycle peritoneal fluid drainage ($P = 0.018$). Patients additionally treated with CIK cells showed a longer median time to progression (TTP; $P = 0.001$) and a superior OS ($P = 0.006$).

Another study that was published in 2008 focuses on the side effects occurring during CIK cell treatment [24]. Sixty elderly patients with advanced gastric cancer were treated with FOLFOX chemotherapy; 29 of them received additional intravenous (i.v.) autologous CIK cell infusions.

Side effects appearing after CIK transfusions included chills (13 patients), fever (9 patients), a general malaise (3 patients), nausea, and vomiting (1 patient). These symptoms could all be managed by symptomatic therapy. The therapeutic results were also quite promising. The total remission

rate in the CIK cell therapy patient group was higher than in the group of patients treated with chemotherapy alone (58.6% versus 45.2%). In the CIK cell-treated group, eight patients developed partial remission (PR), nine moderate remission (MR), seven stable disease (SD), and five progressive disease (PD). In the chemotherapy group, only five patients developed PR, nine MR, seven SD, and even ten PD.

The same research group performed a retrospective study to analyze the correlation between CIK cell therapy and cancer-related death in patients with gastric cancer [25]. One hundred and fifty-six patients were included in this study; 81 patients were treated with FOLFOX chemotherapy alone and 75 with additional CIK cell immunotherapy five times after six cycles of chemotherapy.

The 2-year survival time was significantly longer after additional CIK cell therapy than after chemotherapy alone ($P = 0.007$). The 5-year survival rates showed a clear trend towards a superior survival time for patients treated with CIK cells ($P = 0.0526$). The frequency of CIK cell immunotherapy was found to be significantly associated with the survival of patients ($P = 0.002$).

Another retrospective study evaluates the clinical outcome of 165 advanced gastric cancer patients treated with either FOLFOX chemotherapy or 5-FU/cisplatin chemotherapy; patients in the study group were additionally treated with CIK cell therapy [26]. All patients underwent surgery and received four cycles of adjuvant chemotherapy within one month after surgery. While 112 patients in the control group received no additional treatment, 53 patients in the study group were given CIK cell therapy after four cycles of chemotherapy in a one-month interval. CIK therapy was maintained as long as the patients agreed or until tumor progression occurred. Two to twenty CIK therapy cycles were given with a median of three cycles per patient.

No serious side effects were observed after CIK cell transfer. The 3-year progression-free survival (PFS) and OS rates were higher in the CIK cell therapy group compared to the study group, but the differences were not significant. A significant improvement could be seen for the 5-year PFS (49.1% in the study group versus 24.1% in the control group, $P = 0.026$) and for the 5-year OS (56.6% versus 26.8%, $P = 0.014$). The median PFS was 36.0 months in the CIK treated study group versus 23.0 months in the control group ($P = 0.028$). The OS time was also significantly longer in the study group: 96.0 months versus 32.0 months in the control group ($P = 0.003$). Within the study group, the frequency of CIK cell therapy, the clinical stage, and the followup therapy were found to be the most important factors for PFS and OS ($P < 0.05$).

Again, CIK cell therapy was shown to prevent recurrence and improve survival in combination with surgery and chemotherapy. The authors themselves criticize the retrospective character and the number of patients of this study and wish for a prospective paired study to confirm these results. Nevertheless, this study gives a good insight into the possibilities of CIK cell transfer in the adjuvant therapy of gastric cancer.

These six clinical studies show that the combination of CIK cell therapy with palliative or adjuvant chemotherapy protocols can be a significant step forward in the treatment of advanced gastric cancer. The adoptive transfer of CIK cells was well tolerated,—i.v. as well as i.p.—and prolonged survival in all studies discussed above.

The clinical studies on CIK cells for the treatment of gastric cancer are summarized in Table 1.

3. Pancreatic Cancer

The incidence of pancreatic cancer is relatively low compared to the other types of cancer discussed here. Nevertheless, it belongs to the ten most common causes of death from cancer [1]. Pancreatic adenocarcinoma derived from glandular tissue of the pancreas is the most common type of pancreatic cancer. Among others, alcohol consumption, smoking, and diet are known to be risk factors. Diabetes, chronic pancreatitis, and obesity are also associated with a higher risk of pancreatic cancer. Even infections, for example, with *H. pylori,* have been found to be related to pancreatic cancer [27, 28]. In more than 70% of the cases, pancreatic adenocarcinoma is diagnosed at advanced stages. Standard therapy includes surgery, chemotherapy (mostly with gemcitabine), and radiotherapy, but much effort is made to develop new molecular therapies [29, 30].

Qiu et al. report on a new approach of pancreatic carcinoma-specific immunotherapy using synthesized α1,3-galactosyl epitope-pulsed dendritic cells (DCs) along with CIK cells [31]. In this pilot study, fourteen patients with advanced pancreatic cancer were enrolled and treated with gemcitabine combined with oxaliplatin and radiotherapy.

The aim of this clinical approach was to improve tumor-associated antigen (TAA)-pulsed DC therapy. In humans, there is no α-galactosyl (α-Gal) epitope on the cell membrane, whereas the natural anti-Gal antibody is present in human serum [32]. Hence, the idea behind this study was that the expression of the α-Gal epitope on tumor cells can result in *in situ* binding of natural antibodies. This in turn enhances DC phagocytosis and thereby presentation of TAA to T cells, finally generating an antitumoral immune response. Cancer patients often have low immunity; therefore, CIK cells were used for coculturing with the α-Gal-expressing tumor cell lysate-pulsed DCs.

In a first step, metastatic tumor nodules or lymph nodes were surgically obtained from the patients. Using neuraminidase and recombinant α1,3-galactosyltransferase, α-Gal epitopes were synthesized on freshly isolated tumor cells from these biopsies. The cells were then incubated with natural anti-Gal antibody and finally lysed. DCs, which were induced from peripheral blood mononuclear cells (PBMCs) using granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, and tumor necrosis factor (TNF)-α, were then incubated with the α-Gal-expressing tumor cell lysate. CIK cells were prepared from bone marrow samples of the patients, harvested on day twelve, and co-cultured with the lysate-pulsed DCs in presence of IFN-γ, anti-CD3, and IL-2 for 72 hours. The DC-CIK cell mixtures were then used for i.v. transfusions.

The first injection was applied one week after completion of the conventional therapies. Then, depending on the availability of tumor samples, one to five further injections were

Table 1: Clinical studies applying CIK cells for the treatment of gastric cancer.

Study	Number of patients	Therapy	Results	Conclusions
Jiang et al., 2006 [21]	57	Gastrectomy and chemotherapy; immunotherapy group: additional CIK infusions	Better survival rate of patients in immunotherapy group only in beginning—after 2 yrs no difference in survival between the groups	Combination of chemo- and CIK cell therapy superior to chemotherapy alone in patients with advanced gastric cancer
Shi et al., 2012 [22]	151	Gastrectomy and chemotherapy; immunotherapy group: additional CIK cell therapy	The mean $CD3^+$, $CD4^+$ level and the $CD4^+/CD8^+$ ratio increased in patients' blood up to 2 months after immunotherapy; no severe side effects; OS and DFS significantly better in immunotherapy group	CIK cell therapy can prolong DFS and OS; patients with intestinal-type tumors benefit most from CIK cell therapy as determined by retrospective subgroup analysis
Wang et al., 2013 [23]	42	Chemotherapy; immunotherapy group: additional i.p. perfusion of CIK cells	Reduced volume of 2-cycle peritoneal fluid drainage in immunotherapy group; no serious adverse reactions; prolonged TTP and higher OS in immunotherapy group	Superior efficacy of combination of chemo- and CIK cell therapy for advanced gastric cancer patients with ascites; CIK cell therapy can enhance immunological function and prolong survival
Jiang et al., 2008 [24]	60	Chemotherapy; immunotherapy group: additional CIK cell transfusions	The remission rate was 58.6% in the immunotherapy group and 45.2% in the control group; side effects of CIK cell transfusions were chills, fever, general malaise, nausea, and vomiting	CIK cell therapy can reduce clinical signs of elderly advanced gastric cancer patients; side effects can be treated with conventional therapy
Jiang et al., 2010 [25]	156	Chemotherapy; immunotherapy group: additional CIK cell therapy	Significantly longer 2- and 5-yr survival time	Better survival after CIK cell therapy; increasing the frequency of CIK cell therapy seems to be beneficial for survival
Zhao et al., 2013 [26]	165	Gastrectomy and chemotherapy; immunotherapy group: additional CIK cell therapy	Improved 5-yr PFS and OS in immunotherapy group; within the immunotherapy group, the frequency of CIK cell therapy, clinical stage, and follow-up therapy were the most important factors for PFS and OS	CIK cell therapy can prevent recurrence and improve survival in combination with gastrectomy and chemotherapy

CIK: cytokine-induced killer; OS: overall survival; DFS: disease-free survival; i.p.: intraperitoneal; TTP: time to progression; PFS: progression-free survival.

administered once a week with increasing numbers of cells (2×10^9 to 10×10^9 cells per injection).

No serious side effects are reported. Moderate increases of $CD3^+$ and $CD4^+$ T cells ($P < 0.05$) and significant increases of $CD3^+CD8^+$ T cells, $CD3^+CD45RO^+$ cells, and $CD3^+CD56^+$ cells ($P < 0.01$) could be detected in the patients' peripheral blood one week after the third transfusion, indicating a boosting cellular immunity. These levels returned to the original level (before DC-CIK therapy) six to nine months after the third injection. Moreover, a significant increase in IFN-γ secretion by PBMCs ($P < 0.01$) was detected, which remained increased for the followup period of 24 months. Most patients ($n = 12$) showed a positive delayed-type IV hypersensitivity (DTH) reaction after three injections. The carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 levels decreased in most patients.

The clinical response was evaluated according to RECIST criteria one month after the third injection. Six patients had SD, two PR, and six remained in PD. Interestingly, there were strong correlations between an increasing DTH reaction, a decrease in CEA and CA19-9 levels, respectively, and survival ($P < 0.01$). The four patients who survived the followup period showed strong DTH reactions and significantly increased IFN-γ secretion.

This study gives insight into an innovative strategy on pancreatic cancer therapy. Unfortunately, a control group without CIK therapy is missing, which inhibits definite conclusions. Comparing the clinical efficacy of this DC-CIK approach to CIK-therapy alone is also indispensable.

Beyond this study, only one case report on CIK cell therapy for advanced pancreatic carcinoma is published [33]. An elderly patient was diagnosed with a poorly differentiated adenocarcinoma. One month after resection a nodule emerged at the resection margin. Because the patient was unable to tolerate the side-effects of conventional chemo- and radiotherapy, CIK cell immunotherapy was applied. The patient received four i.v. CIK cell infusions of 5×10^9 cells and two million units of IL-2 per infusion from day one to five.

No adverse reactions were observed and the nodule shrank significantly. The formerly elevated levels of CEA, CA 19-9, and CA-724 also reduced to normal values. Following another twelve CIK cell transfusions, the nodule further decreased and marker levels remained normal. After another sixteen injections, the nodule almost disappeared. Finally, the patient received another four cycles to assure treatment efficacy. Unfortunately, a nodule emerged at the same place only a few months later and CA 19-9 levels were elevated again. Altogether the patient had a relatively long PFS of >19 months and an improved quality of life.

This case report gives a promising view on an individual course of CIK cell therapy in a patient with pancreatic cancer. The patient was at poor health and not able to receive chemo- or radiotherapy. Here, CIK cell therapy proved to be a good if not superior alternative.

4. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common histological type of liver cancer and the fifth most common cause of malignancy worldwide. Liver cancer rates are more than twice as high in men as in women and the second most common cause of cancer-related death in men. The majority of cases occur in developing countries, where liver cancer is strongly related to infections with hepatitis B and C viruses (HBV and HCV). In industrial countries, where the incidence of HCC is increasing, the most common risk factors are alcohol abuse and obesity resulting in fatty-liver disease, liver fibrosis, and finally liver cirrhosis. In 80% of the cases, HCC is developing on top of a liver fibrosis/cirrhosis [1].

In most of the cases HCC is diagnosed in an already advanced stage or with decompensated liver cirrhosis. Therefore, the majority of patients can only be treated with palliative therapies. Despite the evolving new medical treatments such as the tyrosine kinase inhibitor sorafenib, therapy of HCC is still challenging.

In 2000 a study investigating postsurgical recurrence rates in HCC patients was published [34]. HCC patients who had all undergone hepatic resection were randomly divided into two groups: one group received adjuvant cytokine-stimulated lymphocyte immunotherapy; the other group received no additional treatment. Peripheral blood was drawn from the patients one day before surgery and lymphocytes were cultured with IL-2 and anti-CD3 for two weeks. Patients in the immunotherapy group received autologous lymphocytes at weeks two, three, four, twelve, and 24 after surgery.

In the end, 72 patients received all five CIK cell transfers, two received only four courses of CIK therapy because of detection of extrahepatic metastases, and two received only one cycle and refused subsequent cell infusions. Adverse events occurred in 45 patients and were all WHO grade 1 or 2 and self-limiting.

The median time for followup was 4.4 years. The recurrence rate of HCC was significantly lower in the immunotherapy group (59%, 45 patients) than in the control group (77%, 57 patients; $P = 0.01$). Also the time to first recurrence was significantly longer in the immunotherapy group ($P = 0.008$). Hence, adjuvant cell therapy was able to lower the frequency of recurrence and to extend the recurrence-free time after hepatic resection.

Four years later a small clinical trial about CIK cell therapy in HCC patients was published by Shi et al. [35]. They enrolled thirteen patients with diagnosed HCC. All patients had liver cirrhosis and more than twenty years of chronic HBV infection.

Autologous CIK cells were reinfused i.v. at days ten, thirteen, and fifteen of CIK cell culture. Before treatment and ten days after CIK cell therapy, the patients' PBMCs were analyzed using a flow cytometer: percentages of $CD3^+CD8^+$, $CD25^+$, and particularly of $CD3^+CD56^+$ cells were significantly increased ($P < 0.05$). Patients were followedup for up to 108 days after CIK cell therapy. At that time, the composition of lymphocyte subpopulations was still similar to the levels determined ten days after therapy. This indicates that the induced changes in the subpopulations can last for at least 108 days.

As all patients had a background of chronic HBV infection, the influence of CIK therapy on the HBV viral load was of great interest: on average, the HBV content decreased from 1.85×10^6 to 1.41×10^5 copies of DNA/mL three months after therapy. It is well established that DC function is suppressed in patients with HCC and chronic HBV or HCV infections [36]. Therefore, the frequencies of DC1, which induce Th_1 cell differentiation and immunity, and DC2, which induce Th_2 differentiation and immunogenic tolerance, were analyzed during this study [37]. The percentages of both cell types were significantly increased in blood after CIK cell therapy ($P < 0.01$). These results suggest that CIK cells are able to play a major role not only in tumor treatment but also in restriction of viral infections.

In a study by Zhang et al. seventeen patients were treated with autologous CIK cells [38]. Most patients have had postoperative recurrence and were in need of further treatment.

CIK therapy is reported as being safe, effective, and without side effects. Without giving reasons, only the results of one patient are given in detail in this publication. This patient had recurrent HCC with metastases and was treated with i.v. transfusion of 1.3×10^{10} CIK cells. Following the transfusion, the patient had decreased nausea, vomiting, and ascites. After three months a tumor specimen of this patient showed large lymphocyte infiltration—much more

than before treatment. The cells were stained for T cell, T memory cell, and mono/macrophage markers and all cell types were increased after CIK therapy. This probably indicates the induction of an antitumor immune response. Unfortunately, no clinical parameters or results are stated and therefore no conclusions on the clinical effect of the CIK cell transfer can be drawn from this study.

Zhao et al. included 64 HCC patients in a CIK cell therapy study [39]. All patients had undergone transarterial chemoembolization (TACE) and additional radiofrequency ablation (RFA). No residual tumor or extrahepatic metastases could be detected. Each of the 33 patients in the study group received eight autologous CIK infusions every three to four weeks either via the peripheral vein or the hepatic artery. The 31 patients in the control group were given no additional treatment. After a relatively short followup of one year, 29 patients in the study group and 23 patients in the control group were recurrence-free. As in the study discussed above, the HBV DNA content was determined before and after treatment. In the study group, the number of patients with a viral DNA content lower than 1×10^3 increased from 19 patients before treatment to 29 afterwards. In the control group, the viral DNA content of only one patient dropped below 1×10^3.

Again, this study gives an idea of what adoptive CIK cell therapy is capable of; it may reduce recurrence, prolong recurrence-free time, and fight HBV. However, the relatively short followup makes it difficult to draw any substantial conclusions.

The research group conducted a similar study in 2008 [40]. Eighty-five HCC patients were treated by TACE and RFA and were divided into two groups. The 45 patients in the study group received additional CIK cell therapy via the hepatic artery. CIK transfusions were given fortnightly after sequential TACE/RFA, four times as a course of treatment. Thirty-nine patients received eight infusions and six patients received ten infusions. Patients in the control group ($n = 40$) received no additional treatment.

Following the transfusions, eleven patients developed a light fever and shiver; all adverse effects were grade 1 or 2. The proportions of several T cell subsets in the patients' peripheral blood were measured by flow cytometry two weeks after CIK transfusions. $CD4^+$, $CD3^+$, $CD56^+$, and $CD3^+CD56^+$ T cell populations and the $CD4^+/CD8^+$ ratio were significantly increased ($P < 0.05$). Remarkably, the percentages of these cells were lower in patients who experienced recurrence than in patients who were recurrence-free (concerning all 85 patients; $P < 0.05$). Interestingly, the percentage of $CD8^+$ T cells, which was decreased after CIK cell therapy, was increased again in recurrent patients.

During eighteen months of followup all patients survived. Fourteen patients (31.1%) of the immunotherapy group had HCC recurrence compared to 32 patients (80.0%) in the control group ($P = 0.001$). The median recurrence-free survival was significantly longer for patients who received CIK cell transfer ($P = 0.012$). Also the recurrence-free survival rates were significantly higher in the immunotherapy group than in the control group (31 patients = 68.9% versus 8 patients = 20.0%; $P = 0.01$).

When entering the study none of the 85 patients showed active lesions or metastases. This study shows that CIK cell immunotherapy can be an effective adjuvant therapy to improve recurrence and survival prognosis for patients with HCC. Thus, CIK cell therapy might be particularly helpful to eradicate microscopic residual tumor lesions to prevent recurrence and improve survival.

A similar study by Pan et al. focuses on changes in serum alpha-fetoprotein (AFP) levels in patients with HCC after TACE/RFA and CIK therapy [41]. AFP is a well-known tumor-associated marker of HCC. This protein is not only known to play a role in the inhibition of the immune system but also in the promotion of cancer cell growth. The aim of this study was to examine AFP as a potential marker to predict the clinical outcome of the combinational therapy of TACE/RFA and CIK cells.

Six to eight weeks after TACE/RFA therapy the possibility of residual tumor burden was excluded by imaging techniques. Before randomization, two consecutive AFP measurements assured a stable AFP level. Patients were then randomly divided into a control group ($n = 39$) and a study group ($n = 42$); patients within the study group received autologous CIK cell infusions once every week via the common hepatic artery or peripheral veins. At least four infusions were given with $>1 \times 10^{10}$ CIK cells each. Further AFP levels were examined one and four weeks after TACE/RFA and—only in the study group—before each CIK cell transfer and once every four weeks within one year after CIK cell therapy.

Comparing AFP levels during followup to baseline levels before TACE/RFA, no significant decrease in AFP concentration could be observed in the control group. By contrast, AFP concentrations in patients additionally treated with CIK cells gradually decreased during followup. The differences between the two patient groups were significant ($P < 0.05$). Within the control group, nine patients experienced recurrence within one year; six of them had AFP concentrations slightly higher than the baseline level. In the study group three patients developed recurrence within one year, with two of them having AFP levels slightly higher than the baseline level.

In summary, the one-year recurrence rate was 7.14% in the study group, which was significantly lower than in the control group (23.1%; $P = 0.04$). Therefore, CIK therapy was able to reduce short-term tumor recurrence. Serum AFP levels were also reduced in patients after CIK cell transfusions indicating that a sustainable decrease of AFP might be useful to predict the clinical efficacy of CIK cell therapy.

Regarding these promising results, the researchers recently published another retrospective study including 174 HCC patients from January 1999 to April 2012 [42]. Eighty-nine patients in the TACE + RFA group were treated by TACE/RFA alone and 85 patients in the TACE + RFA + CIK group received additional CIK cell therapy. After sequential therapy with TACE and RFA, blood was collected from patients in the CIK group. CIK cells were prepared and reinfused i.v. two weeks later; four to 25 (median = 9) successive CIK cell infusions were given per patient.

No major complications occurred during treatment and the overall frequency of adverse effects was similar in both groups. The evaluation of the clinical efficacy was based on modified Response Evaluation Criteria in Solid Tumors (mRECIST) [43]. Differences in the short-term efficacy (three months after therapy) of the two treatment modalities were not significant. After a median followup of 78 months OS and PFS were calculated for both groups. The median survival time (MST) and the median PFS were significantly longer in the CIK group than in the control group (MST: 56 months versus 31 months, $P = 0.023$; PFS: 17 months versus 10 months, $P < 0.001$). The 3-, 5-, and 10-year OS was also significantly higher in the CIK group ($P \leq 0.005$).

The results of this study suggest that additional CIK cell infusions can be beneficial for patients treated with sequential TACE/RFA. CIK cell therapy can prolong recurrence-free survival, which points to a safe and effective treatment option for patients with HCC.

Hao et al. published a nonrandomized controlled clinical trial combining only TACE with CIK cell therapy [44]. From 2005 to 2008, 146 patients with unresectable HCC were assigned by request either to the TACE group or to the combination group. No significant differences were found in the baseline characteristics between the two groups. At first, all patients were treated by TACE. The 72 patients in the combination group received four additional CIK cell transfusions as one course of CIK treatment with maximally four courses in one patient every year.

According to RECIST criteria, short-term responses were similar in both groups: 18% (13 patients) in the combination group and 14.9% (11 patients) in the TACE group had PR; 81.9% (59 patients) in the combination group and 85.1% (63 patients) in the TACE group had SD. The PFS in the combination group was significantly improved compared to the TACE group; the 6-month, 1-year, and 2-year PFS rates were 72.2%, 40.4%, and 25.3% in the combination group versus 34.8%, 7.7%, and 2.6% in the TACE group ($P < 0.001$). Among other factors, CIK cell therapy and times of TACE before disease progression were associated with PFS and OS as identified by univariate analysis. The median OS was significantly longer in the combination group (31 months) than in the TACE group (10 months; $P < 0.001$).

In this study, CIK immunotherapy was able to prolong PFS and OS in patients with unresectable HCC. Especially the times of TACE combined with CIK cell therapy were critical for the clinical outcome. This might be due to the low residual tumor burden after several courses of TACE on which CIK cells might be most effective.

In a recently published trial, i.p. perfusions of autologous CIK cells were combined with local radio frequency (RF) hyperthermia in 31 patients with advanced HCC [45]. Twice a week all patients received i.p. perfusions of 3.2×10^9 to 3.6×10^9 CIK cells with local RF hyperthermia performed two hours later. This treatment was repeated four times as one course. After one month a new treatment course was performed.

No serious adverse events were observed and mild adverse events resolved without intervention. Levels of peripheral blood lymphocytes, the AFP concentration, and the abdominal circumference were recorded every two weeks. The level of peripheral blood $CD4^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$ cells increased significantly after treatment with CIK cells ($P < 0.05$). The AFP level ($P = 0.001$) and the abdominal circumference ($P = 0.002$) significantly decreased. After a median followup of 8.3 months (range 2–12 months), the median TTP was 6.1 months and the median OS 8.5 months. The 3-, 6-, 9-months, and 1-year survival rates were 93.5%, 77.4%, 41.9%, and 17.4%, respectively.

Although a control group is missing in order to draw definite conclusions, the authors suggest a benefit of this treatment modality for patients with advanced HCC. They also emphasize the necessity of more clinical trials including higher numbers of patients to provide evidence and evaluate combinations with other therapeutic approaches.

A relatively large randomized controlled study on postoperative CIK immunotherapy was conducted from January 2000 to 2002 [46]. The aim of this study was to evaluate the clinical outcome of two different schedules of adjuvant CIK cell therapy. For this purpose, 127 patients with HCC, who underwent radical hepatic resection, were randomly assigned to three groups. The first group (CIK-I) comprised 41 patients who received three courses of CIK cell transfer, while the 43 patients in group CIK-II were given six courses of CIK cell transfer. The control group ($n = 43$) did not receive any postoperative adjuvant therapy. The patients were followedup for a relatively long period of five to seven years.

In total, five patients had to stop CIK therapy due to side effects. Still, no long-dated side effects could be observed. The DFS rates were significantly longer in the CIK-treated groups than in the control group ($P < 0.005$), but there were no significant differences between the CIK-I and the CIK-II groups ($P = 0.345$). Comparing the 5-year DFS rates, the results of the CIK-I and the CIK-II groups are relatively close together (23.3% and 19.4%), while the result of the control group is about half the percentage (11.2%). Interestingly, there were no significant differences in the OS rates ($P = 0.884$). Apart from the treatment modality, liver cirrhosis, tumor size, tumor differentiation, and vascular invasion were identified as significant factors influencing the DFS ($P < 0.05$).

This randomized study was conducted with a relatively high number of patients and a long followup. The authors demonstrate that adoptive CIK cell therapy can prevent or at least delay recurrence of HCC after hepatic resection. However, adjuvant CIK cell therapy does not seem to be able to improve the OS.

Similar to the study about α-Gal-pulsed DCs and CIK cells for the treatment of pancreatic cancer, the same authors tested this strategy on HCC [47]. Eighteen patients with clinical stage III HCC were included in this study and treated with conventional therapies. Nine patients were enrolled in the study group and nine patients in the control group.

Autologous tumor cells were synthesized with α-Gal epitopes, then bound by natural anti-Gal antibody, and finally lysed. DCs were induced from PBMCs and co-cultured with this α-Gal epitope-expressing HCC tumor cell lysate. CIK cells were co-cultured with these DCs.

CIK cell transfusions in the study group started three days after completion of the chemo- or radiotherapy and were then given every week for two to seven times.

CIK application was safe as no serious side effects were observed. Tumor-specific CIK cell therapy significantly prolonged survival—the median survival of the study group was 17.1 months versus 10.1 in the control group ($P = 0.00121$). Increases in $CD3^+$, $CD4^+$, $CD45RO^+$, $CD8^+$, and $CD56^+$ cells could be detected in the patients' peripheral blood after CIK therapy.

In summary, this innovative strategy has great potential for specific and individual tumor therapy. It would yet be interesting to compare the clinical effect of these tumor-specific CIK cells to standard CIK cells in order to elucidate whether the coincubation with the α-Gal-pulsed DCs gives a significant benefit.

The clinical trials on CIK cells in HCC therapy are summarized in Table 2.

5. Colorectal Cancer

Colorectal cancer belongs to the most common and deadliest types of cancer [1]. On the one hand, the incidence of colorectal cancer is decreasing in some countries, for example, in the United States. This decrease is due to an increase in detection and removal of precancerous lesions. On the other hand, the incidence is increasing in some other countries, for example, in several Asian and Eastern European countries, probably due to several lifestyle factors such as increasing obesity and smoking. In general, nonlifestyle risk factors for colorectal cancer include age, genetic predisposition, and chronic inflammatory bowel disease.

To our knowledge the only clinical trial with CIK cells including colorectal cancer patients was published by Schmidt-Wolf et al. in 1999 [48]. Here, CIK cells were transfected with a plasmid containing the IL-2 gene. Ten patients with metastatic disease were included in this study—seven patients with colorectal cancer, two patients with lymphoma, and one patient with renal carcinoma. The patients received one to five i.v. infusions of IL-2-transfected CIK cells and five infusions of untransfected CIK cells; the second treatment cycle followed three weeks after the first one.

CIK cell therapy was well tolerated; only three patients developed WHO grade 2 fever. Transfected cells could be detected in the patients' blood for up to two weeks after immunotherapy. Moreover, significantly increased serum levels of IFN-γ, GM-CSF, and transforming growth factor-β (TGF-β) were measured ($P < 0.05$). Also an increase in the cytotoxicity of PBLs was observed following CIK cell therapy.

The clinical evaluation in this study was based on comparison of CT scans before and after treatment and on the WHO Handbook for reporting results of cancer treatment [49]. At the beginning of this study, all ten patients were in PD. After treatment six patients remained in PD, three patients had SD, and one patient had a complete response (CR) with no signs of tumor for at least four weeks. In conclusion, this clinical trial proved CIK cell administration to be safe and promising for patients with colorectal cancer.

6. Conclusions and Future Prospects

The twenty clinical studies discussed here prove CIK cell therapy to be an effective treatment option—after or along with conventional therapies—for patients with gastrointestinal tumors. In addition, as it was shown to be safe and not to induce major adverse effects, CIK cell therapy is a valuable alternative for patients not able or willing to tolerate side effects of conventional chemotherapy [24, 33]. Within the different studies, CIK cells were applied either via peripheral veins, i.p., or via the common hepatic artery and all approaches were well tolerated. Most probably, the best application depends on the tumor side itself and on the prevailing circumstances; for example, in case a catheter is introduced for TACE, the application of CIK cells through the same catheter is apparent. Still, as CIK cells are easily applicable i.v., this therapeutic approach is theoretically useful for most types of tumors.

Several studies showed that after CIK cell application the levels of $CD3^+$, $CD4^+$, $CD3^+CD8^+$, $CD3^+CD45RO^+$, and $CD3^+CD56^+$ cell populations and the $CD4^+/CD8^+$ ratio in the patients' blood increased, which indicates a boosting cellular immunity induced by CIK cell transfusions [22, 31, 35, 45]. The secretion of IFN-γ by PBMCs and the IFN-γ serum level, respectively, were also shown to increase after CIK cell therapy [31]. In the study of Qui and colleagues the increased INF-γ secretion lasted for the followup period of two years and elevated $CD3^+$, $CD4^+$, $CD3^+CD8^+$, $CD3^+CD45RO^+$, and $CD3^+CD56^+$ levels returned to normal six to nine months after CIK cell therapy [31]. Shi and colleagues detected significantly increased levels of $CD3^+CD8^+$, $CD25^+$ and $CD3^+CD56^+$ cells during the whole followup period of up to 108 days [35]. Remarkably, in the study of Weng and colleagues the percentages of $CD4^+$, $CD3^+$, $CD56^+$, and $CD3^+CD56^+$ cells were lower in recurrent patients [40]. Therefore, the immunomonitoring of these T-cell subtypes seems to be critical to predict the clinical response to CIK-cell therapy as increases in these T-cell subtypes suggest a prolonged TTP.

Some types of cancer such as HCC are closely associated with underlying viral infections (HBV, HCV) making the viral load an important prognosis factor. Therefore, one therapeutic goal is to decrease the viral load in order to improve the clinical outcome. In some clinical studies on CIK cell therapy for HCC, the viral load of HBV has been determined before and after CIK transfusions in order to evaluate the effect of CIK cells on viral replication. After CIK cell therapy, the HBV copies of DNA/mL decreased. This indicates a positive effect of CIK cells on viral infections and thus a clear benefit for patients with tumors and chronic viral disease [35, 39].

In summary, CIK cell therapy provides a promising approach in cancer therapy. CIK cells have a favorable biology with non-MHC-restricted tumor targeting and uncomplicated isolation and cultivation. In all studies, CIK cell therapy was well tolerated and superior to conventional therapies alone. There are several questions yet to be elucidated, for example, the optimal application schedule and the best therapeutic combination with conventional treatment modalities.

Table 2: Clinical studies applying CIK cells for the treatment of HCC.

Study	Number of patients	Therapy	Results	Conclusions
Takayama et al., 2000 [34]	150	Resection; Immunotherapy group: additional infusions of lymphocytes activated *in vitro* with rIL-2, and anti-CD3	Recurrence: 59% in immunotherapy group versus 77% in control group; TTP: 2.8 yrs in immunotherapy group versus 1.6 yrs in control group	Immunotherapy lowered risk of recurrence by 41%; the difference in OS was not significant; safe and feasible treatment
Shi et al., 2004 [35]	13	i.v. CIK transfusions	Increased proportions of $CD3^+CD8^+$, $CD25^+$, and $CD3^+CD56^+$ cells in peripheral blood up to 108 d after immunotherapy; median HBV viral load decreased from 1.85×10^6 to 1.41×10^5 copies of DNA/mL in 3 months	CIK cells can efficiently improve the immunological status of HCC patients; CIK cells played important role in antiviral and antitumoral treatment
Zhang et al., 2005 [38]	17	Resection; CIK cell transfusion	Only one case described: decreased ascites; improvement of nausea, and vomiting; large lymphocyte infiltration in tumor	Significant enhancement of antitumor immunity; perform CIK therapy to eradicate remaining tumor cells after operation
Zhao et al., 2006 [39]	64	TACE/RFA; immunotherapy group: additional CIK infusions i.v. or via hepatic artery	After 1 yr followup: 29 of 33 patients in immunotherapy group and 23 of 31 patients in control group were recurrence-free; in 29 patients in the immunotherapy group and in only 1 patient in the control group the HBV DNA content was $<1 \times 10^3$	CIK therapy can prolong the recurrence-free time and fight HBV; CIK therapy after TACE/RFA is an effective therapeutic strategy for HCC
Weng et al., 2008 [40]	85	TACE/RFA; immunotherapy group: additional CIK infusions via hepatic artery	Increased proportions of $CD3^+$, $CD4^+$, $CD56^+$, and $CD3^+CD56^+$ cells and the $CD4^+/CD8^+$ ratio—percentages were lower in recurrent patients than in nonrecurrent patients; recurrence: 31.1% in immunotherapy group versus 85.0% in control group	CIK cell therapy can reduce recurrence and improve survival rates; CIK transfusions can boost immunity of HCC patients
Pan et al., 2010 [41]	83	TACE/RFA; immunotherapy group: additional CIK cell transfusions i.v. or via common hepatic artery	Downtrend of AFP only in immunotherapy group; 1-yr recurrence rate 7.14% in immunotherapy group versus 23.1% in control group; percentage of patients with HBV DNA content $<1 \times 10^3$ copies/mL was 73.5% in the immunotherapy group versus 9.1% in the control group	CIK cell transfusions can decrease the 1-yr recurrence rate of HCC patients and reduce serum AFP levels, which may serve as a useful marker to predict clinical outcome after immunotherapy and TACE/RFA
Huang et al., 2013 [42]	174	TACE/RFA; immunotherapy group: additional i.v. CIK cell infusions	Significantly longer OS and PFS in immunotherapy group	Combination of TACE/RFA and CIK cell therapy is safe and can be an effective treatment modality
Hao et al., 2010 [44]	146	TACE; immunotherapy group: additional i.v. CIK cell transfusions	1-yr and 2-yr PFS rates: 40.4% and 25.3% in the immunotherapy group versus 7.7% and 2.6% in the control group; 1-yr and 2-yr OS rates: 71.9% and 62.4% in the immunotherapy group versus 42.8% and 18.8% in the control group; the times of TACE and CIK cell transfusions were independent prognostic factors for PFS and OS	Adjuvant CIK cell therapy can greatly improve the efficacy of TACE and prolong PFS and OS in HCC patients

Table 2: Continued.

Study	Number of patients	Therapy	Results	Conclusions
Wang et al., 2013 [45]	31	RF hyperthermia; i.p. CIK cell perfusions	Significant increases in levels of $CD4^+$, $CD3^+CD8^+$, and $CD3^+CD56^+$ cells in peripheral blood; AFP and abdominal circumference decreased; median TTP: 6.1 mo; 1-yr survival rate: 17.4%; median OS: 8.5 months	I.p. perfusions of CIK cells combined with local RF hyperthermia are safe, can improve immunology, and prolong survival of HCC patients
Hui et al., 2009 [46]	127	Resection; immunotherapy group I: additional 3 courses of CIK therapy; immunotherapy group II: additional 6 courses of CIK therapy	DFS rates significantly higher in CIK-treated groups than in control group; no statistical significance between immunotherapy group I and group II; no statistical significance in OS between the 3 groups	Postoperative CIK cell therapy can prolong DFS but not the OS rates; valuable therapeutic strategy for HCC patients to prevent recurrence
Qiu et al., 2011 [47]	18	Resection, radio-, chemo-, and interventional therapies; immunotherapy group: additional transfusion of CIK cells previously cocultured with α-Gal epitope-pulsed DCs	Survival was significantly prolonged: 17.1 months in the immunotherapy group versus 10.1 months in the control group; all patients in the immunotherapy group had systemic cytotoxicity in response to tumor lysate, decreased serum AFP, and increased levels of $CD8^+$, $CD45RO^+$, and $CD56^+$ cells in peripheral blood	CIK therapy was safe and effective; new therapeutic approach has great potential in tumor therapy

CIK: cytokine-induced killer; HCC: hepatocellular carcinoma; rIL-2: recombinant Interleukin-2; anti-CD3: anti-CD3 antibody; TTP: time to progression; OS: overall survival; i.v.: intravenous; HBV: hepatitis B virus; TACE: transarterial chemoembolization; RFA: radiofrequency ablation; AFP: alpha fetoprotein; PFS: progression-free survival; i.p.: intraperitoneal; DFS: disease-free survival; α-Gal: α1,3-galactosyl; DC: dendritic cell.

A few of the abovementioned studies are retrospective studies and many authors stated that more large-scale randomized controlled studies are needed. Indeed, these retrospective studies only give an idea of the effectivity of CIK cell therapy while the improved data quality of prospective clinical studies results in a higher significance. The great advantage of CIK cell therapy is that it is a personalized therapeutic approach—unfortunately, this makes CIK cell therapy very cost- and time-intensive, hampering the conduction of large prospective randomized trials.

There are many variations in the methodology and in the clinical evaluation between the research groups, which impede the comparison of the trials. The *ex vivo* protocols for the generation of CIK cells are heterogenous. For example, Jiang et al. generate CIK cells by addition of IFN-γ and IL-2 on day 0, anti-CD3 antibody and IL-1α on day 1, and then IL-2 every third day [21]. In contrast, Li et al. add anti-CD3 antibody, IL-1α, and IFN-γ at day 0, IL-2 after 24 hours, and then IFN-γ and IL-2 every five days [33]. Shi and colleagues do not use any IL-1 at all but add only IFN-γ at day 0 and anti-CD3 antibody and IL-2 at day 1 [35]. The same applies for the concentration of cytokines and the time of incubation till harvesting. Often not even information about the resulting phenotype composition in the CIK cell cultures is given, which makes the drawing of definite conclusions even more difficult. The International Registry on CIK Cells (IRCC) was established to collect data about CIK cell therapy and to set new standards on the report of results from CIK cell application [50]. Making the generation of CIK cells, the treatment, and the reporting of results more uniform will definitely push the application of CIK cell therapy forward and result in advantageous treatment options for cancer patients.

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

Activation of Dendritic Cells by the Novel Toll-Like Receptor 3 Agonist RGC100

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Toll-like receptor (TLR) 3 agonists emerged as attractive candidates for vaccination strategies against tumors and pathogens. An important mechanism of action of such agonists is based on the activation of TLR3-expressing dendritic cells (DCs), which display a unique capacity to induce and stimulate T-cell responses. In this context, it has been demonstrated that targeting of TLR3 by double-stranded RNA such as poly(I:C) results in potent activation of DCs. Major disadvantages of poly(I:C) comprise its undefined chemical structure and very poor homogeneity, with subsequent unpredictable pharmacokinetics and high toxicity. In the present study, we evaluated the physicochemical properties and biological activity of the novel TLR3 agonist RGC100. RGC100 has a defined chemical structure, with a defined length (100 bp) and molecular weight (64.9 KDa) and a good solubility. RGC100 is stable in serum and activates myeloid DCs through TLR3 targeting, as evidenced by gene silencing experiments. Activation of mouse and human myeloid $CD1c^+$ DCs by RGC100 leads to secretion of several proinflammatory cytokines. In addition, RGC100 improves the ability of $CD1c^+$ DCs to stimulate T-cell proliferation. Due to its physicochemical properties and its immunostimulatory properties, RGC100 may represent a promising adjuvant for prophylactic and therapeutic vaccination strategies.

1. Introduction

In the initial phase of infection, the innate immune system generates a rapid and potent inflammatory response. This response aims at blocking dissemination of the infectious agent, with subsequent activation of T cells and B cells that mount the acquired immune response against the pathogen [1]. Recognition of pathogen-related components by immune cells occurs through pathogen recognition receptors (PRR). PRRs are present on cell surfaces, in endosomes, or in cytosol. Toll-like receptors (TLR) represent an important family of PRRs [2, 3]. They are expressed on various subsets of immune cells such as dendritic cells (DCs) [4]. DCs are professional antigen-presenting cells that play an important role in the induction and maintenance of innate and adaptive immune responses [5, 6]. Due to their functional properties and prominent expression of Toll-like receptors, DCs represent promising candidates for TLR agonist-based vaccination strategies against tumors and pathogens [7, 8].

Expression of TLR3 has been evidenced in $BDCA1^+$ myeloid DCs (mDCs), human-monocyte-derived DCs (MoDCs) but not in plasmacytoid DCs [9–13]. Double-stranded RNA (dsRNA) is a ligand of TLR3 [14]. It is recognized as a pathogen-associated molecular pattern (PAMP), triggering innate immune response through the interaction with TLR3 expressed by DCs [15–17]. Of note, a variety of cancer cells have been reported to express TLR3. Upon triggering of TLR3 in tumor cells, apoptosis and/or antitumoral effect occur [18–21].

Polyinosinic-polycytidylic acid poly(I:C) is a potent activator of innate immunity [14, 22]. Poly(I:C) activates DCs through combined targeting of various innate immunity

pathways, including TLR3. Major disadvantages of poly(I:C) comprise its undefined chemical structure and very poor homogeneity, resulting from its manufacturing process [23]. Poly(I:C) is composed of a mix of single-stranded and double-stranded RNA molecules ranging from about 1.5 to 8 kb [22], imperfectly annealed as dsRNA or single-stranded RNA. This is mainly due to limited solubility and difficult reconstitution of poly(I:C) that requires heating (50–60°C) and slow cooling over many hours to achieve reannealing of both poly(I) and poly(C) strands. As a consequence, poly(I:C) has a reported toxicity in clinical trials, ranging from hypersensitivity to coagulopathy, renal failure, or systemic cardio-vascular failure [24]. A further problem of dsRNA compounds such as poly(I:C) are their rapid degradation in body fluids by RNAses, with a reported half-life of few minutes [25, 26] and subsequent unpredictable pharmacokinetics of degradation products. Optimization of physicochemical properties of poly(I:C) has led to generation of derivatives that have increased stability in body fluids (such as polyICLC), [27] or reduced toxicity through reduced stability in body fluids (such as poly(I:C_{12}U) [28, 29]. Poly(I:C) and its derivatives are produced under GMP conditions for intravenous administration and have been tested in various clinical trials [28–30].

In the present study, structure, analytical profile and biological activity of the novel TLR3 agonist RGC100 are presented. RGC100 displays a very well defined chemical structure, length and molecular weight, a good solubility and serum stability, being able to activate DCs in a dose-dependent manner by specifically targeting endosomal TLR3.

2. Materials and Methods

2.1. Physicochemical Analysis. Analysis of RGC100 length and integrity was performed on 12% native PAGE. DNA marker (Fermentas, Germany) was used to illustrate molecular size distribution and RNA staining was achieved by using Stains-all (Alfa Aesar, USA). Analysis of poly(I:C) was performed on 1% native agarose gel electrophoresis. Two different poly(I:C) compounds were used: poly(I:C) with a low molecular weight (LMW, 0.2–1 kb, Invivogen, USA), and poly(I:C) with a high molecular weight (HMW, 1.5–8 kb, Invivogen, USA). RNA marker (Promega, Germany) was used to illustrate molecular size distribution.

Physical characterization of RGC100 in solution was performed by size-exclusion chromatography (SEC) with UV, refractive index (RI), and right angle light scattering (RALS) detection on the Viscotek TDAmax (Malvern, UK). A sample volume of 125 μL (~100 μg RGC100) was injected to a Superdex 200 10/300 column (GE Healthcare, USA) and SEC was performed by using phosphate buffer saline.

2.2. Immunomagnetic Isolation of CD1c$^+$ DCs and CD3$^+$ T Lymphocytes. Blood samples were obtained with informed consent from healthy donors. The study was approved by the institutional review board of the University Hospital of Dresden (no. EK 27022006). Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Biochrom, Germany) density centrifugation. Subsequently, human CD1c$^+$ DC were isolated from freshly prepared PBMCs by immunomagnetic negative depletion and positive selection according to the manufactures instructions (Miltenyi Biotec, Germany). CD3$^+$ T cells were isolated from PBMCs by negative depletion using immunomagnetic separation according to the manufacturer's instructions (Miltenyi Biotec, Germany). To analyze the purity of the cell preparations, CD1c$^+$ DCs were stained with PE-conjugated anti-CD1c$^+$ and FITC-conjugated anti-CD19 antibodies and CD3$^+$ T cells with PE-conjugated anti-CD2 and FITC-conjugated anti-CD3 antibodies. Purity was determined by FACS analysis, which was performed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

2.3. T-Cell Proliferation Assay. CD1c$^+$ DCs (1×10^4/well) were cocultured with autologous CD3$^+$ T cells (1×10^5 cells/well) in the presence or absence of 50 μg/mL RGC100 or poly(I:C) in round-bottomed 96-well plates. Before coculture, T-cells were stained with 1 μM cell proliferation dye eFluor 670 (eBioscience, Germany). Cells were incubated for 8 days, harvested and T cell proliferation was analyzed by flow cytometry, which was performed on a FACSCalibur flow cytometer (BD Biosciences).

2.4. Cells and Cytokine Assays. JAWS II cell line was obtained from American Type Culture Association (ATCC, USA). JAWS II is an immortalized immature myeloid DC line derived from C57BL/6 mice, which displays a similar phenotypic profile as resting bone-marrow-derived DCs (BMDCs) [31]. Cells were plated in round-bottomed 96-well plates at 5×10^4/well in DMEM with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (100 U/mL). Cells were incubated with RGC100 (Riboxx, Germany) at different concentrations with or without preincubation with chloroquine (Invivogen, USA). After 16 h, supernatants were collected and the concentration of cytokines and chemokines was determined by ELISA according to manufacturer's instructions (Qiagen, Germany).

To assess the toxicity of siRNA on JAWs II DCs, a cell proliferation assay was performed as previously described [32]. Briefly, JAWS II DCs were seeded into a 96-well plate with 5×10^4 cells/well. A serial dilution of siRNA at a concentration ranging from 200 nM to 12.5 nM was added to the cells. After 24 h, 100 μL of MTS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added to each well. After 2 h, absorbance of the solution was measured at 490 nm and the CC_{50} value was determined.

Human CD1c$^+$ DCs were plated in round-bottomed 96-well plates at 2.5×10^4/well in RPMI 1640 medium containing 10% human AB serum (CCPRO, Germany), 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin (Biochrom, Germany). Then, cells were stimulated with RGC100 or poly(I:C) (Sigma-Aldrich, Deutschland) at different concentrations. After 24 h, supernatants were collected and the concentration of IL-1β,

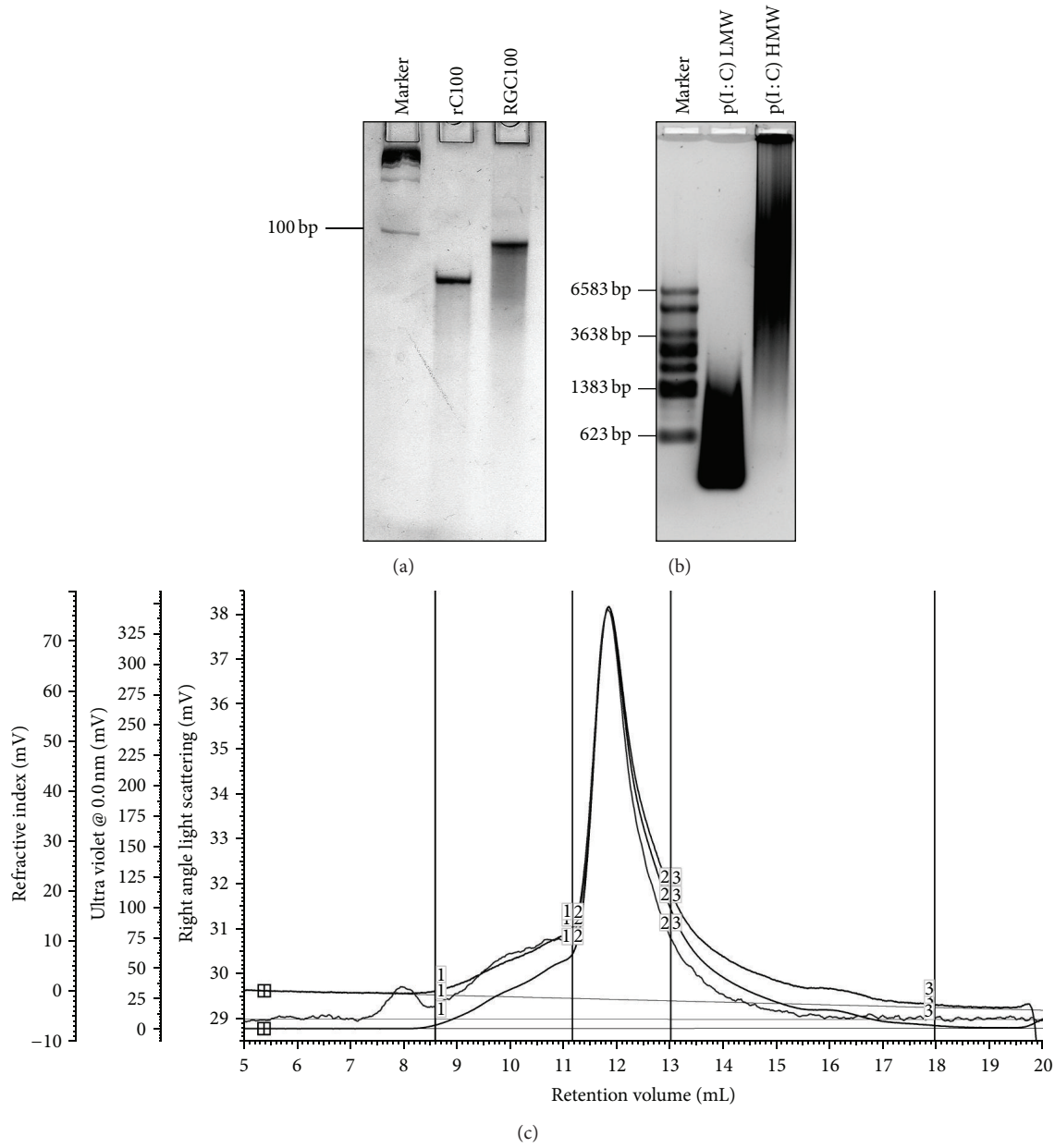

Figure 1: Determination of physicochemical properties of RGC100. (a) Analysis of RGC100 by 12% native PAGE. RGC100 displays a length of 100 bp as indicated. It consists of 100 rC bases paired to 100 rG bases, perfectly annealed in a double strand. As a reference, a 100 mer consisting of homopolymeric cytidine is shown. (b) Analysis of poly(I:C) by 1% native agarose gel electrophoresis. p(I:C) LMW: poly(I:C) with a low molecular weight. p(I:C) HMW: poly(I:C) with a high molecular weight. (c) Analysis of RGC100 by size-exclusion chromatography (SEC) with UV, RI and RALS detection. Data analysis provides information about molecular size and polydispersity (Mw/Mn = 1.015).

IL-6, and TNF-α was determined by ELISA according to the manufacturer's instructions (BD Biosciences).

2.5. Gene Silencing of TLR3. Gene silencing was performed using IBONI siRNA (Riboxx, Germany) targeting TLR3 (5′-CTCGGCCTTAATGAAATTGAA-3′) and a nontargeting siRNA (Riboxx, Germany). Therefore, JAWS II cells were plated in round-bottomed 96-well plates at 5×10^4/well and incubated at 37°C (5% CO_2) for 16 h. Then, IBONI siRNA (Riboxx. Germany) was mixed to riboxxFECT transfection reagent (Riboxx, Germany) according to manufacturer's instructions and the mix was added to the wells at a concentration of 20 nM. At 6 h after transfection, RGC100 was added and the cells were incubated for 16 h. Subsequently, cells and supernatants were harvested. RNA was extracted from cells using the RNeasy kit (Qiagen, Germany) and used for subsequent qRT-PCR. Supernatants were used for cytokines

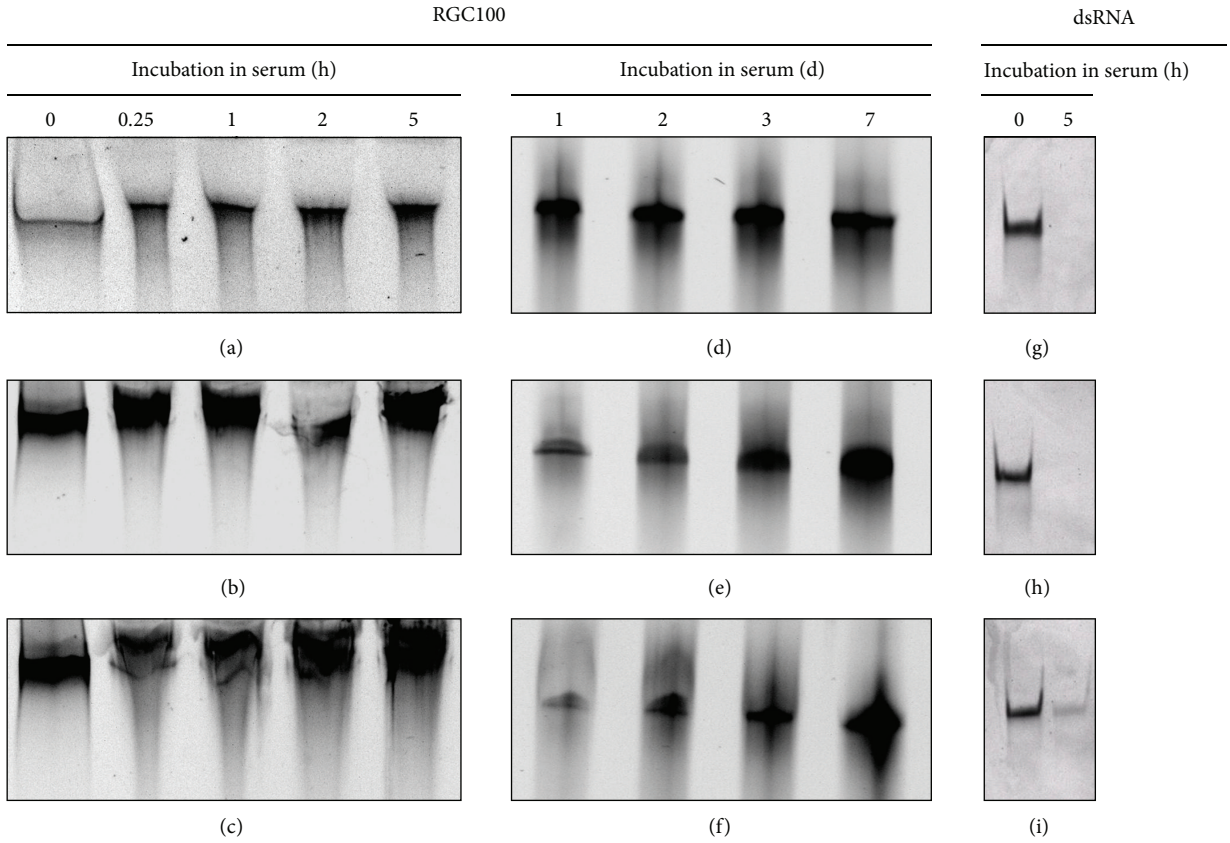

Figure 2: Assessment of the stability of RGC100 in FCS, mouse serum, or human serum. RGC100 (1.6 μM) was incubated in 80% serum at 37°C and samples were analyzed on 12% native PAGE at indicated time points (h, hours and d, days). The corresponding untreated dsRNA (0 h) is shown as a reference. (a) Incubation of RGC100 in FCS up to 5 h. (b) Incubation of RGC100 in mouse serum up to 5 h. (c) Incubation of RGC100 in human serum up to 5 h. (d) Incubation of RGC100 in FCS up to 7 days. (e) Incubation of RGC100 in mouse serum up to 7 days. (f) Incubation of RGC100 in human serum up to 7 days. (g) Incubation of dsRNA (25 bp) used as a control in FCS for 5 hours. (e) Incubation of dsRNA (25 bp) used as a control in mouse serum for 5 hours. (f) Incubation of dsRNA (25 bp) used as a control in human serum for 5 hours.

measurement with ELISA according to the manufacturer's instructions (Qiagen, Germany). qRT-PCR was performed on LightCycler using QuantiTect Primer assays (Qiagen, Germany) for mouse TLR3 and mouse β-actin and QuantiTect SYBR Green RT-PCR kits (Qiagen, Germany) according to instructions of manufacturer.

2.6. Serum Stability Assays. RGC100 (1.6 μM) was incubated in 80% FCS, mouse serum or human serum at 37°C from 1 h to 7 days. FCS and mouse serum were purchased Invitrogen (Germany) and Sigma-Aldrich (Germany) respectively. Human serum was collected from a blood donor. Integrity of RGC100 was assessed through analysis on native 12% PAGE. As an indicator, RGC100 not incubated in serum was used. As a control for effective degradation of dsRNA by serum nucleases, a dsRNA (1.6 μM) of 25 bp was incubated in FCS, mouse serum or human serum for 5 h and analyzed on native 12% PAGE.

2.7. Melting Point Analysis. Analysis of RGC100 (3 μM) was performed on a LightCycler (Roche, Switzerland) using the riboxx LIGHT Kit (Riboxx, Germany) according to manufacturer's instructions.

2.8. Statistical Analysis. Student's t-test was performed to evaluate the significance of the results. Values of $P < 0.05$, were considered as significant.

3. Results and Discussion

3.1. RGC100 Has Defined Chemical Structure and Good Solubility. Design of RGC100 was performed based on the knowledge of structural and biological characteristics of TLR3 agonists. Crystal structure of the ectodomain of TLR3 with its dsRNA ligand [33] have shown that dsRNA of ~ 45 bp in length is sufficient for activation of TLR3 [34]. Most importantly, interaction of TLR3 ectodomains occurs only with the ribose backbone, indicating that triggering of TLR3 is not RNA sequence specific [33]. Hence, length of dsRNA is the major determinant of TLR3 triggering [35].

The choice of sequence composition of RGC100 was based on previous studies on biological activity *in vivo*

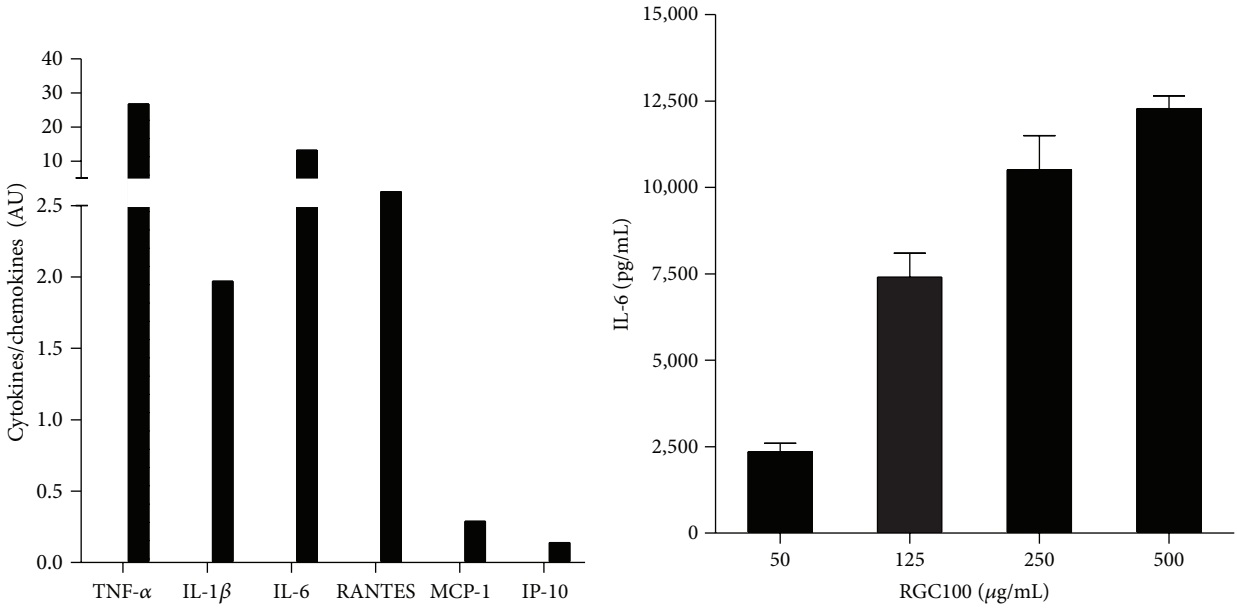

Figure 3: Activation of JAWS II DCs by RGC100 in a dose-dependent manner. (a), cytokine and chemokine profile of JAWS II DCs activated by RGC100. RGC100 was incubated with JAWS II DCs for 16 h at a concentration of 250 μg/mL. Secretion of cytokines was measured by ELISA. Negative control consists of supernatant of cells incubated in the absence of RGC100. Values of negative control have been subtracted from the values represented on the graph. Values shown are mean ± SEM of two independent measures. (b), Dose-dependent activation of JAWS II DCs by RGC100. RGC100 was incubated with cells for 16 h at the indicated concentrations. Secretion of IL-6 was measured by ELISA. Values shown are mean ± SEM of two independent measures. Negative control consists of supernatant of cells incubated in the absence of RGC100. Values of negative control have been subtracted from the values represented on the graph.

of a polyguanidinic-polycytidinic compound (poly(G:C)). Poly(G:C) has been reported to have the same interferon-inducing and antiviral activity as poly(I:C) [36]. Importantly, poly(G:C) displays an up to 12.7-fold higher LD_{50} in comparison to poly(I:C) when administrated intravenously to mice (200 mg/kg versus 15.8 mg/kg, resp.) [36]. In rabbits, the LD_{50} of poly(G:C) administrated intravenously is up to 4.5 fold higher than poly(I:C) (1 mg/kg versus 0.22 mg/kg, resp.) [36].

Taking these experimental observations on length and sequence composition into consideration, we have designed RGC100 that bears a length of 100 bp, and consists of 100 rC paired to 100 rG. Analysis by native PAGE and SEC with UV, RI and LS detection showed that RGC100 displays a defined physicochemical structure. RGC100 has an observed molecular weight of 64.6 kDa (MW_{calc} = 64.9 kDa) with low polydispersity (Figures 1(a) and 1(c)). Melting point of RGC100 was 91.6°C.

The defined chemical structure and good solubility of RGC100 are of importance to reduce potential toxic effects of TLR3 agonists. As observed for poly(I:C), the homogeneity of the compound plays an essential role in the genesis of toxicity. Poly(I:C) is a polydisperse and heterogeneous compound (Figure 1(b)) due to its polymeric macromolecular structure, being a mixture of single poly(rI) and poly(rC) as well as dsRNAs poly(I:C) of different lengths. This high chemical heterogeneity induces unpredictable pharmacokinetics [35] that translate into severe toxic side effects observed in clinical trials, such as coagulopathies, hypersensitivity reactions, renal failure, and even chock [24]. Heterogeneity of chemical structure of poly(I:C) leads to uncontrolled and combined signaling of at least three innate immunity pathways, namely TLR3, RIG-I, and/or MDA-5 [22]. Indeed, signaling of TLR3 is triggered by dsRNA with a length of more than 50 bp [34, 35], whereas signaling of RIG-I is stimulated by dsRNA of a length of 300–1000 bp [22, 37], and signaling of MDA-5 is activated by dsRNA of more than 1000 bp [22, 37, 38]. Moreover, the presence of ssRNA in the mixture resulting from imperfect annealing triggers TLR7 [33]. Taken together, the toxicity of poly(I:C) relates mainly to its heterogeneous composition and undefined chemical structure, with subsequent unpredictable pharmacokinetics and biological activity [35].

The advantages of using a TLR3 agonist such as RGC100 displaying defined physicochemical properties such as solubility and homogeneity, as well as precise chemical structure, length and molecular weight have been already highlighted by others [35]. An additional important advantage of RGC100 is the ability to fine-tune its potency for immune cell activation, by varying the length of the dsRNA compound. As reported previously [35], activation of TLR3 pathway *in vivo* depends mainly on the length of dsRNA, not the nucleotide sequence. Hence, RGC100 offers in addition to a highly defined chemical structure, the possibility to optimize the selectivity index as to immunological potency and toxicity

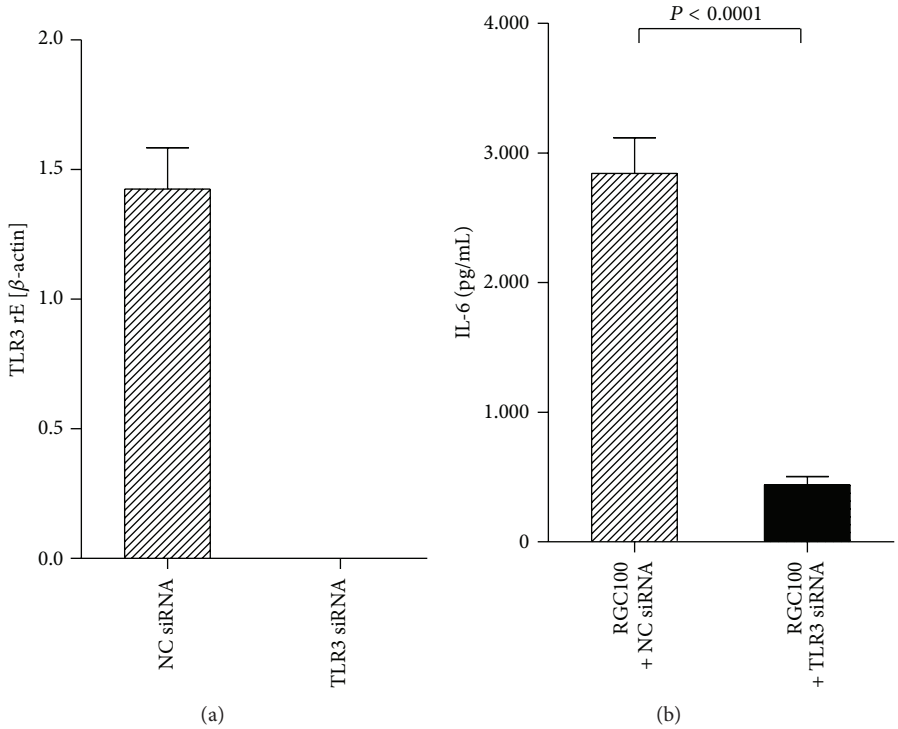

Figure 4: Inhibition of activation of JAWS II DCs by RGC100 using siRNA targeting TLR3. Cells were treated with siRNA targeting TLR3, then incubated with RGC100 at the indicated concentrations. RNA was extracted and supernatant was harvested. (a) Relative expression of TLR3 mRNA in cells treated with siRNA targeting TLR3 or with a nontargeting siRNA (NC siRNA). mRNA levels were normalized to β-actin mRNA. rE, relative expression. (b) Secretion of IL-6 was measured by ELISA. Values shown are mean ± SEM of two independent measures. Negative control consists of supernatant of cells incubated in the absence of RGC100 and siRNA. Values of negative control have been subtracted from the values represented on the graph.

Table 1: Comparison of the physicochemical and functional properties of RGC100 and poly(I:C).

	Length	Molecular weight	Chemical structure	Biostability[a]	Agonist of
RGC100	100 bp	64.6 KDa	dsRNA	7 days	TLR3
Poly(I:C)	~1500–8000 bp	~1020–5440 KDa	dsRNA ± ssRNA	<5 minutes	TLR3, RLRs[b]

[a]Biostability measured as resistance to serum nuclease; [b]RLR: RIG-I-like receptors.

for a specific indication. These physicochemical and functional properties differentiate RGC100 clearly form poly(I:C) (Table 1).

3.2. RGC100 Is Stable in Serum. Stability of RGC100 was examined in serum. RGC100 was incubated with FCS, mouse serum and human serum, and its integrity was assessed on native PAGE over time. As shown in Figures 2(a) to 2(f), RGC100 is stable in FCS, mouse and human serum up to 7 days. In contrast, a dsRNA of 25 bp used as a control is degraded in 5 h (Figures 2(g) to 2(i)), and poly(I:C) incubated with FCS was completely degraded in less than 5 minutes (see Supplemental Figure S1) available online on http://dx.doi.org/10.1155/2013/283649. This is not surprising, because as reported by others, dsRNA is usually degraded in serum within minutes [25, 26]. Degradation is due to nuclease activity of serum RNAses [25, 26]. The increased stability of RGC100 in serum can be explained by its GC content of 100%. Experimental data has shown that GC rich sequences display tight base stacking in dsRNA structure [39], with subsequent increase in duplex stability [39]. Increasing stability of duplex has been shown to increase half-life of dsRNA in serum. For instance, locked nucleic acids that increase stability of duplex confer resistance of dsRNA to serum nucleases [40]. Also, chemical modifications in ribose backbone (i.e., 2′-O-Methyl) that increase duplex stability improves its resistance to serum nucleases [25]. Our findings suggest that RGC100 displays physical properties making it suitable for *in vivo* applications.

3.3. RGC100 Activates Mouse Myeloid DCs. DCs display an extraordinary capacity to induce and expand $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T cells [8, 41]. $CD8^+$ CTLs efficiently destroy tumor cells, whereas $CD4^+$ T cells promote the antigen-presenting capacity of DCs and provide help for the activation and proliferation of $CD8^+$ CTLs. Besides their unique ability to induce and stimulate T-cell responses, DCs efficiently improve the immunomodulatory and cytotoxic

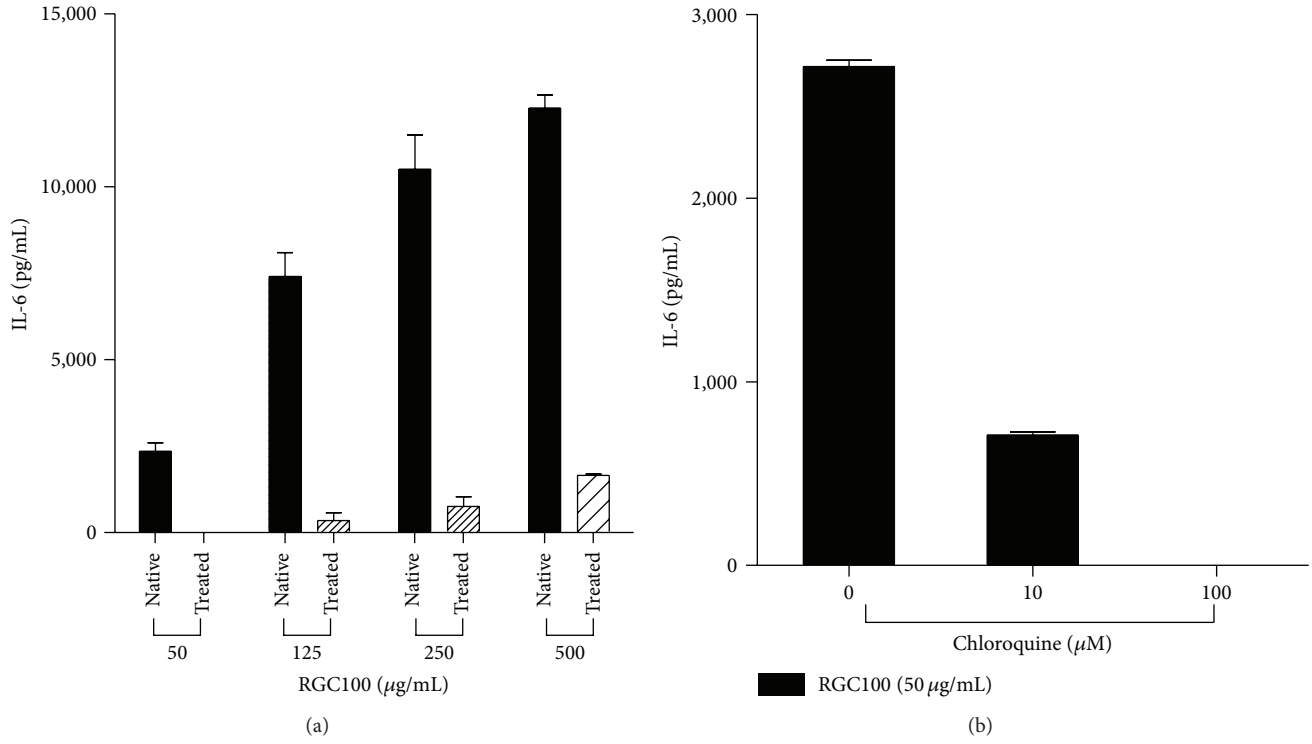

Figure 5: Inhibition of activation by RGC100 of JAWS II DCs through chloroquine. (a) Cells were first treated with chloroquine (treated, 100 μM) or not (native), then incubated with RGC100 at the indicated concentrations. (b) Cells were first treated with chloroquine at the indicated concentrations, then incubated with RGC100 (50 μg/mL). Secretion of IL-6 was measured in supernatant by ELISA. Values shown are mean ± SEM of two independent measures. Negative control consists of supernatant of cells incubated in the absence of RGC100. Values of negative control have been subtracted from the values represented on the graph.

potential of natural killer cells, which essentially contribute to the elimination of virus-infected and tumor cells [42]. Due to their various immunostimulatory properties, DCs evolved as promising candidates for vaccination strategies against tumors and pathogens [7, 43].

In the present study, we investigated the impact of RGC100 on TLR3-expressing murine JAWS II cells, representing immature myeloid DCs, which have been used in studies focusing on antitumor and pathogen-specific immunity [31]. JAWS II cell line was expanded immediately after reception from supplier and frozen in aliquots. Cells were maintained in culture no more than 4 weeks. These procedures were done in order to ensure that no phenotypic drift occurs, as recommended by others [44]. As shown in Figure 3(a), incubation of JAWS II DCs with RGC100 resulted in secretion of various cytokines and chemokines. High levels of TNF-α, IL-6, and IL-1β were observed, as expected for activation of DCs. However, upon stimulation of JAWS II cells by RGC100, secretion of Type I interferon was not detected. In a further step, dose-response profiles of RGC100 were examined. As shown in Figure 3(b), efficient activation of JAWS II DCs was achieved over a range of concentrations from 50 to 500 μg/mL.

3.4. RGC100 Is a Ligand of Endosomal TLR3. To explore the mechanism of action of RGC100, knockdown of TLR3 in JAWS II DCs was performed. In order to prevent cytotoxicity resulting from off-target effects of siRNA, the CC_{50} of the siRNA was assessed in a cell proliferation assay. The CC_{50} of siRNA was >200 nM. Consequently, the concentration used to knockdown TLR3 (20 nM) was chosen to be 10-fold lower than the CC_{50}. Additionally, the siRNA used in this assay displays a specific design that prevents off-target effects, as previously reported [45]. As shown in Figure 4, silencing of TLR3 expression in JAWS II DCs inhibited activation by RGC100, indicating that TLR3 is the ligand of RGC100.

In a further step, we examined whether endosomal acidification is essential for activation of JAWS II DCs by RGC100. For this purpose, cells were treated by chloroquine followed by incubation with RGC100. As shown in Figure 5(a), inhibition of the endosomal acidification by chloroquine impaired stimulation of JAWS II DCs by RGC100 in a dose-dependent manner (Figure 5(b)), indicating that endosomal uptake is essential for activation by RGC100.

3.5. RGC100 Activates Human Myeloid Dendritic Cells. To get novel insights into the impact of RGC100 on the immunostimulatory properties of TLR3-expressing native human DCs, we investigated whether RGC100 promotes the release of proinflammatory cytokines by $CD1c^+$ DCs in comparison to poly(I:C). Therefore, $CD1c^+$ DCs and $CD3^+$ T cells were immunomagnetically isolated from blood of healthy donors

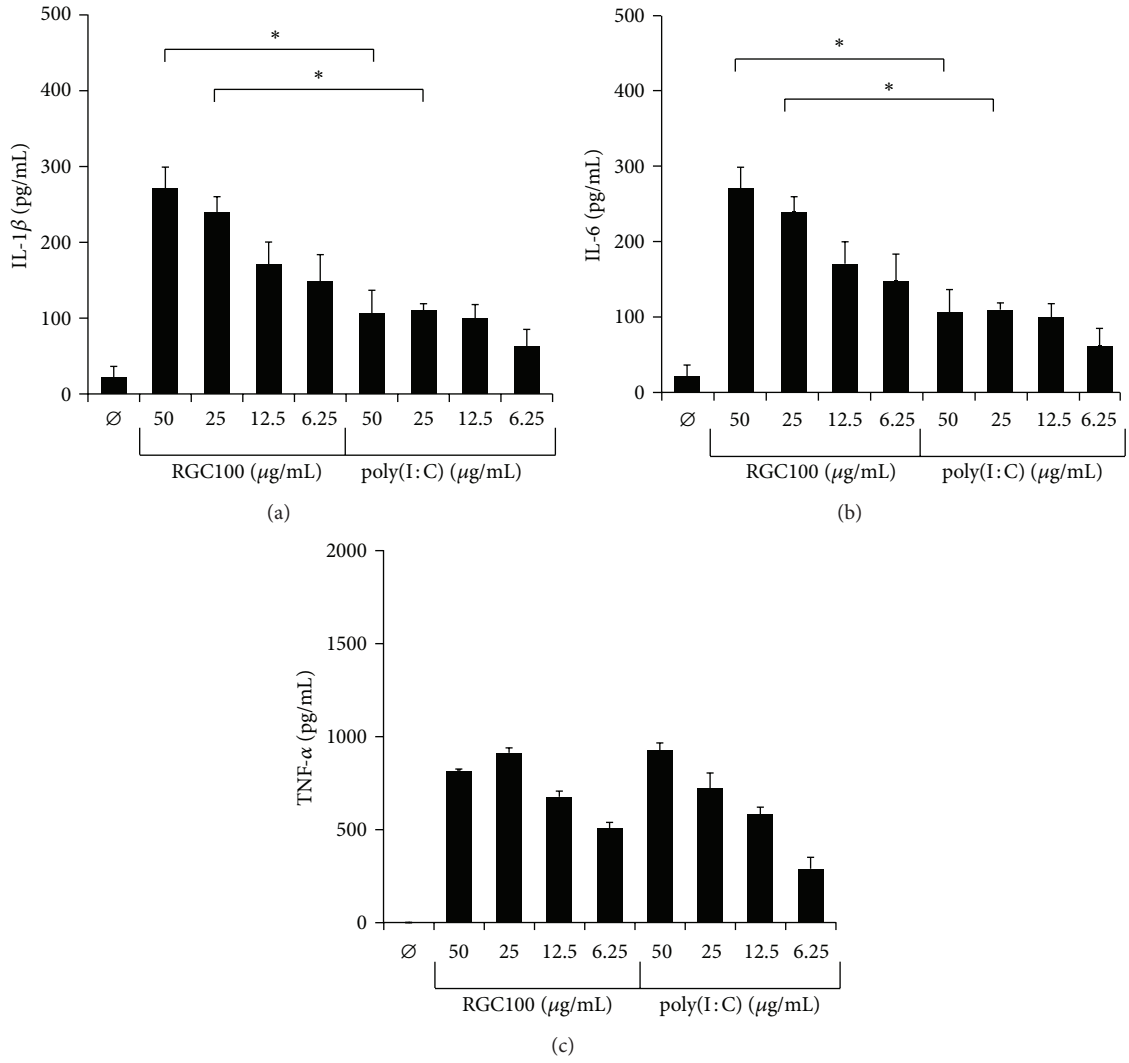

Figure 6: Activation of human myeloid CD1c$^+$ DCs by RGC100 and poly(I:C). Freshly isolated CD1c$^+$ DCs were cultivated in the presence or absence of RGC100 or poly(I:C). After 24 h, supernatants were harvested and the concentration of IL-1β, IL-6, and TNF-α was determined by ELISA as indicated. The results of one representative donor out of three performed with similar results are demonstrated. Values represent the mean ± SEM of triplicate samples and asterisks indicate a statistically significant difference ($P < 0.05$).

and maintained in the presence or absence of RGC100 and poly(I:C). The purity of isolated CD1c$^+$ DC and CD3$^+$ T cells was >90% as assessed by flow cytometric analysis (supplemental Figure S2). As shown in Figure 6, both TLR-3 agonists efficiently stimulate the production of IL-1β and IL-6 by CD1c$^+$ DCs. Interestingly, compared to poly(I:C), RGC100 has a significantly enhanced capacity to promote IL-1β and IL-6 by CD1c$^+$ DCs, whereas the ability to stimulate TNF-α secretion was comparable between the TLR-3 agonists (Figure 6). In further experiments, we evaluated the impact of RGC100 on the ability of CD1c$^+$ DCs to stimulate the proliferation of T cells. As depicted in Figure 7, RGC100 and poly(I:C) displayed a similar potential to augment CD1c$^+$ DC-mediated T cell proliferation. These results indicate that the novel TLR3 agonist RGC100 efficiently stimulates the release of TNF-α, IL-1β and IL-6 by CD1c$^+$ DCs and improves their capacity to promote T cell proliferation.

4. Conclusions

In the present study, experimental data on physicochemical properties and biological activity of the novel TLR3 agonist RGC100 are presented. RGC100 has optimal physicochemical properties, such as defined chemical structure and stability in serum. RGC100 activates murine myeloid DCs through targeting of endosomal TLR3, resulting in secretion of pro-inflammatory cytokines in a dose-dependent manner. In addition, RGC100 efficiently augments the secretion of pro-inflammatory cytokines by native human CD1c$^+$ DCs and improves their capacity to promote T-cell proliferation. Based

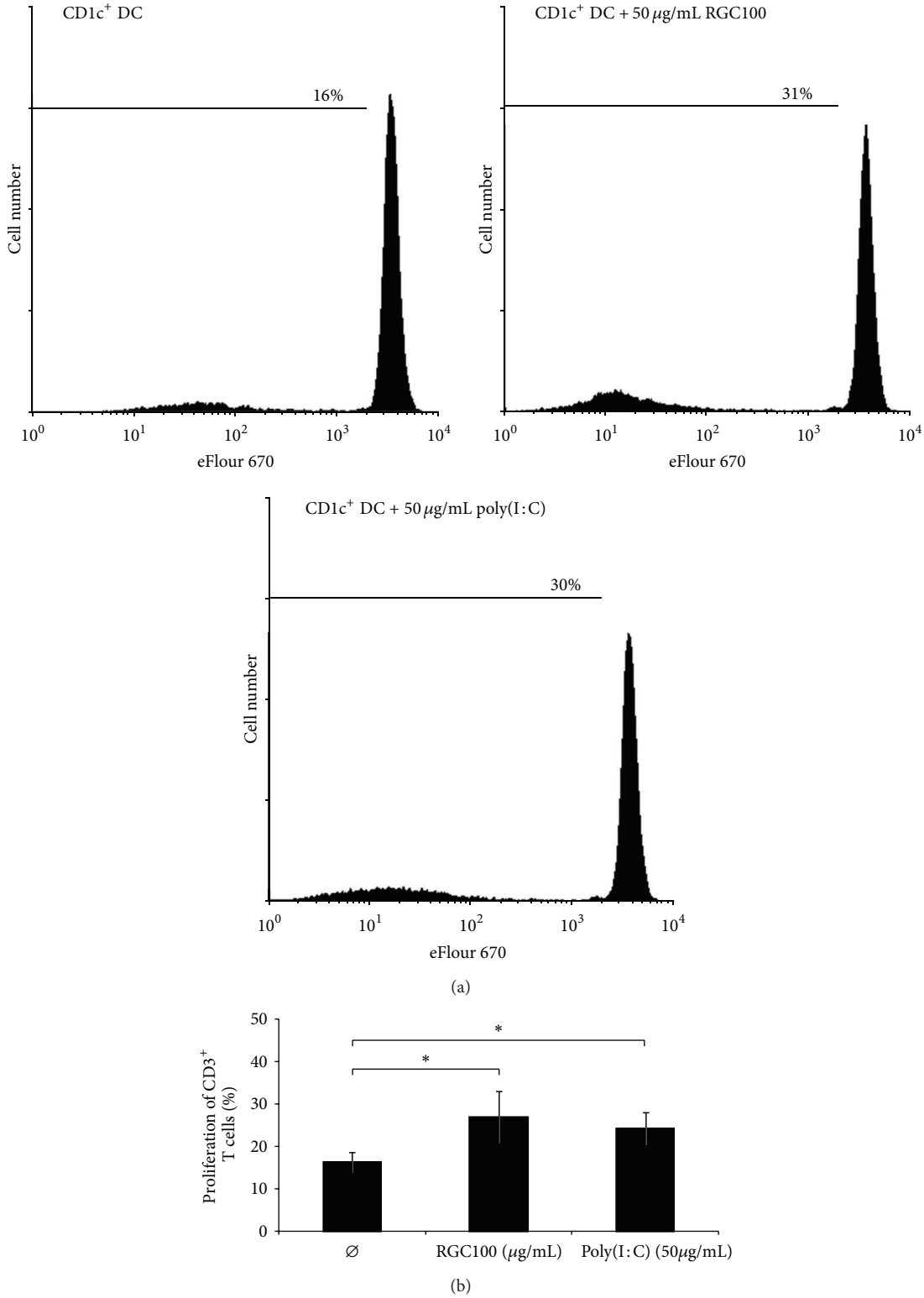

Figure 7: Impact of RGC100 and poly(I:C) on CD1c$^+$ DC-mediated T-cell proliferation. CD1c$^+$ DCs were cocultured with autologous T cells in the presence or absence of 50 μg/mL RGC100 or poly(I:C). Before coculture, T cells were stained with cell proliferation dye eFluor 670. Cells were incubated for 8 days and harvested and T cell proliferation was determined by flow cytometry. (a) The results of one representative donor out of three performed with similar results are depicted. (b) The results of three different donors are presented as mean ± SEM. Asterisks indicate a statistically significant difference ($^*P < 0.05$).

on these properties, RGC100 may represent a promising candidate for prophylactic and therapeutic vaccination strategies against tumors and pathogens.

Disclosure

Kai Naumann, Christiane Petzold and Jacques Rohayem are employees of Riboxx GmbH, Radebeul, Germany. RGC100 is covered by two PCT patent families (pending).

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Research Article

Combinations of TLR Ligands: A Promising Approach in Cancer Immunotherapy

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Toll-like receptors (TLRs), a family of pattern recognition receptors recognizing molecules expressed by pathogens, are typically expressed by immune cells. However, several recent studies revealed functional TLR expression also on tumor cells. Their expression is a two-sided coin for tumor cells. Not only tumor-promoting effects of TLR ligands are described but also direct oncopathic and immunostimulatory effects. To clarify TLRs' role in colorectal cancer (CRC), we tested the impact of the TLR ligands LPS, Poly I:C, R848, and Taxol on primary human CRC cell lines (HROC40, HROC60, and HROC69) *in vitro* and *in vivo* (CT26). Taxol, not only a potent tumor-apoptosis-inducing, but also TLR4-activating chemotherapeutic compound, inhibited growth and viability of all cell lines, whereas the remaining TLR ligands had only marginal effects (R848 > LPS > Poly I:C). Combinations of the substances here did not improve the results, whereas antitumoral effects were dramatically boosted when human lymphocytes were added. Here, combining the TLR ligands often diminished antitumoral effects. *In vivo*, best tumor growth control was achieved by the combination of Taxol and R848. However, when combined with LPS, Taxol accelerated tumor growth. These data generally prove the potential of TLR ligands to control tumor growth and activate immune cells, but they also demonstrate the importance of choosing the right combinations.

1. Introduction

Since the last decades of cancer research, numerous approaches have been initiated aiming at activating cytotoxic immune reactions against tumors. Besides targeting the adaptive immune system, stimulators of the innate immune system gained much attention. In this context and resulting from their strong immune stimulatory capacity, ligands for Toll-like receptors (TLRs) were extensively studied. TLRs are a family of pattern recognition receptors. They have a key position in the first-line defense against pathogens by recognizing specific pathogen-associated molecular patterns, conserved structures expressed by pathogens. Furthermore, they bind to endogenous damage-associated molecular patterns. These molecules are released by stressed or dying cells [1]. Upon ligand binding, TLR-signaling leads to inflammation and antimicrobial responses, thus priming adaptive immune responses [2]. Besides components directly originating from bacteria or viruses, synthetic substances like Poly I:C (ligand for TLR3) or Resiquimod (R848, ligand for TLR7/8) were extensively studied either as single substance in experimental cancer models or as vaccine adjuvant in clinical trials [3–5].

TLRs are primarily expressed by cells belonging to the innate immune systems' arm, that is, dendritic cells (DCs) and monocytes. The observation that TLRs are functionally expressed in several types of tumors, however, hints towards another tumor-promoting role [6]. Recent evidence suggests that they act as double-edged sword in tumorigenesis.

Even more, several studies revealed adverse effects of TLRs on carcinogenesis. Kundu et al. described LPS-induced malignant transformation of benign prostate epithelia [7]. The group of Schmaußer found that TLRs on malignant gastric carcinoma cells enabled interaction with pathogens and subsequently enhanced cell proliferation [8]. Some additional studies substantiated a tumor growth and malignancy-promoting effect of TLRs overexpressed on tumor cells. These include employment of TLR4 signaling by breast and

colorectal cancer cells [9, 10], as well as flagellin-induced activation of TLR5 on gastric cancer cells [11].

On the other hand, at least as many studies revealed antitumoral effects of TLR ligands by inducing tumor cell apoptosis/necrosis or activating immune cells. Direct oncopathic effects on different tumor entities have been described for Poly I:C (TLR3 agonist) and Imiquimod (TLR7 agonist) [12–14]. Hence, similar to what is known about the immune system as a whole, TLRs are capable of both inhibiting and promoting cancer.

Although the TLR expression patterns and their effects are well understood on immune cells, their functional relevance in tumorigenesis and resulting immunological changes remain to be fully elucidated. Further studies are needed to clarify their function in tumor biology and to evaluate their therapeutic potential which will finally help to establish effective treatment schedules. Therefore, we here tested TLR ligands for treatment of colorectal carcinomas (CRC) *in vitro* and *in vivo*. Experiments revealed strongest oncolytic effects in the presence of a functional immune system. Hence, these findings underscore the rationale for using TLR ligands in cancer immunotherapy—either alone or as combinations; preferably together with conventional chemotherapy.

2. Material and Methods

2.1. Tumor Cell Lines and TLR Ligand Treatment. The CRC cell lines HROC40, HROC60, and HROC69 (all three microsatellite stable) were established in our lab from patients subsequent to operation and for analyses passages 25–35 were used. Cells were maintained in full medium: DMEM/HamsF12 supplemented with 10% fetal calf serum, glutamine (2 mmol/L), and antibiotics (medium and supplements were purchased from PAA, Cölbe, Germany). The cells were seeded at the appropriate density for each cell line in both 96-well or 24-well plates and incubated 24 h prior to TLR ligand treatment. For all *in vitro* experiments, the following TLR ligands and their combinations were used in the concentrations 0.01, 0.1, 1, and 10 μM: Taxol, R848 (Enzo Life Sciences, Lörrach, Germany), LPS (Sigma-Aldrich, Hamburg, Germany), and Poly I:C (InvivoGen, San Diego, CA, USA). All substances were applied once. Antitumoral effects were examined after 24, 48, and 72 h of incubation.

2.2. RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR. Total RNA from tumor cells was isolated with TRIzol reagent according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA from 2 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Target cDNA levels of human cell lines were analyzed by quantitative real-time PCR using TaqMan Universal PCR Master Mix and predesigned TaqMan gene expression assays, Hs00152933_m1 (TLR3), Hs00152939_m1 (TLR4), Hs00152971_m1 (TLR7), Hs00152972_m1 (TLR8), Hs00152973_m1 (TLR9), and Hs99999905_m1 (GAPDH, housekeeping gene control) in an ABI Prism 7000 sequence detection system (Applied Biosystems). PCR conditions were as follows: 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C. TLR expression by the murine CRC cell line CT26 was analyzed using SibirRoxHot Master Mix (Bioron, Ludwigshafen, Germany). The mRNA levels of target genes were normalized to GAPDH. Primer pairs used in real-time PCR were the following: TLR3 5′-GGTCCCCAGCCTTCAAAGAC-3′ and 5′-ACGAAGAGGGCGGAAAGGT-3′, TLR4 5′-ACCTGGCTGGTTTACACGTC-3′ and 5′-CTGCCAGAGACATTGCAGAA-3′, TLR7 5′-CCACAGGCTCACCCATACTTC-3′ and 5′-GGGATGTCCTAGGTGGTGACA-3′, GAPDH 5′-CATGGCCTTCCGTGTTCCTA-3′ and 5′-CCTGCTTCACCACCTTCTTGAT-3′. Reactions were performed in triplicate wells. The general expression level of each sample was considered by calculating ΔCT (ΔCt = $Ct_{target} - Ct_{GAPDH}$). Expression patterns were classified as strong, moderate, low, or absent in comparison to normal immune cells (i.e., human dendritic cells and murine macrophages). These cells served as standard and quality control in each qRT-PCR.

2.3. MTS and Flow Cytometric Cell Viability Analysis. Experiments were performed in 96-well plates in triplicate and replicated at least three times. MTS (Promega, Mannheim, Germany) was mixed with PMS (Sigma-Aldrich) and 20 μL of this mix was added to each well. After incubation of cells at 37°C for at least 1 h, the absorbance was measured at 492 nm on a LP400 ELISA reader (Anthos Mikrosysteme, Krefeld, Germany). Direct TLR ligand effects on tumor cell viability were additionally characterized by flow cytometry. Cells were treated as described above. All cells (adherent plus cells in supernatant) were harvested and stained with 2 μM Calcein-AM (Sigma Aldrich). Fluorescent microsphere beads (1.4×10^5 beads/mL, Polysciences, Germany) were added to the samples in a final volume of 200 μL. A gate was set in the FSC/SSC on the beads, and all living cells (Calcein-AM positive) per 5.000 beads were counted. Experiments were performed in 24-well plates in duplicates and replicated at least four times. Percentages of proliferating/viable and total number of cells were calculated compared to untreated control cells, that is, without TLR ligand application. Samples were analyzed on a FACSCalibur Cytometer (BD Pharmingen). Data analysis was performed using CellQuest software (BD Pharmingen).

2.4. Lymphocyte Preparation and Coculture Experiments. Peripheral blood lymphocytes (PBL) were obtained from healthy volunteers following Ficoll density-gradient centrifugation. The cytotoxicity mediated by TLR ligand-stimulated immune cells on CRC cell lines was examined by direct coculture experiments. Tumor cells were seeded in duplicate into 24-well plates (1×10^4/well) and incubated overnight. Medium was removed and fresh medium containing PBLs (1×10^6 PBL/well, ratio 1 : 100) with or without TLR ligand was added. Following a 48 h or 72 h incubation period, PBLs were removed and tumor cells were harvested by trypsinization. Prior to FACS analysis, fluorescent microsphere beads (1.4×10^5 beads/mL, Polysciences, Germany) were added to the samples in a final volume of 200 μL. One gate was placed

on tumor cells in the FSC/SSC to exclude lymphocytes. A second gate was set in the FSC/SSC on the beads, and all living tumor cells per 5.000 beads were counted. A representative plot is given in Figure 4(c). Data are given as X-fold number of tumor cells compared to untreated controls with PBLs. Another control consisted of tumor cells without PBL addition.

2.5. Flow Cytometric Phenotyping of PBL. For phenotypic analysis of human PBLs, cells were harvested after TLR ligand stimulation as described before (72 h). Controls were incubated in complete medium without any TLR ligand. Cells were washed and stained with directly FITC-, PE-, or APC-labeled mAbs against CD3, CD4, CD8, CD16/56, CD25, CD62L, CD69, and CD71 (each 1 μg, ImmunoTools, Friesoythe, Germany) for 30 min at 4°C. Then, cells were washed twice and resuspended in 200 μL PBS. Negative controls were stained with the appropriate isotypes. Cells were analyzed by flow cytometry as described above. For each sample, 20.000 events were measured. To overcome interindividual differences between single donors, data of untreated control PBLs were set as 1 and data of TLR ligand stimulated cells were given as X-fold increase.

2.6. In Vivo Tumor Models and Treatment Regimen. Experiments were performed on female 8–10-week-old Balb/c mice weighing 18–20 g. Mice were bred in the university's animal facility and maintained under specified pathogen-free conditions. All animals were fed standard laboratory chow and given free access to water. Experiments were performed in accordance with the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, vol. 25, no. 28, 1996). Tumor challenge was performed by subcutaneous (s.c.) injection of 5×10^6 CT26 cells into the right hind leg. Tumor growth was routinely controlled at least twice a week and tumor volume was estimated according to the formula: $V = \text{width}^2 * \text{length} * 0.52$. After tumor establishment, mice were divided into experimental groups ($n = 7$ per group) each treated with one of the following substances/combinations: Taxol (20 mg/kg bw), R848 (60 mg/kg bw), LPS (2 mg/kg bw), Irinotecan (20 mg/kg bw), Taxol + R848, Taxol + LPS, and R848 + LPS. Treatment was performed two times a week for a total of three weeks. As control, tumor-carrying mice received equivalent volumes of PBS (saline, $n = 7$). Tumor-carrying mice (treatment, control) were sacrificed at day 21 or when they became moribund before the tumor volume reached 2.000 mm^3. Blood samples were taken on day 10 of therapy. At the end of each experiment, blood samples, tumor material, spleen, and mesenteric lymph nodes were removed from all animals for further analysis.

2.7. Flow Cytometry of Blood and Spleen Cells. Flow cytometry was performed with leukocytes from peripheral blood during and after therapy using the following fluorescein-isothiocyanate- (FITC-) and phycoerythrin- (PE-) conjugated rat anti-mouse monoclonal antibodies (mAbs): CD3ε FITC, CD62L PE (1 μg, ImmunoTools), CD11b FITC, CD11c FITC, CD19 FITC, CD4 PE, CD8 PE, Gr1 PE (1 μg, Miltenyi Biotec, Bergisch-Gladbach, Germany), and CD166 PE (1 μg eBiosciences, Frankfurt, Germany) followed by lysis of erythrocytes (FACS Lysing Solution, BD Pharmingen). Negative controls consisted of lymphocytes stained with the appropriate isotypes (BD Pharmingen). Samples were analyzed as described above.

Table 1: TLR expression on CRC cell lines and immune cells.

	TLR3	TLR4	TLR7	TLR8
HROC40	++	++	+	−
HROC60	++	++	+	−
HROC69	−	++	+	−
DC	+++	+++	+++	+++
CT26	+++	+	+	n.d.
Macrophages	+	+++	+++	n.d.

ΔCT values were compared between cell lines and different target genes (+++ strong expression, ++ moderate expression, + low expression, − no expression, and n.d. not done).

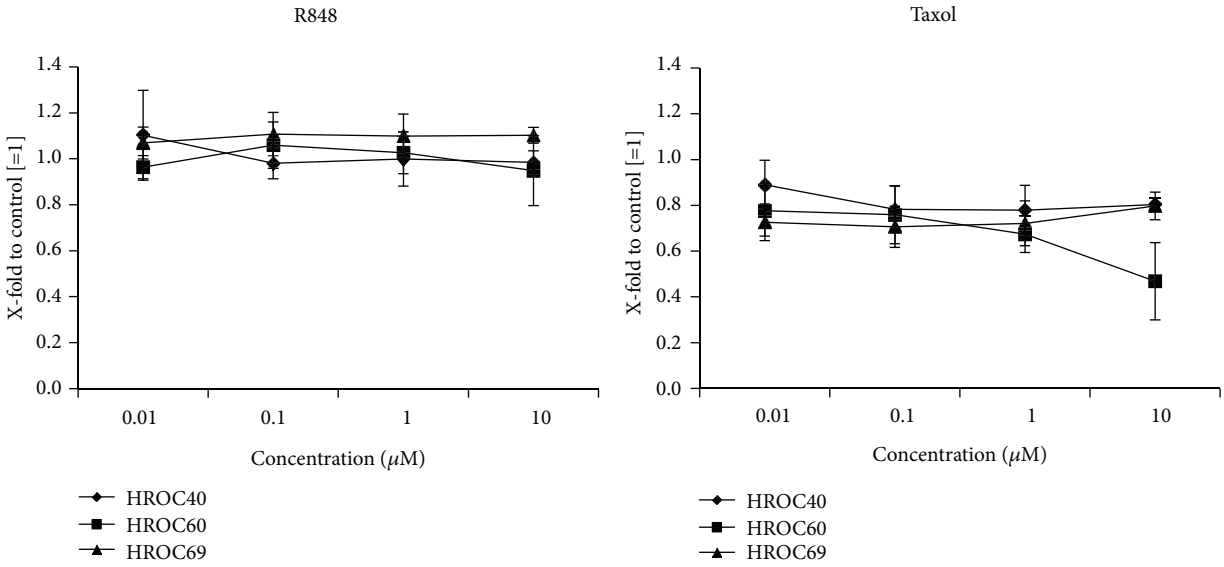
2.8. Statistical Analysis. All values are expressed as mean ± SE for *in vitro* data and mean ± SEM for tumor growth data. After proving the assumption of normality, differences between controls and experimental samples were determined by using the unpaired Student's t-test. If normality failed, the nonparametric Mann-Whitney U-Test was applied. The tests were performed by using Sigma-Stat 3.0 (Jandel Corporation, San Rafael, CA, USA). The criterion for significance was set to $P < 0.05$.

3. Results

3.1. TLR Expression on CRC Cell Lines. As a starting point for this study, the expression of TLRs was analyzed by qPCR on a set of ultra-low-passage CRC cell lines established in our lab. According to the TLR ligands chosen for the subsequent functional analyses, TLR3 (Poly I:C), TLR4 (LPS, Taxol), TLR7, and TLR8 (both R848) were examined (Table 1). TLR8 was not expressed at all, TLR7 was expressed at low levels by all cell lines; TLR4 showed moderate expression in HROC40, HROC60, and HROC69 cells compared to expression patterns of DCs. Similarly, TLR3 expression varied between cells.

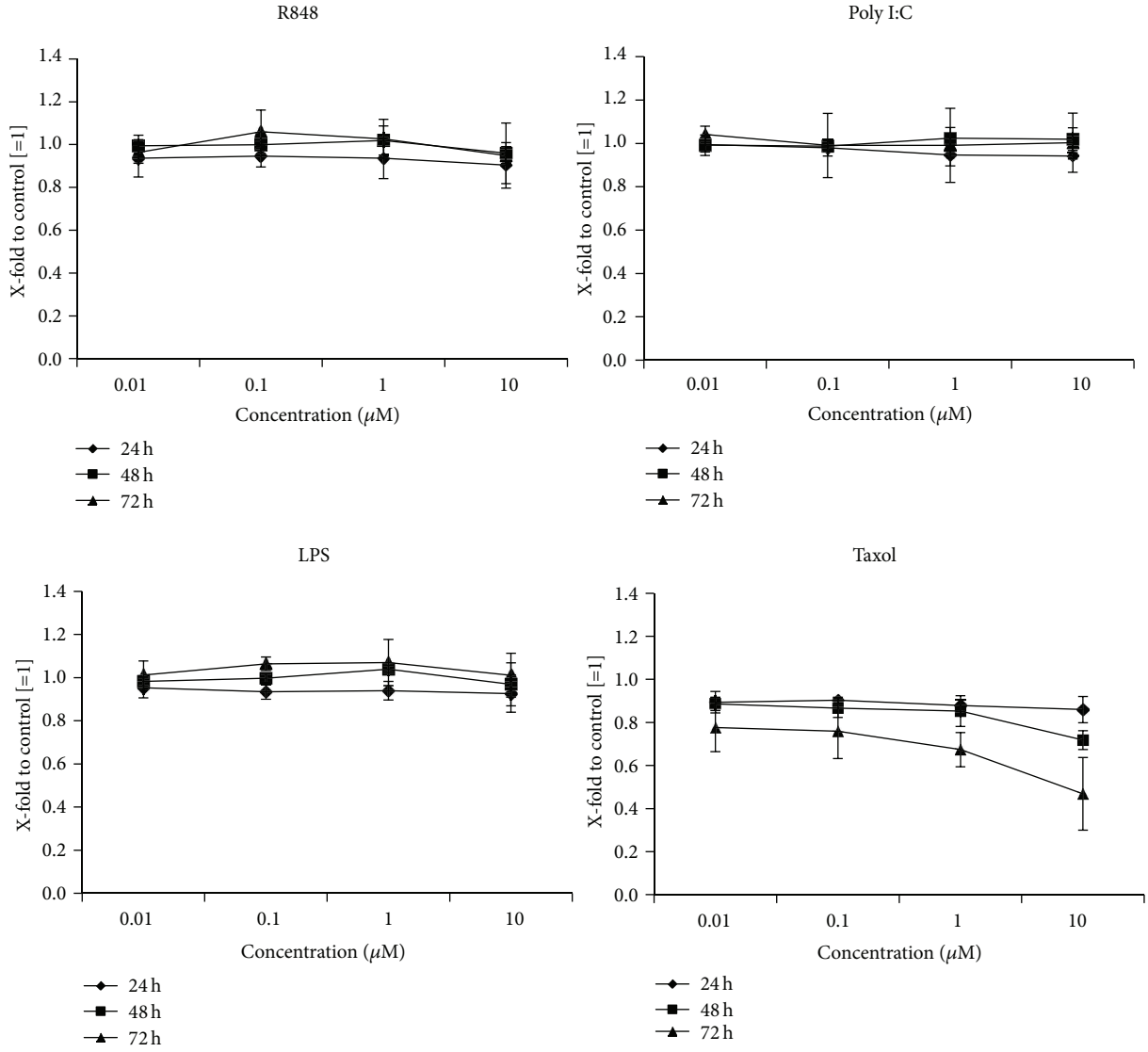
3.2. Direct Effects of TLR Ligands on CRC Cells. To evaluate direct effects of TLR ligands R848, LPS, Poly I:C, and Taxol on CRC cells, the three primary tumor cell lines HROC40, HROC60, and HROC69 were treated with increasing concentrations, ranging from 0.01 μM to 10 μM. Readout was performed after 24, 48, and 72 hours using a standard MTS assay. In each experiment and at every given time point, untreated cells served as controls.

The ligands for TLR7/8 (R848), TLR4 (LPS), and TLR3 (Poly I:C) exerted no significant antiproliferative effects (data not shown and exemplary results for R848 after 72 hours in



(a)

(b)



(c)

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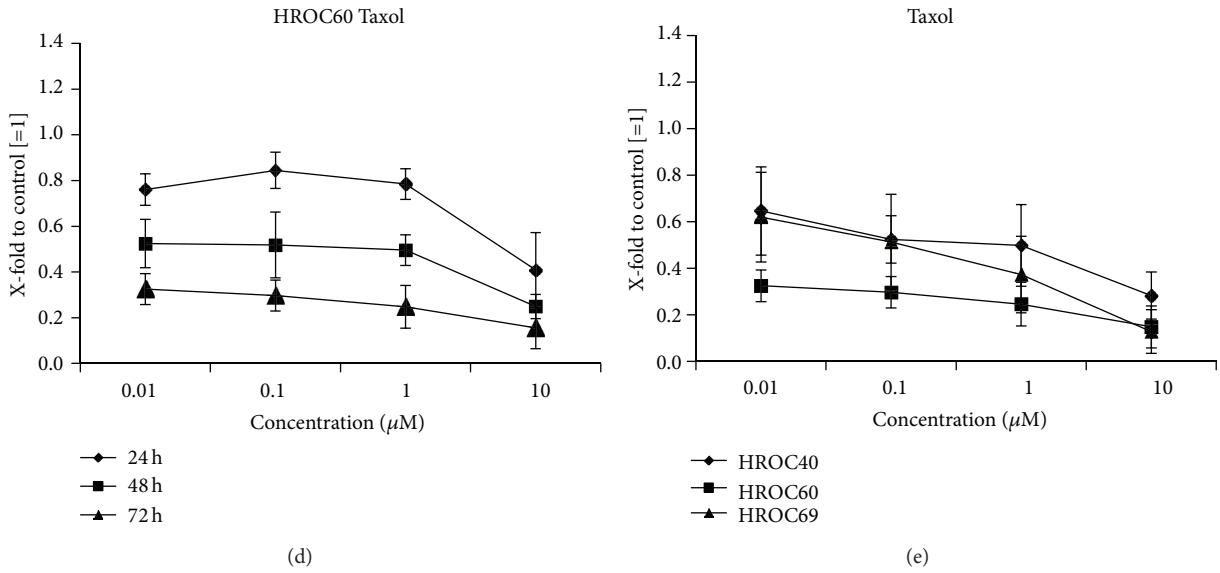


Figure 1: Direct cytotoxicity of TLR ligands towards CRC cell lines. Tumor cells were treated with increasing concentrations of the TLR ligands (a) R848 and (b) Taxol for 72 h. Cell viability was assessed by standard MTS assay. Results for the HROC60 cell line reached statistical significance ($P < 0.05$ versus control). (c) HROC60 cells were treated with TLR ligands for 24, 48, and 72 h with different concentrations (0.01–10 μM). (d) HROC60 cells were treated with Taxol for 24, 48, and 72 h with different concentrations (0.01–10 μM). Antitumoral effects were determined by a flow cytometric assay. Given results reached significance ($P < 0.05$ versus control). (e) The effect of increasing Taxol concentrations on CRC cell lines was assessed after 72 h incubation by flow cytometry. Results for HROC60 cells reached statistical significance at all concentrations; significant growth inhibition of HROC40 and HROC69 cells was obtained at 10 μM and 1 μM ($P < 0.05$ versus control). Untreated cells without TLR ligand were set as 1 and all data are given as X-fold increase. Experiments were performed in duplicate and repeated at least three times. Values are given as mean ± SD; $P < 0.05$ versus control; t-test.

Figure 1(a)). Taxol was the only cell growth inhibiting drug (Figure 1(b)); however, HROC40 and HROC69 displayed nearly complete resistance but in the highest dose (10 μM), Taxol inhibited growth of HROC60 cells up to >50%. The metabolic activity was determined by a MTS assay and generally tended to decrease in a time- and dose-dependent manner (Figure 1(c)).

To prove these data, number and viability of CRC cells were analyzed after TLR ligand treatment by a flow cytometric assay. In principle, this test confirmed the MTS data in that Taxol was the only TLR ligand tested with direct antitumoral potential. Again, a clear time and dose dependency was observed in comparison to untreated control cells (Figure 1(d)). Antitumoral effects were generally more pronounced when compared to the results of the MTS assays. And here, Taxol exerted effects not only towards HROC60, but also against all three cell lines tested (Figure 1(e)).

To analyze if any synergism of the TLR ligands on direct antitumoral effects can be observed, all possible combinations of the substances were tested in the lowest concentration (0.01 μM). Readout was again performed by flow cytometry measuring the proportion of living cells in comparison to untreated controls. The antitumoral effect of Taxol towards HROC69 and HROC60 was slightly increased by any additional substance (Figures 2(a) and 2(b)). However, no increase could be observed for the cell line HROC40 (Figure 2(c)). Incubation with three or four substances showed no further enhancement of this effect (Figure 2). Similar to the results of the single agents, none of the combinations without Taxol exerted any antitumoral effect on the tumor cell lines (Figure 2).

3.3. Immune Stimulation by TLR Ligands. TLR ligands exert direct immune stimulatory effects. To further elucidate their impact on PBLs in our setup, we performed a series of *in vitro* experiments. PBLs were either stimulated with single substances (all concentrations) or their combinations (each 0.01 μM). As expected, TLR ligands directly stimulated immune cells. In detail, most pronounced effects were observed for R848. This substance activated immune cells in a dose-dependent manner (Figure 3(a), upper panel). Numbers of $CD25^+$ and $CD69^+$ activated cells increased upon TLR stimulation (Figure 3(a)). Likewise, proportions of $CD16^+CD56^+$ were elevated (Figure 3(b)). Hence, NK cells were identified as the main responding cell population. Poly I:C and Taxol exerted weaker though still stimulating effects, however, only at low concentrations. By contrast, LPS-mediated influences on PBLs could largely be neglected. When analyzing TLR ligand combinations, no further boost of immune stimulation was observed.

3.4. Enhancement of TLR Ligand Mediated In Vitro Effects by Lymphocytes. The above results demonstrated no effects of the TLR ligands R848, LPS, and Poly I:C but a strong influence of Taxol on CRC cells. Since the main antitumoral effects of TLR ligands are likely to base on immune stimulation, we

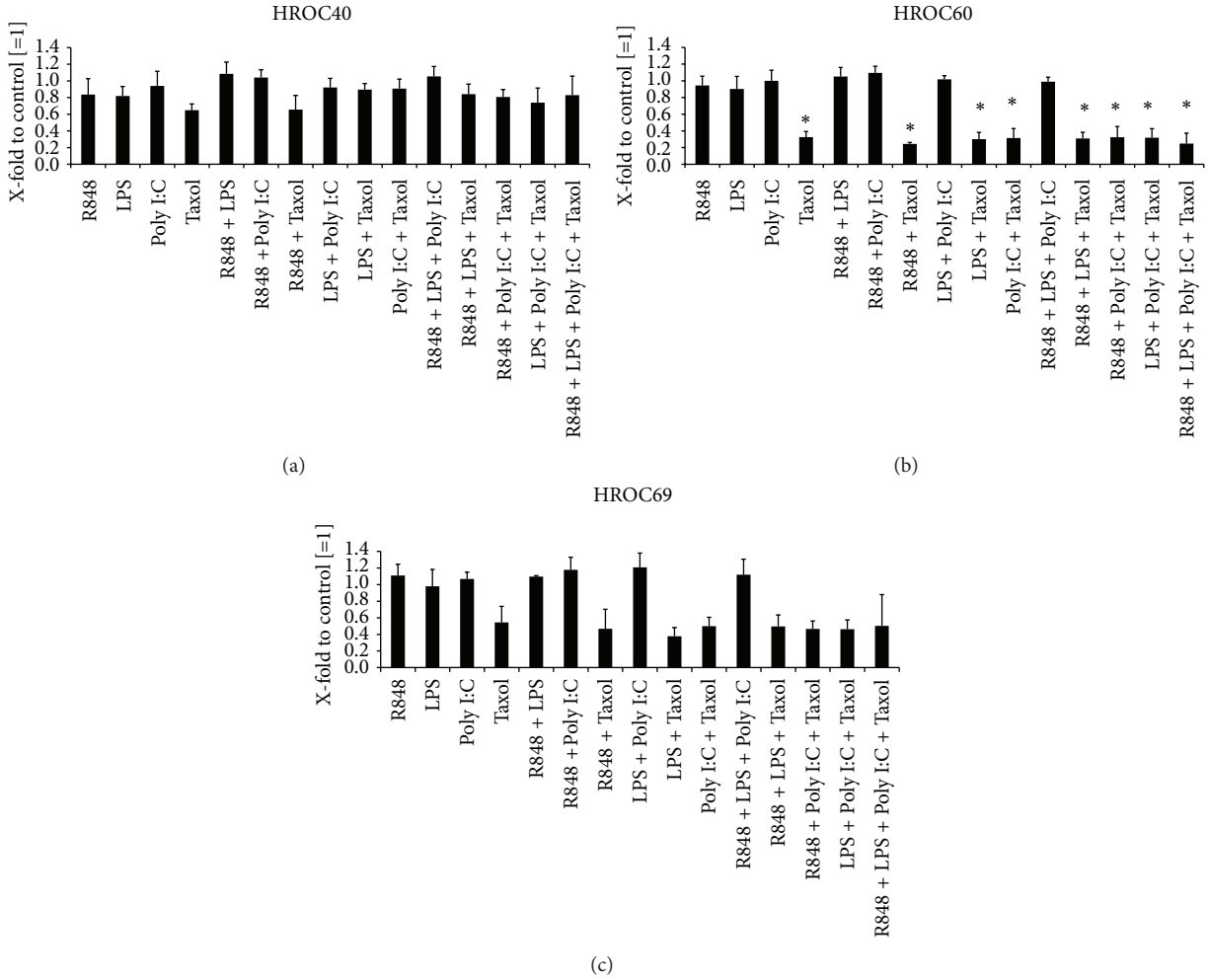


Figure 2: Direct cytotoxicity of TLR ligand combinations towards CRC cell lines. (a) HROC40, (b) HROC60, and (c) HROC69 cells were treated with all possible combinations between R848, LPS, Poly I:C, and Taxol for 72 h in a concentration of 0.01 μM. Antitumoral effects were determined by flow cytometry. Results after treatment with single TLR ligands (0.01 μM) are additionally shown. Untreated cells without TLR ligand were set as 1 and all data are given as X-fold increase. Experiments were performed in duplicate and repeated at least three times. Values are given as mean + SD; $P < 0.05$ versus control; t-test.

next analyzed the effects of TLR-stimulated immune cells on CRC cell lines. The latter were coincubated with PBL (ratio 100 : 1, PBL to tumor cell) from five healthy volunteers in the presence of TLR ligands (0.01 μM–10 μM). Tumor cells alone and together with PBL served as controls.

After 48 and 72 h of incubation, numbers of surviving tumor cells were determined using flow cytometry. Two of the donor's PBL showed strong reactions towards the CRC cell lines even in the absence of TLRs which must be considered as alloreactivity. Consequently, these were excluded from further analysis. Additionally, the results from the experiments without PBL addition are given to simplify comparability (Figure 4).

Activation of immune cells by R848 resulted in strong cytotoxicity towards HROC69 (76%–93% killing versus control (tumor cells + PBL); Figure 4(a)). In this setting, a strong dose dependency could be observed with favorable effects in higher concentrations (data not shown). The CRC cell line HROC60 was moderately affected by R848 while HROC40 showed no susceptibility to immune-mediated antitumoral effects. The TLR ligand LPS exerted no effect on tumor cells in this coculture experiment. Poly I:C treatment led to reduced cell numbers when incubated with PBL and HROC69 (versus control: tumor cells + PBL). Interestingly, addition of PBL to tumor cells in the presence of the chemotherapeutic agent Taxol mediated strong oncopathic effects (Figure 4(a)). Numbers of surviving tumor cells fell to 5% compared to the control (tumor cells + PBL), and hence this coculture setting even enhanced the strong cytotoxic effects achieved by monotherapy (for comparison, please see Figure 1(d)).

Taken together, these data indicate elevated antitumoral effects by TLR ligands due to immune cell stimulation. However, tumor-cell-specific differences in vulnerability towards immune-mediated lysis were aparent. Taxol was the only substance leading to appreciable levels of cell number reduction for all cell lines.

Next, combinations of TLR ligands were added to tumor and immune cells. To identify potential synergistic effects, all

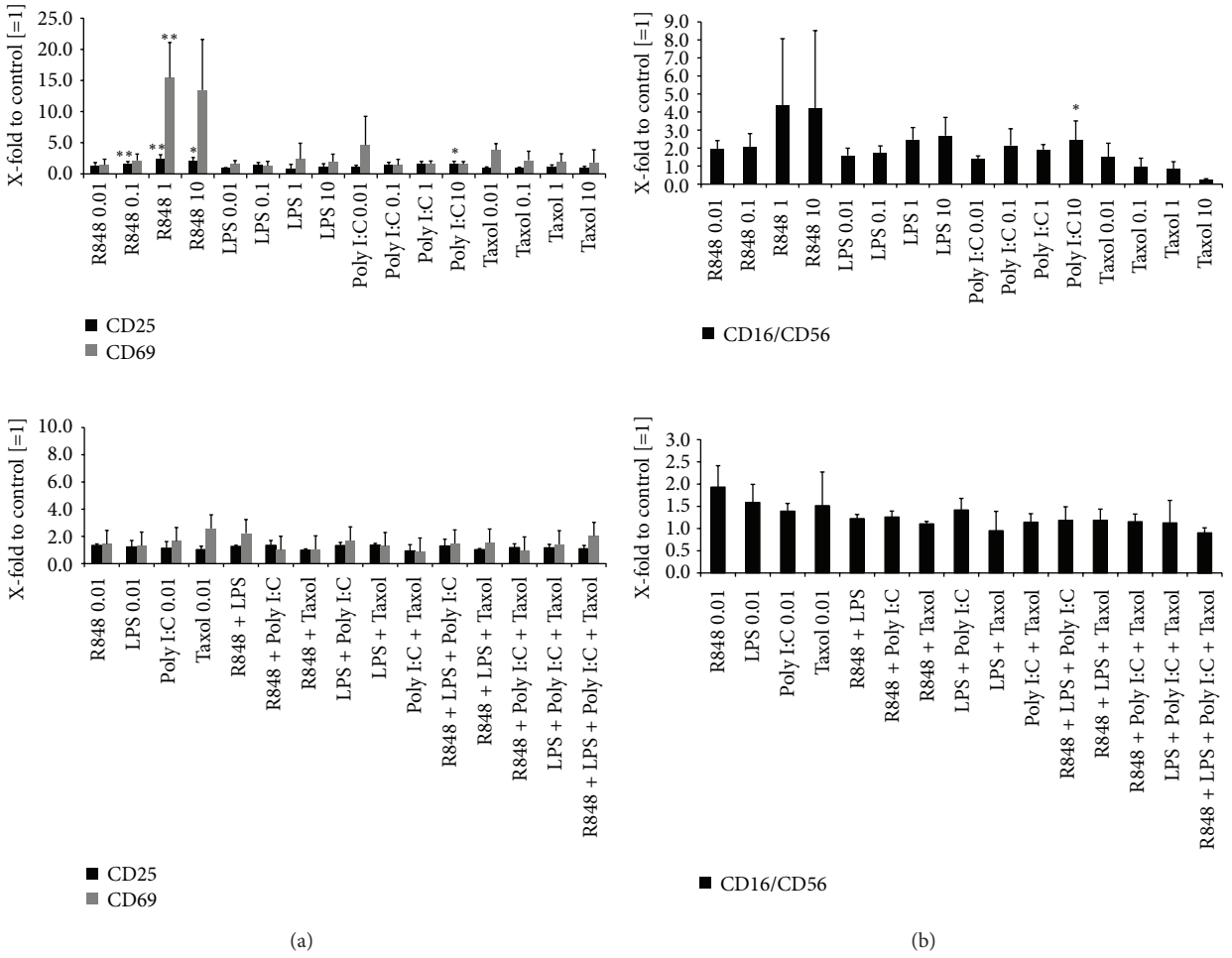


FIGURE 3: Flow cytometric phenotyping of TLR ligand stimulated PBLs. PBLs were either incubated with single TLR ligands (0.01 μM; (a) and (b) upper panel) or in the presence of TLR ligand combinations (0.01 μM; (a) and (b) lower panel) for 72 h. (a) Activation of PBLs following TLR stimulation as given by positive staining for CD25 and CD69. (b) $CD16^+CD56^+$ NK cells were the main responding cell population. Data of untreated control PBLs were set as 1 and all data were given as X-fold increase. Values are presented as mean + SD; $P < 0.05$ versus control; *t*-test.

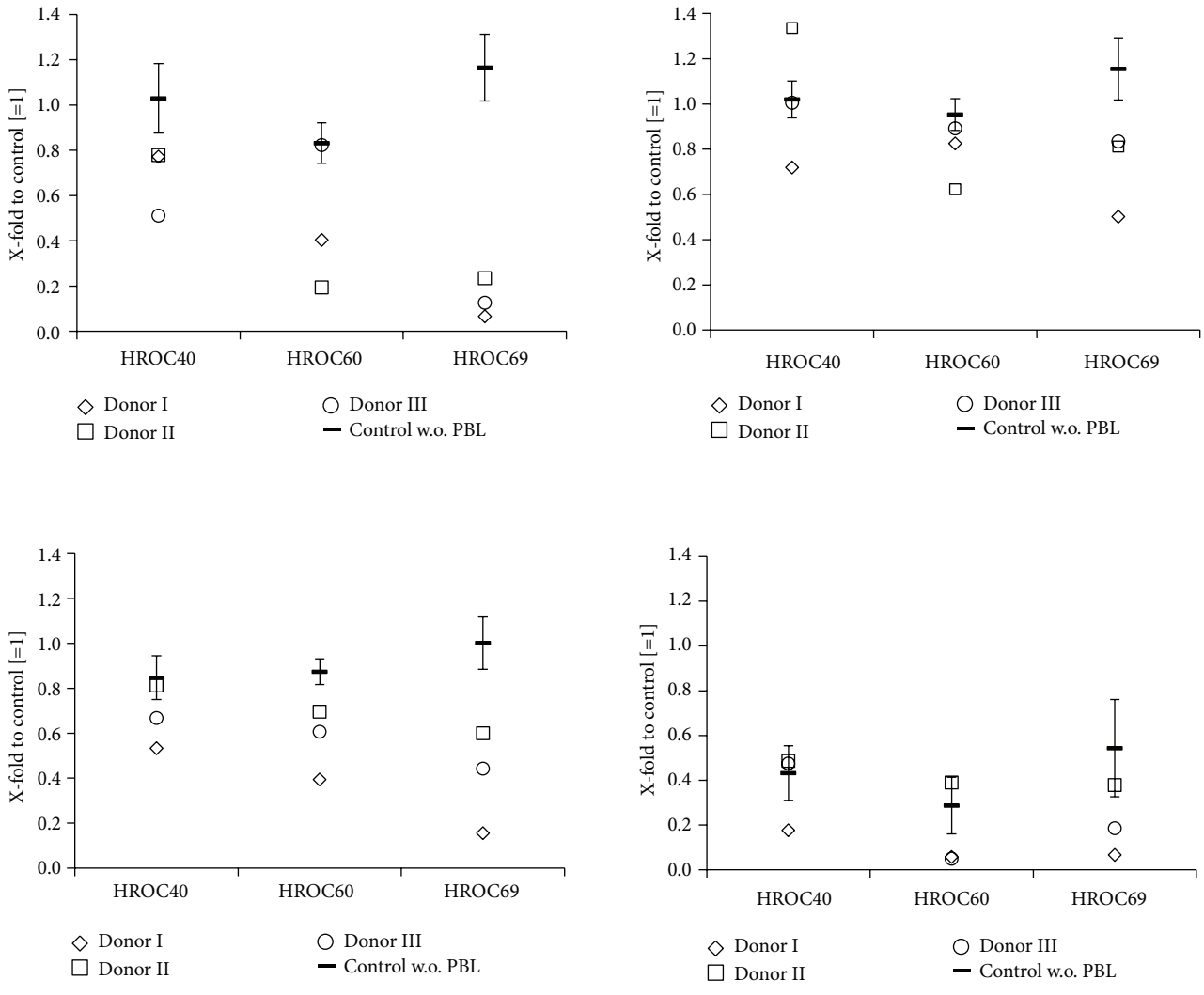
possible TLR ligand combinations were considered (concentration of each TLR ligand: 0.01 μM). Exemplary results are given in Figure 4(b). In this setting, most pronounced effects were obtained after Taxol/Poly I:C treatment, which was, however, comparable to Taxol monotherapy. Hence, TLR-combinations did not enhance immune-mediated oncolysis. By contrast, some combinations even tended to dampen antitumor responses (e.g., R848 + Poly I:C).

3.5. Impact of TLR Ligands on CRC Tumor Growth In Vivo. To finally prove the antitumoral effects of TLR ligands on CRC, an *in vivo* experiment was performed using the well-established CT26 tumor model. *In vitro*, this murine CRC cell line was sensitive towards Taxol but to no other TLR ligand used in this study (data not shown).

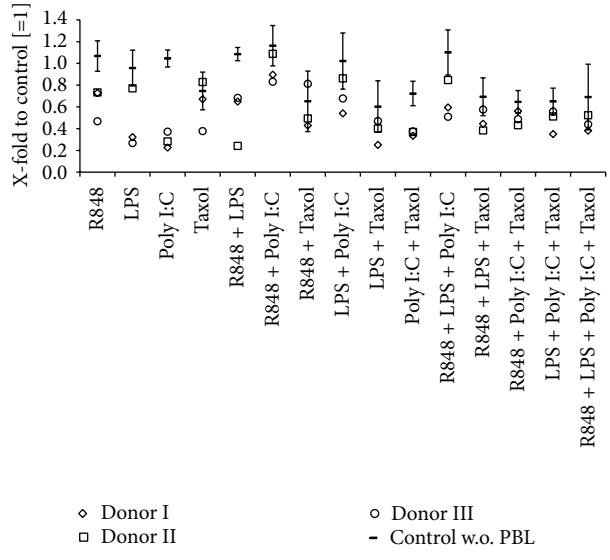
When tumors reached 50–100 mm^3, experimental treatments were performed by biweekly i.p. applications of Taxol, R848, LPS, or TLR combinations (Figure 5). In order to better appraise TLR-mediated growth inhibition in this model, one group of animals was treated with the topoisomerase I inhibitor Irinotecan, a clinically approved drug that is standard to treat CRC patients. Control mice received equivalent volumes of the solvent alone (saline).

All treatment protocols, except two (Poly I:C and the combination of Taxol + LPS), mediated at least slight growth inhibition. R848 had strongest antitumoral potential within the TLR ligand monotherapy groups (compared to Taxol > LPS, versus control). Rather unexpectedly, Poly I:C exhibited a very strong tumor growth promoting activity and animals had to be redeemed by suffering at day 7 of therapy. The strongest antitumoral effects were obtained following Taxol and R848 therapy. This combination was even better in controlling tumor growth than Irinotecan. The combination of R848 + LPS slightly prevented tumor growth. However, no additive or synergistic effects could be obtained compared to single LPS or Taxol therapy.

3.6. Correlation of Immune Status with Course of Tumor Growth. Finally, the involvement of the immune system in



(a)



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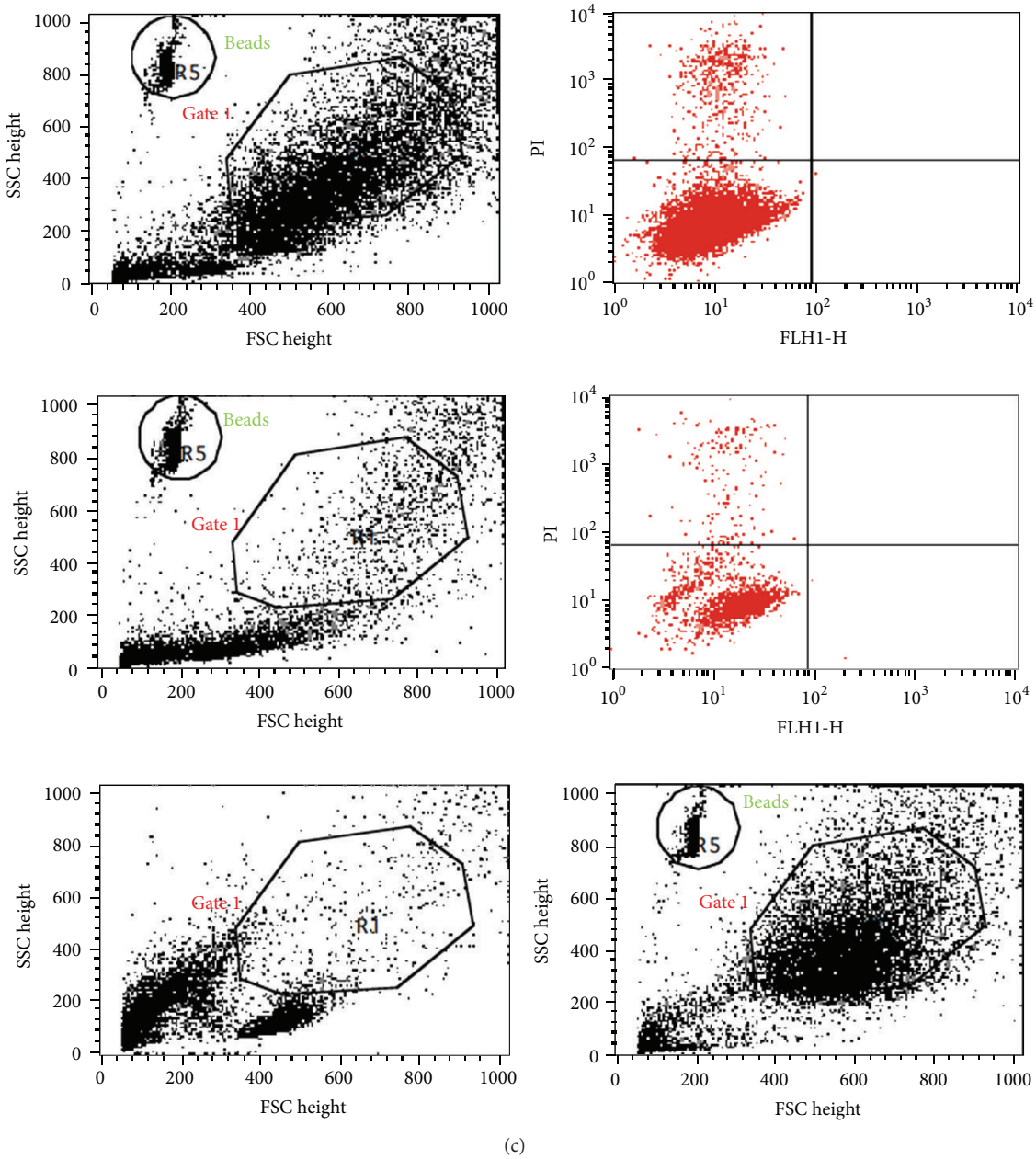

Figure 4: Coculture experiments. Tumor cells were cocultured with PBLs of three different healthy donors in the presence of (a) single TLR ligands (10 μM) or in the presence of (b) TLR ligand combinations (0.01 μM) for 72 h. Thereafter, numbers of viable tumor cells were quantified by flow cytometry using microsphere beads as calibrator. Tumor cells without TLR ligand were set as 1 and all other data were given as X-fold increase. (b) Results for single substance treatment (0.01 μM) are additionally shown. ((a), (b)) For each approach, cells treated with the particular TLR ligand but without PBL are shown. (c) Representative dot plot illustrating the gating strategy for quantification of tumor cells. Shown are FACS data of HROC69 cells treated with PBL alone (control, upper panel) and treated with PBL + R848 (10 μM, middle panel) for 72 h. Dead cells present in the gated tumor cells (Gate1) were excluded by staining with propidium iodide (PI^+ cells; upper left quadrant in the right upper and middle blots). To illustrate the reliability of the gating-based separation of tumor cells and lymphocytes after coculture, FACS data of PBL alone and HROC69 cells alone are shown in addition (lower panel).

tumor growth control *in vivo* was examined. Blood samples from treated and control animals were analyzed on day 10 after start of therapy (Figure 6(a)) and upon therapy completion at day 17 (Figure 6(b)). Additionally, potential activation of immune cells in spleens of treated animals was studied (Figure 6(c)).

None of the treatment protocols mediated significant immunological changes, except for a massive increase in activated circulating $CD166^+$ (ALCAM) immune cells accompanied by decreased levels of CD62L (L-selectin) cells. Especially significant elevations of $CD166^+$ cells could be observed in the blood after 10d for treatment with Irinotecan

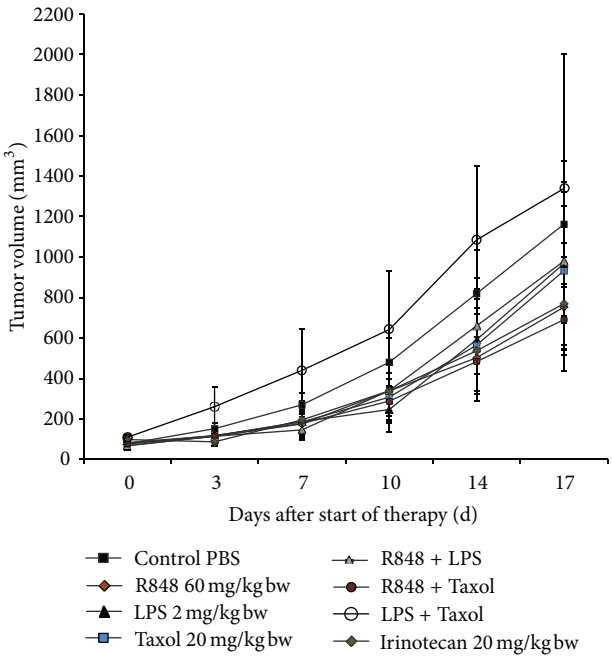

Figure 5: Tumor growth control *in vivo*. Growth kinetics of CT26 tumors in animals following injection of TLR ligands, their combinations, or Irinotecan. Therapy was performed by repetitive i.p. application of substances twice a week for a total of six times ($n = 7$). Control animals received equivalent volumes of PBS ($n = 7$). Values are given as mean ± SEM. None of the treatments reached statistical significance (P values d17 versus control: R848 0.232; Taxol 0.655; LPS 0.705; Irinotecan 0.340; R848 + Taxol 0.252; R848 + LPS 0.680; Taxol + LPS 0.789).

($P = 0{,}032$) and R848 + LPS ($P = 0{,}035$) as well as for LPS ($P = 0{,}004$) at day 17 in the spleen. Additionally, CD62L elevation achieved significance at day 17 in the spleen for the treatment groups R848 + Taxol ($P = 0{,}002$) and Taxol + LPS ($P = 0{,}038$). Both markers indicate T-cell activation. In case of L-selectin, proteolytic cleavage of cell surface molecules (=L-selectin shedding) or downregulation on the mRNA level possibly explains this observation best. Interestingly, this finding correlates with the antitumoral *in vivo* results, indicating involvement of these cell populations in tumor growth control.

However, we did not observe any other immune stimulatory effects at the given time points.

4. Discussion

In recent years, the old concept of fighting tumors with microbial agents has been rekindled by us and others [15–20]. This idea is to induce tumor regression both by direct oncolysis and indirectly through immune stimulation. To develop the approach further, we here explored the potential of defined TLR ligands as therapeutic agents. Therefore, we chose ligands for TLR3 (Poly I:C), TLR4 (LPS, Taxol), TLR7, and TLR8 (both R848). Those TLR ligands underwent extensive clinical investigations, clinical investigations. However, most studies focused on the immunostimulatory capacity of these molecules and their application as adjuvants along with tumor and virus vaccines [21–23]. Mechanistically, TLR ligands exert their antitumoral effect via activation of several cell types, including DCs and T cells [24, 25]. Due to their supposed direct antitumoral potential, TLR ligands are now tested as immunotherapeutic agents as well [26].

First, expression of relevant TLR receptors was analyzed on our freshly established, ultra-low-passage CRC tumor cell lines. Though expression pattern differed between cell lines, three out of the four receptors were detected (TLR3, 4, and 7). As expected, expression levels were comparably low (i.e., versus immune cells). By using cell lines in low passage (<40), most characteristics of primary tumor cells are retained. In those, varying TLR patterns have been found not only among normal/neoplastic cells (e.g., upregulation of TLR3/4 in tumors), but also within a single tumor [27]. However, this implies an important albeit unclarified role of TLRs in CRC biology. Therein, TLR expression is special, since the colon is continuously subjected to bacterial antigens and live bacteria. There is a fine line between physiological homeostasis of the commensal flora and stimulating tumor growth under conditions of chronic inflammation [28].

To shed light on the direct effect of TLR activation, primary CRC cells were here treated with the respective TLR ligands. Those were applied as single agents or in combinations to (i) mimic whole bacteria/viruses and (ii) to analyze if any synergisms in antitumoral action can be achieved. None of the employed substances mediated significant growth inhibition or tumor apoptosis/necrosis—neither as single agent nor in combination—some of them even tended to promote tumor growth (e.g., R848 + LPS; Figures 1 and 2). The sole exception was Taxol, a widely-used chemotherapeutic drug that additionally binds to TLR4. This finding is somehow interesting, since TLR4 expression often correlates with chemoresistance and metastasis [29]. Though we also observed interindividual differences, all cell lines responded to this drug (Figure 1). Antitumoral effects were slightly enhanced by adding another TLR ligand (Figure 2). However, combinations without Taxol did not mediate growth inhibition. This observation fits well with recent data from the literature. Emerging evidence suggests a dual role of TLR ligands, in which they simultaneously trigger both pro- and antitumoral effects depending on the applied molecule [7, 12, 13, 30]. In multiple myeloma cells, induction of autocrine interleukin-6/-18 production accounts for enhanced proliferation upon TLR activation [30, 31]. O'Leary and coworkers even described increased metastatic potential of colon cancer cells upon LPS stimulation via Nox1-mediated redox signaling [32]. By contrast, flagellin-induced TLR5 activation mediates tumor regression [33].

Despite their conflicting direct effects on tumor cells, TLR ligands are, to the most part, potent immune stimulators. In line with this well-established fact, we here observed boosted antitumoral effects in an *in vitro* coculture setting resembling aspects of a competent immune system (Figure 3). Effects were cell-line and substance specific—HROC40 cells generally tended to be more resistant than the other cell lines. Of note, R848 and Taxol proved most effective in stimulating immune-mediated tumor cell lysis, while LPS and Poly

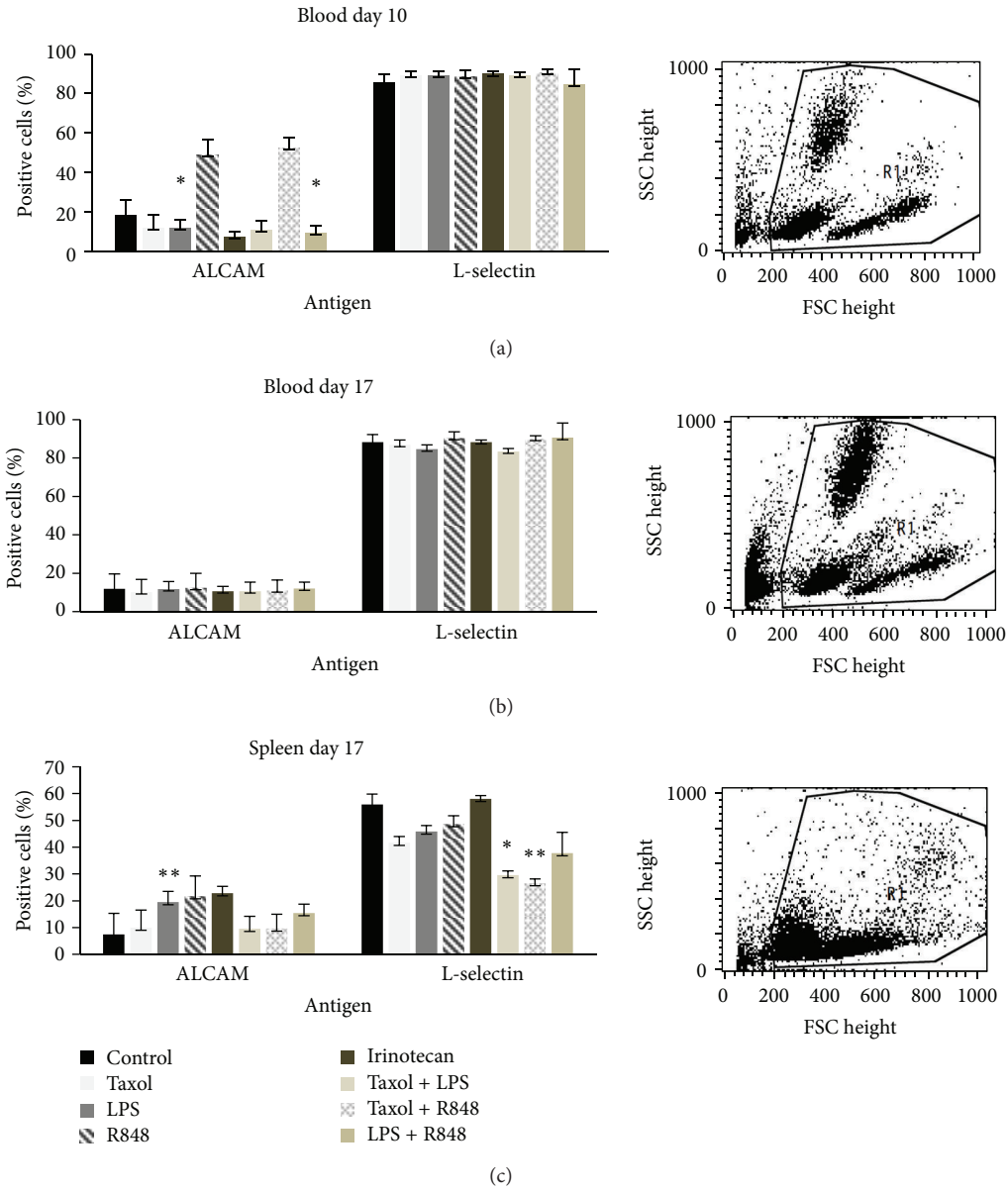

Figure 6: Analysis of leukocytes (a) during therapy and ((b), (c)) after necropsy from Balb/c mice. Blood samples of animals were taken on day 10 after start of therapy. At necropsy, blood samples and spleens were obtained and analyzed by flow cytometry. Given are the percentages of CD166 and CD62L positive cells. Control animals received equivalent volumes of PBS ($n = 7$). Values are given as mean + SD; $P < 0.05$ versus saline; t-test. Significant data for CD166 (ALCAM) were achieved in the blood at d10 in animals treated with Irinotecan ($P = 0.032$) and R848 + LPS (0.035) and additionally in the spleen at d17 in the LPS group ($P = 0.004$). For CD62L (L-Selectin), significance was reached in the spleen at d17 for R848 + Taxol ($P = 0.002$) and Taxol + LPS (0.038).

I:C did not work in our hands. Their supposed immune-stimulating potential was presumably not strong enough to negotiate the tumor cells' natural immunosuppressive capacity [34].

The TLR7/8 activator R848 is clinically approved for immunotherapy of skin tumors [35]. In patients, R848 treatment induces inflammatory cytokine secretion by macrophages and myeloid DCs as well as IFN-α release by plasmacytoid DCs [36]. Additional mechanisms include activation of NK cells. Besides, Taxanes mediate immunostimulatory effects against neoplasms, supporting the idea of a TLR ligand tumor vaccine. Experiences gathered in clinical studies demonstrated that Taxol enhances NK- and lymphokine activated killer cell functions [37]. The observed oncolytic effects in the present study were most likely also due to activation of NK or NK-like cells, whose tumor attacking potential is widely accepted [38–40].

To test this theory, immunotherapy with TLR ligands was performed in a syngeneic tumor model. Mice challenged with murine CT26 tumor cells received repetitive injections

of TLR ligands. The route of application and intervals of treatment have been found to be crucial for an effective therapeutic schedule [36]. Topical application of TLR ligands is usually highly effective, whereas their systemic application met with limited success [41, 42]. However, for potential clinical application in CRC patients, systemic, repetitive injection is the method of choice. With this regimen, we observed at least partial growth retardation in our tumor model (Figure 4). Of note, monotherapy with R848 was as effective as Irinotecan, a first- and second-line standard therapeutic for advanced or recurrent CRCs [43]. The best combination used here was made of Taxol and R848, yielding >50% growth inhibition. Antitumoral effects were accompanied by massively increased levels of activated circulating $CD166^+$ immune cells, that is, activated T cells and monocytes (Figure 5). This finding is consistent with the *in vitro* coculture results on human CRC and immune cells. Based on these *in vitro* findings, far better antitumoral results may be expected when testing this treatment approach in humanized mice, since TLRs are differently expressed in mice and humans [44]. This was also true for our human and mouse CRC cell lines. The exact mechanism of how Taxol and R848 act complementary and mutually reinforce antitumoral responses remains elusive. One may speculate that Taxol primarily inhibits direct tumor growth by interfering with the cell cycle. R848 on the other hand stimulates the immune system (primarily $CD166^+$ cells). Both agents boost antitumoral immune responses that finally control tumor growth.

A rather unexpected finding of the current study was the tumor-promoting activity of Poly I:C and the combination of Taxol + LPS. This was evident from the beginning of therapy. Tumors rapidly grew, became necrotic, and tended to ulcerate. In case of Taxol + LPS, this may best be explained by some kind of antagonism, in which both substances compete for the same TLR or intracellular signaling. Also, tumor or immune cells may respond with secretion of tumor-growth-promoting and immunosuppressive cytokines (e.g., IL10) [45]. These mechanisms abrogate the antitumoral effects of the single substances and strengthen tumor development.

Therefore, TLR tolerance, characterized by a state of immune unresponsiveness, can be waived [36]. Moreover, since this was in contrast to the *in vitro* results, we can only speculate that the reasons for fostering of *in vivo* tumor growth by combinatorial treatment with these agents lie in the specific inter- and intracellular environment or may partly be attributable to the differences between human and mouse TLRs.

Lastly, though TLR ligands are critical for first-line tumor therapy, there are many arguments in favor for their immunotherapeutic application: (i) single substances or combinations are ideal immune stimulators: both antigen-presenting (especially DCs) and effector cells ($CD8^+$ T and NK cells) are functionally activated; (ii) conjugation to antigenic peptides is technically easy to perform; (iii) antibody-mediated cellular cytotoxicity is enhanced by increasing Fc-γ receptor expression; and thus treatment with monoclonal antibodies might be improved; and (iv) given their synthetic nature, they can be produced under GMP conditions and as a matter of fact, most ligands are already clinically approved.

5. Conclusion

Data presented herein prove the therapeutic potential of TLR agonists mediating both tumor inhibition and activation of immune effectors. Thus, they are very promising candidates for optimization of immune-based therapies, including applications as single agents or in combinations for active unspecific therapies, adjuvant standard regimens or in addition to cell-based immunotherapies. Our data also concern the Janus face character of TLR agonists and subsequent studies will further elucidate the exact balance between pro- and antitumoral activities of TLR agonists as single agents but especially of combinations.

Abbreviations

PBL: Peripheral blood lymphocytes
TLR: Toll-like receptor
CRC: Colorectal carcinoma
DC: Dendritic cell.

Authors' Contribution

Saskia Stier and Claudia Maletzki contributed equally to this work.

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Research Article

Haemophilus parasuis Subunit Vaccines Based on Native Proteins with Affinity to Porcine Transferrin Prevent the Expression of Proinflammatory Chemokines and Cytokines in Pigs

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The expression of chemokines (CCL-2 and CXCL-8) and cytokines (IL-1α, IL-1β, IL-6, TNF-α, and IL-10) was evaluated by RT-qPCR in colostrum-deprived pigs vaccinated and challenged with *Haemophilus parasuis* serovar 5. Two vaccines containing native proteins with affinity to porcine transferrin (NPAPTim and NPAPTit) were tested, along with two control groups: one inoculated with PBS instead of antigen (challenge group (CHG)), and another one nonimmunized and noninfected (blank group). The use of NPAPTim and NPAPTit resulted in complete protection against *H. parasuis* (no clinical signs and/or lesions), and both vaccines were capable of avoiding the expression of the proinflammatory molecules to levels similar to physiological values in blank group. However, overexpression of all proinflammatory molecules was observed in CHG group, mainly in the target infection tissues (brain, lungs, and spleen). High expression of CCL-2, CXCL-8, IL-1α, IL-1β, and IL-6 can be considered one of the characteristics of *H. parasuis* infection by serovar 5.

1. Introduction

During infection *Haemophilus parasuis* needs to establish replicative niches in a host that possesses a robust innate immune system, which is an evolutionary ancient form of host defense in multicellular organisms [1]. When *H. parasuis* succeeds in overcoming this response via depletion of specific cell populations [2], Glässer's disease, a severe systemic disorder, develops in pigs.

Proinflammatory cytokines, such as interferon-α (IFN-α), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8, are produced during the acute stage of bacterial infection [3–5] and play a pivotal role in the modulation and orchestration of the innate immune response. Each of these molecules has a number of different local and systemic effects, such as macrophage activation [6], increase in adhesion molecules on the vascular endothelium [7, 8], induction of acute phase proteins [9], and neutrophil chemotaxis and activation [10]. Some bacterial molecules as LPS can induce the production of proinflammatory cytokines, which results in fever, hypotension, inadequate tissue perfusion, metabolic acidosis, septic shock, and organ failure [11]. In order to regulate the severity of the inflammatory response, immune system has some cytokines, such as IL-10, which are capable of inhibiting a large amount of cytokines and chemokines secreted by macrophages and activated monocytes [12, 13].

Little is known about the interaction between *H. parasuis* and cytokine expression in pigs, which hinders the understanding of Glässer's disease pathogenesis and the development of effective vaccines. We evaluated in this study the capability of a subunit vaccine to inhibit the transcription of a set of chemokines and cytokines directly related to inflammation, in systemic and target infection organs. Colostrum-deprived pigs [14] were used as animal model in order to avoid the endemic colonization by *H. parasuis* in the upper respiratory tract of suckling piglets that have contact with their sows.

2. Materials and Methods

2.1. Vaccines, Experimental Groups and Immunization. Two vaccines based on native proteins with affinity to porcine transferrin (NPAPTim and NPAPTit) [15] were compared. NPAPTim contained 400 μg of NPAPT antigen adjuvanted with Montanide IMS 2215 VG PR (Seppic Inc., Paris, France) in a 1 : 4 ratio. NPAPTit contained the same antigen but potentiated with neuraminidase from *Clostridium perfringens* (type VI) at a concentration of 100 mU/mL. Twenty colostrum-deprived pigs were randomly assigned to four experimental groups. The NPAPTim group ($n = 6$) received 2 mL of NPAPTim vaccine by intramuscular injection at 28 and 49 days of age, while the NPAPTit group ($n = 6$) received the same volume of NPAPTit vaccine by intratracheal injection at the same days. The challenge control (CHG) group ($n = 4$) received 2 mL of PBS intramuscularly at the same days. These three groups were challenged intratracheally at 63 days with a lethal dose (3×10^8 CFU) of *H. parasuis* Nagasaki strain (serovar 5). The fourth group ($n = 4$) comprised pigs that were maintained as nonimmunized, noninfected blank group. All experiments were conducted in accordance to the guidelines of the University of León Ethical Committee. Surviving pigs were humanely euthanized at 78 days. More details of this schedule have been already reported [15].

2.2. Sample Collection and RNA Extraction. This description is based on the MIQE guidelines [16]. After necropsy, small pieces (≤0.5 cm) from brain, lungs, spleen, and mediastinal, tracheobronchial, and mandibular nodes from each pig were collected aseptically and maintained in RNA later solution (Ambion-Applied Biosystems, Life Technologies, USA) overnight at 4°C. After centrifugation, the supernatant was discarded, and the samples were stored at −80°C. Total cellular RNA was purified from 30 mg of tissue of each sample, using the RNAspin Mini RNA Isolation Kit (GE Healthcare, Spain). Samples were maintained at −80°C in 40 μL of RNase free water. Immediately before performing the cDNA synthesis, the concentration and quality (integrity) of the total RNA were checked by a capillary electrophoresis system, using the Agilent 2100 Bioanalyzer and the RNA 6000 Pico Chip Kit. All RNA obtained had a RNA integrity number (RIN) higher than 7 (scale 1 to 10).

2.3. cDNA Synthesis. The first-strand cDNA synthesis was carried out by reverse transcription (RT) from total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen Life Technologies, USA). Briefly, 2 μg of total RNA was primed with 1 μL of random hexamers (50 ng/μL) and 1 μL of annealing buffer and was completed until 8 μL with RNase free water. It was incubated at 65°C for 5 min and then immediately placed on ice for 1 min. Afterwards, 10 μL of 2 × First-Strand Reaction Mix and 2 μL of SuperScript III/RNaseOUT Enzyme Mix were added to the tube on ice and incubated at 25°C for 10 min, at 50°C for 50 min and at 85°C for 5 min, to stop the reaction. The resulting cDNA was diluted 10 times and stored at −80°C until use.

2.4. Primers and Probes Design. The set of primers and hydrolysis probes used to determine the expression of chemokines (CCL2 and CXCL-8), cytokines (IL-1α, IL-1β, IL-6, TNF-α, and IL-10), and reference genes (GAPDH, β-actin, cyclophilin) were designed for this study or obtained from previous reports (Table 1). Hydrolysis probes were labeled with 6-carboxy-fluorescein (FAM) at the 5′ end and the Black Hole Quencher (BHQ_1 or BHQ_2) at the 3′ end.

2.5. Development of a PCR Internal Control. The cDNA obtained from tracheobronchial nodes of CHG group was amplified using the primers described in Table 1, including a final step of 72°C for 30 min. The PCR-specific product from every gene was cloned into the pGEM-T-Easy vector (Promega, Spain) according to the manufacturer's instructions.

2.6. Quantitative Real-Time PCR (qPCR) Analysis. Quantitative real-time PCRs were carried out in 96-well plates (LightCycler 480 Multiwell Plates 96, white, -Roche-) in a total volume of 20 μL containing 500 nM of each primer, 100 nM of each specific probe, 1 × Light Cycler Probes Master (Roche) and 5 μL of cDNA. The plates were sealing with an adhesive foil (Roche), and then the PCRs were run on a LightCycler 480 II System (Roche) using the following thermocycling conditions: an initial denaturation step (95°C, 10 min) followed by 50 cycles of 95°C for 10 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension). qPCRs were run in triplicate for better accuracy and reproducibility. No template controls (NTC) from RT and qPCR were additionally included. Calibration curves for each chemokine, cytokine and reference gene were obtained using tenfold serial dilutions of a plasmid DNA containing the specific nucleotide sequence (PCR internal control) of each gene. The relative quantification of each gene was calculated automatically by LightCycler Relative Quantification Software, using the ΔΔCT-Method (Roche). The PCR efficiencies are shown in Table 1.

2.7. Statistical Analysis. NPAPTim, NPAPTit, and blank groups, whose pigs remained alive until the end of the experiment, were compared with each other. CHG group was not included in the comparative analysis because these animals died at a different time point. Descriptive statistics (mean, standard error), normality (Shapiro-Wilk), and

Table 1: Oligonucleotides used in qPCR analysis.

Genes	Sense	Sequences (5′ to 3′)	Product	Efficiency (%)	Reference
IL1-α	→	GTGCTCAAAACGAAGACGAACC	110 bp	99.5 ± 0.5	Duvigneau et al. [30]
	←	CATATTGCCATGCTTTTCCCAGAA			
	→	**FAM**-TGCTGAAGGAGCTGCCTGAGACACCC-**BHQ1**			
IL1-β	→	CCAAAGGCCGCCAAGATATAA	75 bp	100 ± 0.2	Arce et al. [31]
	←	GGACCTCTGGGTATGGCTTTC			
	→	**FAM**-CTGACTTCACCATGGAAGTCCTCTCTCCCTAAG-**BHQ2**			
TLR-4	→	GCCATCGCTGCTAACATCATC	108 bp	99.7 ± 0.2	This study
	←	CTCATACTCAAAGATACACCATCGG			
	→	**FAM**-CAAAAGTCGGAAGGTTATTGTCGTGGTGTC-**BHQ1**			
IL-6	→	CTGGCAGAAAACAACCTGAACC	94 bp	98.6 ± 0.2	Duvigneau et al. [30]
	←	TGATTCTCATCAAGCAGGTCTCC			
	→	**FAM**-TTGAACCCAGATTGGAAGCATCCGTCTTTT-**BHQ1**			
IL-10	→	CGGCGCTGTCATCAATTTCTG	89 bp	100.4 ± 0.5	Duvigneau et al. [30]
	←	CCCCTCTCTTGGAGCTTGCTA			
	→	**FAM**-AGGCACTCTTCACCTCCTCCACGGC-**BHQ1**			
TNF-α	→	GCCCTGGTACGAACCCATCTA	91 bp	99.1 ± 0.3	Arce et al. [31]
	←	CAGATAGTCGGGCAGGTTGATCTC			
	→	**FAM**-CCAGCTGGAGAAGGATGATCGACTCAGT-**BHQ2**			
CCL-2	→	ACCAGCAGCAAGTGTCCTAAAG	92 bp	99.4 ± 0.7	Arce et al. [31]
	←	TCCTGGACCCACTTCTGCTT			
	→	**FAM**-AGCAGTGATCTTCAAGACCATCGCGG-**BHQ2**			
CXCL-8	→	TTCGATGCCAGTGCATAAATA	120 bp	99.8 ± 0.8	Arce et al. [31]
	←	TGACAAGCTTAACAATGATTTCTGAA			
	→	**FAM**-CATTCCACACCTTTCCACCCCAAATTTATC-**BHQ2**			
GAPDH	→	ACATGGCCTCCAAGGAGTAAGA	106 bp	99.2 ± 0.6	Duvigneau et al. [30]
	←	GATCGAGTTGGGGCTGTGACT			
	→	**FAM**-CCACCAACCCCAGCAAGAGCACGC-**BHQ1**			
cyclophilin	→	TGCTTTCACAGAATAATTCCAGGATTTA	77 bp	100.0 ± 0.1	Duvigneau et al. [30]
	←	GACTTGCCACCAGTGCCATTA			
	→	**FAM**-TGCCAGGGTGGTGACTTCACACGCC-**BHQ1**			
β-actin	→	CTCGATCATGAAGTGCGACGT	114 bp	101.0 ± 0.3	Duvigneau et al. [30]
	←	GTGATCTCCTTCTGCATCCTGTC			
	→	**FAM**-ATCAGGAAGGACCTCTACGCCAACACGG-**BHQ1**			

homoscedasticity (Bartlett's test) were determined. The analysis of cytokines expression levels was performed using one-way analysis of variance (ANOVA). Statistical means were contrasted by Tukey test ($P < 0.05$) using the Statistix 8.0 (Analytical Software, Roseville, MN, USA) [17]. The GraphPad Prism statistical program, version 5.0 (San Diego, CA, USA) was used for the figures.

3. Results and Discussion

3.1. Clinical Results. All pigs from the NPAPTim and NPAPTit groups survived until the end of experiment, and no clinical symptoms were observed. As could be expected, all pigs from CHG group died after the challenge, showing the clinical symptoms of Glässer's disease: high temperature, limb incoordination, swollen joints, severe dyspnea, and coughing. Clinical signs and histopathological findings have been extensively reported in Frandoloso et al. [15].

3.2. Evaluation of Endogenous Gene Expression. Three reference genes were tested due to different cell composition of the organs compared (brain, lungs, spleen, and lymph nodes). Cyclophilin showed constantly the highest homogeneous level of expression in all organs, with an average quantification cycle (Cq) of 21.5 ± 1.5, whereas β-actin and GAPDH expression levels were lower and less constant in all tissues, with a Cq mean of 26.0 ± 2.2 and 27.0 ± 3.3, respectively. On the basis of these results, cyclophilin was used as reference gene for normalization of data.

3.3. Chemokine Expression. No significant changes in transcript levels of CCL-2 (Figure 1(a)) and CXCL-8 (Figure 1(b)) genes were detected between NPAPTim, NPAPTit, and blank groups. There were only two exceptions: that of mediastinal and that of tracheobronchial nodes, for which the NPAPT groups showed a CCL-2 expression of about three- and seven-fold higher, respectively, than that of blank group ($P < 0.05$). However, CHG group transcribed high CCL-2 amounts in all tissues (Table 2). In lungs and draining lymph nodes, the CCL-2 transcription was approximately 20 and 50 times higher, respectively, than that of blank group. The origin of this chemokine could be justified by the activation of alveolar macrophages after *H. parasuis* contact or by the damage induced by this pathogen on epithelial cells. In the brain of CHG pigs, a high CCL-2 level was also observed (approximately 150 times higher than those of NPAPT and blank groups, Figure 1(a) and Table 2), which could be related to the presence of macrophages among the inflammatory cells seen in the histopathological study of this organ, whose lesions were compatible with meningitis [15]. Similarly, increased CCL-2 levels in the spleen and lymph nodes in CHG group seem to suggest a cellular proinflammatory immune response related to the activation of resident macrophages and dendritic cells, because the recruitment of inflammatory monocytes is dependent mainly on CCL-2 [18]. This overall increase in CCL-2 levels was coincident and could explain the elevation in the number of activated monocytes ($CD172\alpha^{+}CD163^{+}$) in the peripheral blood [2].

CXCL-8 has been characterized as the main neutrophil activating chemoattractant chemokine [10]. The high CXCL-8 level measured in the brain of CHG pigs (about 50 times higher than that of blank group, Figure 1(b) and Table 2) could explain the great amount of neutrophils present in inflammatory infiltrates of this organ, as it has been already reported [19]. Bouchet et al. [19, 20] tested the *in vitro* ability of *H. parasuis* to induce IL-8 expression in porcine tracheal and brain vascular microendothelial cells and showed that both dead and alive *H. parasuis* were capable of producing high IL-8 levels. In our *in vivo* study, a correlation could be established between the severity of the suppurative meningitis [15] and the brain CXCL-8 expression. We also observed that the CXCL-8 expression after *in vivo* infection was very similar to that reported for the *in vitro* experiment by Bouchet et al. [19, 20]. This result could indicate that the number of *H. parasuis* organisms reaching the brain was also similar to that used in *in vitro* infection, thus suggesting for the first time that *H. parasuis* could multiply and disseminate very fast in the natural host and that the *in vitro* model using brain and tracheal cells is adequate to perform studies about the expression of proinflammatory molecules. Identical with CCL-2 expression, CXCL-8 was also more transcribed in the tissues associated with the site of inoculation of *H. parasuis* (Table 2).

3.4. Cytokine Expression. IL-1α levels were quite similar for all groups, except for CHG animals (Figure 1(c) and Table 2). However, the two vaccinated groups expressed significantly higher IL-1β levels than that of the blank group in lungs ($P < 0.05$, Figure 1(d)). A significantly higher rise in IL-1β expression was also observed in tracheobronchial nodes in NPAPTim group ($P < 0.05$). A previous study reported by our group [5] showed a high IL-1α expression in the brain of a pig dying as a consequence of Glässer's disease. In the current report, we wanted to know how IL-1β responded to infection, and our results allowed us to assure that pigs suffering from Glässer's disease expressed high amounts of this cytokine. The greatest IL-1β expression in CHG group was measured in lungs, where the levels were about 50 and 25 times higher respectively than those of blank and NPAPT groups. A more marked difference was observed when comparing the IL-1β expression in the brain in CHG group (more than 500 times higher) with that measured in NPAPT groups (Figure 1(d) and Table 2). IL-1β expression has been correlated with pathologies in which inflammatory manifestations are seen [21]. Our results suggest that IL-1α and IL-1β could play an important role in the inflammatory response developed against *H. parasuis*, because both molecules can bind with the same signaling receptor and then induce the propagation of the inflammatory response [21].

The vaccinated groups showed an IL-6 concentration about five times higher in mediastinal nodes than that of blank group ($P < 0.05$, Figure 1(e)). The highest IL-6 expression in these three groups was detected in the secondary lymph tissues, being lower in lungs and especially in brain (Figure 1(e)). CHG group showed considerable IL-6 increases compared to the three other groups, between approximately six and 40 times higher depending on the tissue tested (Figure 1(e) and Table 2). IL-6 has been directly related to the synthesis of acute phase proteins and, in this respect, high levels of pig MAP, C-reactive protein and haptoglobin in the serum have been reported in pigs that died after experimental *H. parasuis* infection [22, 23]. On the other hand, IL-6 plays an important role in Th2 lineage differentiation, being abundantly produced by antigen presenting cells [24]. The increase detected in mediastinal nodes in NPAPT groups could be associated to the stimulation and development of a Th2 response but not to a proinflammatory situation, mainly because low transcriptions of CXCL-8, IL-1 isoforms and TNF-α were observed in this tissue. Otherwise, the systemic transcription of high IL-6 levels measured in CHG group during *H. parasuis* pathogenesis could be due to the direct stimulation of this pathogen on mononuclear phagocytes, epithelial cells, and vascular endothelial cells [25].

Significantly lower values ($P < 0.05$) were detected in TNF-α expression in blank group in mandibular nodes when compared with any of the vaccinated groups (Figure 1(f)), while in the remaining tissues these levels remained similar. Moreover, the TNF-α levels increased markedly in CHG group (Table 2), especially in the secondary lymph tissues, where the expression was more than twelve times higher compared with any of the healthy groups (Figure 1(f) and Table 2). The TNF-α values observed in vaccinated and blank pigs suggest the existence of a basal expression level of this cytokine in swine, such as that observed in mice [26] and guinea pigs [27]. Biologically, the combination of TNF-α and IL-1 can induce changes in endothelial cells of

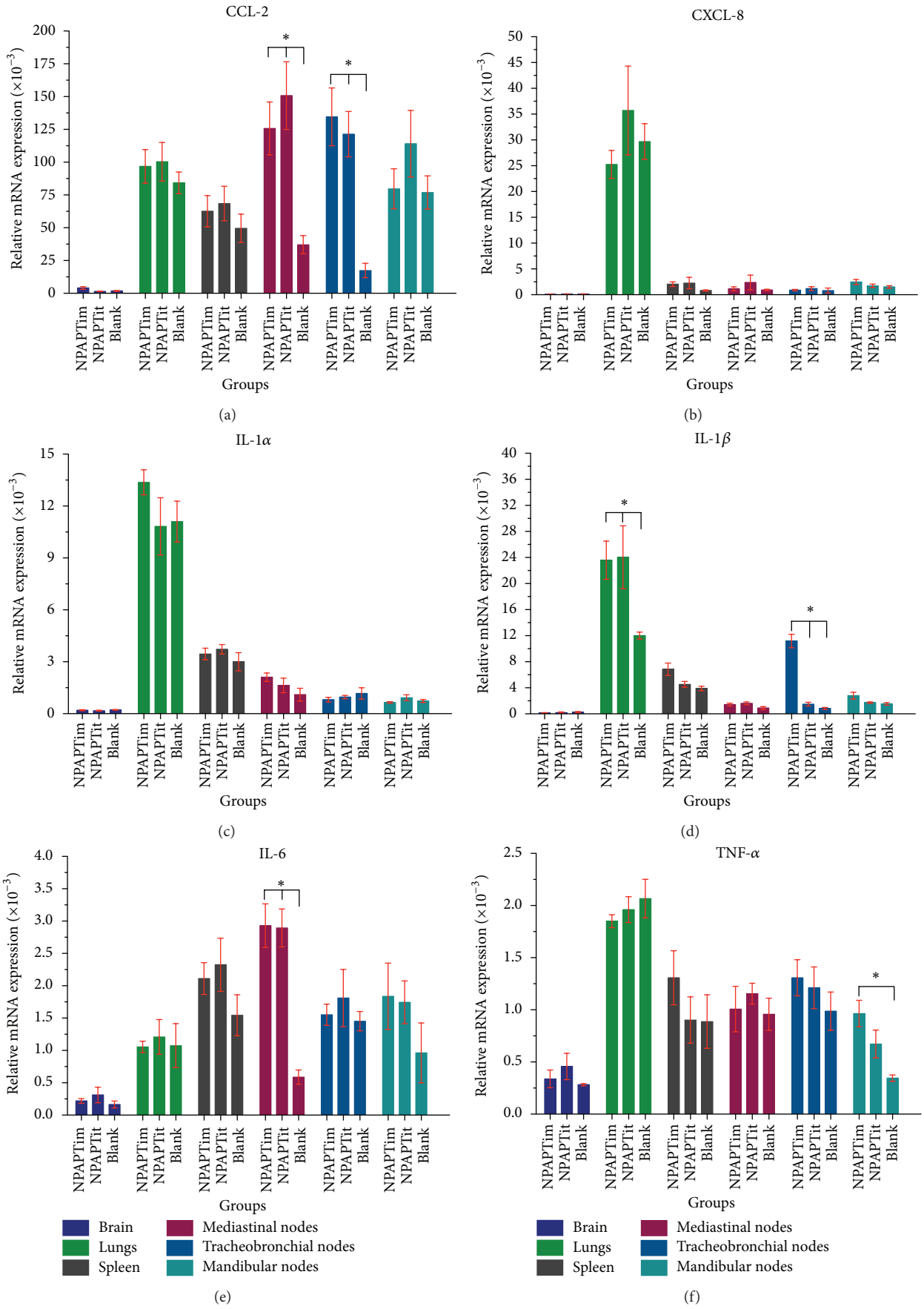
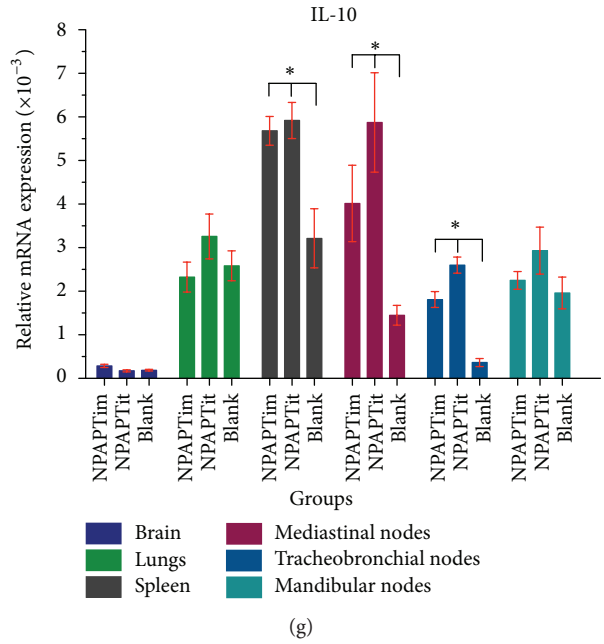


Figure 1: Continued.



(g)

Figure 1: Relative mRNA expression rates of chemokines and cytokines from different tissues in NPAPTim, NPAPTit and blank groups. *indicates significant differences between groups ($P < 0.05$).

Table 2: Expression of proinflammatory molecules from different tissues in CHG group (mean ± standard error).

Molecules*	Tissue					
	Brain	Lung	Spleen	Mediastinal nodes	Tracheobronchial nodes	Mandibular nodes
CCL-2	204.2 ± 40.2	1673.2 ± 96.5	441.7 ± 118.2	901.5 ± 58.9	948.8 ± 73.8	257.1 ± 45.2
CXCL-8	55 ± 3.6	887.6 ± 34.2	12.7 ± 1.6	431.5 ± 74.3	470.7 ± 104.4	17.3 ± 2.4
IL-1α	57.7 ± 5.8	365.4 ± 45.6	35.3 ± 1.7	142.1 ± 20.7	15.3 ± 3.2	15.5 ± 2.1
IL-1β	141.5 ± 11.4	615.4 ± 76.1	92.7 ± 2.8	184.1 ± 32.2	119.2 ± 13.1	63 ± 12.2
IL-6	2.5 ± 0.4	37.4 ± 5.9	20.8 ± 3.7	35.8 ± 1.8	115.1 ± 20.42	13.6 ± 0.7
TNF-α	10.5 ± 0.7	10.9 ± 1.1	19 ± 3.2	75.1 ± 5.4	22.2 ± 4.3	16.5 ± 0.6
IL-10	17.5 ± 1.7	48.1 ± 6.6	10.8 ± 1.8	6.4 ± 1.7	7.1 ± 0.8	12.1 ± 1.5

*Relative mRNA expression ($\times 10^{-3}$).

small vessels, thus increasing the adherence of inflammatory cells. In addition, a TNF-α increase could cause edema and vascular thrombosis [28]. These findings allow us to relate the high expression of IL-1 isoforms, and TNF-α with the edema, lung congestion and vascular thrombosis observed microscopically in a previous report in CHG pigs [15].

The two vaccinated groups showed transcription levels statistically higher than the blank group for IL-10 in spleen and mediastinal and tracheobronchial nodes ($P < 0.05$, Figure 1(g)). However, these values were at least thrice lower than those measured for CHG group, except for mediastinal nodes (Table 2). Taking into account the regulative effect exerted by IL-10 on the different cells belonging to innate and adaptive systems, it could be suggested that the elevation observed in CHG group would be a regulative action of the secretion of proinflammatory cytokines. On the other hand, Go et al. [29] showed that B cells cultivated *in vitro* and stimulated with IL-10 increased the class II MHC expression on the surface of B cells. The increase of IL-10 expression in spleen in NPAPT pigs could be explained by this fact, mainly because these animals showed hyperplasia in the white pulp, characterized by an increase of the spleen germinal centers and by a peripheral increase in B cells, and in the expression of SLAIIDR molecules [2].

4. Conclusion

The experimental infection of colostrum-deprived pigs with *H. parasuis* Nagasaki strain induced an overregulation of the expression of proinflammatory chemokines and cytokines related to innate immunity. Because of the differences in the expression between the protected (NPAPTim and NPAPTit) and nonprotected (CHG) groups, CCL-2, CXCL-8, IL-1α, IL-1β, and IL-6 may be considered the best inflammatory markers to *H. parasuis* infection in target organs. Finally, the protection conferred by NPAPT vaccines was enough to prevent an inflammatory reaction mediated by CCL-2, CXCL-8, IL-1α, IL-1β, IL-6, TNF-α, and IL-10, making NPAPT antigen

a suitable candidate to control Glässer's disease caused by *H. parasuis* Nagasaki strain.

Conflict of Interests

The authors declare that they have no conflict of interests with Cycler Probes Master (Roche) and Analytical Software (Roseville, MN, USA).

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Research Article

CD8 Knockout Mice Are Protected from Challenge by Vaccination with WR201, a Live Attenuated Mutant of *Brucella melitensis*

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CD8+ T cells have been reported to play an important role in defense against *B. abortus* infection in mouse models. In the present report, we use CD8 knockout mice to further elucidate the role of these cells in protection from *B. melitensis* infection. Mice were immunized orally by administration of *B. melitensis* WR201, a purine auxotrophic attenuated vaccine strain, then challenged intranasally with *B. melitensis* 16M. In some experiments, persistence of WR201 in the spleens of CD8 knockout mice was slightly longer than that in the spleens of normal mice. However, development of anti-LPS serum antibody, antigen-induced production of γ-interferon (IFN-γ) by immune splenic lymphocytes, protection against intranasal challenge, and recovery of nonimmunized animals from intranasal challenge were similar between normal and knockout animals. Further, primary *Brucella* infection was not exacerbated in perforin knockout and Fas-deficient mice and these animals' anti-*Brucella* immune responses were indistinguishable from those of normal mice. These results indicate that CD8+ T cells do not play an essential role as either cytotoxic cells or IFN-γ producers, yet they do participate in a specific immune response to immunization and challenge in this murine model of *B. melitensis* infection.

1. Introduction

Brucellosis causes loss of livestock productivity and threatens human health worldwide [1]. The threat is most pronounced in developing nations, but even Europe and North America remain at significant risk [2, 3]. The predominant *Brucella* species in both animal and human infections is *Brucella melitensis* [4]. A vaccine for *B. melitensis* for use in humans would be a boon to millions of agriculture workers worldwide [5] and may be an important goal for protection against bioterrorism [6].

To date, the most successful brucellosis vaccine preparations (used in livestock species) have been modified live derivatives of virulent *Brucella* [7, 8]. However, some of these vaccines are pathogenic in humans [9]. Additionally, modified live vaccines may induce abortion to livestock if administered during pregnancy or to other animals in contact with pregnant animals [10, 11]. Despite being perhaps less efficacious, subunit vaccines may have safety advantages over live attenuated candidates. Delineation of the immune mechanisms responsible for vaccine-induced protection may focus subunit vaccine development by suggesting potential immune correlates and adjuvants tailored to evoke a desired response.

Immunization with *B. melitensis* WR201, an attenuated purine auxotroph, protects mice against intranasal challenge with virulent *B. melitensis* 16M [12]. Protection is associated with production of anti-lipopolysaccharide (LPS) antibodies

and production of IFN-γ by antigen-stimulated immune spleen cells. The contribution of CD8+ T cells in protection has not been examined in this model.

Antibody to the O-polysaccharide of *Brucella* LPS has been firmly established as an important mediator of anti-*Brucella* effects in murine models of secondary immunity [13, 14]. However, cellular immunity also plays a key role [15, 16]. The production of IFN-γ is essential for protection, clearance, and survival in the face of virulent *Brucella* challenge in the mouse model [17]. IFN-γ is produced *in vivo* predominantly by CD4+ T cells and to a lesser extent by CD8+ T cells [16, 18, 19]. Both CD8+ and CD4+ T cells respond specifically to *B. abortus* in mice, CD8+ T cells may function as specific cytotoxic cells in brucellosis caused by *B. abortus* [16, 20, 21], and one study indicated that immune modulation could result in an effective CD8+ T-cell role in secondary immunity [22]. On the other hand, other studies seem to indicate that the role of CD8+ T cells is relatively minor in the immune response to *B. abortus* [17, 23]. One virulence mechanism of both *B. abortus* [24] and *B. melitensis* [25] may be evasion of CD8+ T cell adaptive immunity, and *B. melitensis* epitopes of CD8+ T cell IFN-γ production and cytotoxicity have been identified [26]. In contrast, CD8+ T cells appear to be dispensable in a model of primary *B. melitensis* infection [27]. The study reported here further elucidates the role of CD8+ T cells in brucellosis by evaluating the requirement for the cell type in secondary immunity resulting from modified live organism immunization in a mouse model of *B. melitensis*.

We found that CD8+ T cells from immunized mice specifically produce significant amounts of IFN-γ *in vitro*. However, we also found that these cells are not essential for clearance of attenuated or virulent *B. melitensis* nor for WR201-induced protection against intranasal challenge. Moreover, the key CD8+ T cell mediators of cytotoxicity (perforin and Fas) appear to play no role in elimination of *B. melitensis* in these studies. These data indicate a more limited role for CD8+ T cells in secondary immunity to *B. melitensis* than what has been suggested from previously published work using *B. abortus*.

2. Materials and Methods

2.1. Bacteria and Bacterial Products. B. melitensis 16M and WR201 from our culture collection were prepared as described previously [12]. WR201 from stocks frozen in 50% glycerol was incubated overnight in *Brucella* broth in a shaker flask at 37°C. One mL aliquots of this culture were then plated on *Brucella* agar and incubated at 37°C for an additional three days. The bacterial "lawn" was then scraped from the agar surface, resuspended in 0.9% sodium chloride solution (saline), pelleted, washed twice with saline, and adjusted based on optical density to 5×10^{11} colony forming units (CFU)/mL in saline. In our experience this is the safest and most convenient method by which to obtain brucellae at these high concentrations. On the other hand, *B. melitensis* 16M from stocks frozen in 50% glycerol was incubated overnight in *Brucella* broth in a shaker flask at 37°C, pelleted, washed with saline, and diluted to 3.3×10^5 CFU/mL—a concentration easy to obtain directly from broth. CFU concentration was verified by serial dilution and plating on *Brucella* agar. Rough *Brucella* lysate (RFBL) and *Brucella* LPS were prepared as previously described [12].

2.2. Immunization and Challenge of Mice. Six-week-old C57BL/6, B6.129S2-cd8a^{tm1Mak} (CD8 knockout), C57BL/6-Pfp^{tm1Sdz} (perforin knockout), and B6.MRL-Fas^{lpr} (Fas receptor deficient mutant) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). One pair of experiments, which used males, was conducted to evaluate kinetics of immunization and challenge in CD8 knockout compared to normal C57BL/6 mice. A subsequent pair of experiments, using females, compared the kinetics of clearance of WR201 from CD8 and perforin knockout, Fas mutant, and normal C57BL/6 mice. Animals were housed in animal biosafety level 3 facilities. Immunization and challenge procedures were performed as previously described [28]. Briefly, mice were acclimated for one week, then gavaged with 200 μL 2.5% sodium bicarbonate followed by 10^{11} CFU WR201 also in 200 μL. Sham-immunized mice received an equal volume (200 μL) of sodium bicarbonate and saline. Seven or eight weeks following immunization, mice were either euthanized to obtain tissues for *in vitro* assays or challenged. For challenge, mice were anesthetized with 0.3 mg xylazine and 1 mg ketamine. 1×10^4 CFU 16M in 30 μL were then administered dropwise into the external nares with a micropipette.

2.3. Determination of Bacterial Infection and Immune Responses. Blood was obtained by cardiac puncture from mice euthanized by CO_2 narcosis and allowed to clot. Serum was separated by centrifugation and sterilized by filtration through 0.2 micron filters. Anti-LPS antibody titer was determined by ELISA as previously described [12]. Organs were processed and CFU-per-organ determined by serial dilution and plating as previously described [29].

2.4. Cytokine Production. In some experiments, production of IFN-γ by antigen-stimulated spleen cells from immunized or sham-immunized mice was determined as previously described [12], except for the following: total spleen cells pooled from groups of 7 mice were incubated at 5×10^6/well in 24 well tissue culture plates in 2 mL RPMI-1640 tissue culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10 μg/mL gentamicin with or without 2 μg/mL concanavalin A (conA) or 2 μg/mL RFBL. After the entire mononuclear cell population was incubated together for 24 hrs (in order to simulate the cytokine milieu that occurs *in vivo*), nonadherent cells were collected, pelleted at 1200 rpm in a clinical centrifuge (Sorvall) for 7 minutes, and separated using the MACS separation system (Miltenyi Biotech) or resuspended in fresh medium and set aside on ice (unseparated cells). Separated CD8 and CD4+ T cells and unseparated cells were then replaced at the original concentration of mononuclear cells on the adherent spleen cells and incubation was continued for additional 48 hours in order to allow for cytokine production

from individual T cell subtypes. The same cells that had been incubated with conA or RFBL were again incubated with these stimulants during this additional 48-hour period and unstimulated cells were again incubated with medium only. Culture supernatant fluids were then collected and sterilized by filtration through 0.2 micron filters. IFN-γ concentration was determined by ELISA as previously described [12].

2.5. Flow Cytometry. The purity of CD4 and CD8+ T cell preparations was assessed by direct two-color immunofluorescence staining. Cells were frozen in 1% dimethyl sulfoxide in cell culture medium and stored at −80°C until the day they were stained. Cells were warmed to 4°C, concentrated by centrifugation, then resuspended in 4% methanol-free formaldehyde. Cells were incubated in formaldehyde for one hour to ensure sterility, then concentrated and washed with 0.1% bovine serum albumin in phosphate buffered saline (PBS/BSA). Cells were preincubated for 15 min at 4°C with purified rat anti-mouse CD16/CD32 (Mouse FC Block) (Pharmingen) to reduce nonspecific binding. Then cells were stained for 30 min at 4°C with CD4 FITC and CD8 PE antibodies and matched isotype IgG (Pharmingen). The staining was followed by washing with PBS/BSA in order to remove unbound antibody. Cells were resuspended in PBS/BSA prior to acquisition on the flow cytometer. 10,000 events were acquired on a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and analyzed using CellQuest (Becton Dickinson) software. Data on the percentage of positive cells were obtained by setting a quadrant marker for nonspecific staining.

2.6. Statistical Methods. In the immunization and challenge studies, data from two separate but identical experiments were combined. The intensity of organ infection at 2 weeks after oral immunization, when most organs contained brucellae, was expressed as mean +/− SD $\log_{10}$ CFU. Statistical significance of differences in means was determined by Student's t-test. When the raw CFU per organ was zero, the log transformed value was assigned a value of zero, but this value was used only for graphical representation, not for statistical comparison. At all time points after immunization when some animals had cleared infection from the harvested organs, the frequency of infection in each organ was determined and the significance of differences between groups was assessed using Fisher's exact test. Additionally, CFU/spleen at that time point was analyzed descriptively. IFN-γ concentration was expressed as mean of triplicate or duplicate samples and analyzed descriptively.

3. Results

3.1. Clearance of WR201. In both repetitions of the experiment that we conducted to determine clearance of immunizing strain and subsequent protection against challenge infection, WR201 persisted for 8 weeks in spleens of 2 of 5 CD8 knockout mice but was cleared from all 5 normal (C57BL6/J control) animals at this time point. Intensity of infection was less than 100 CFU in the infected spleens. Using combined data from these 2 experiments, this difference is significant ($P = 0.044$, Fisher's exact test). No other organs from either group were infected at this time point. In another pair of experiments, we immunized groups of 10 female perforin knockout, Fas mutant, CD8 knockout, and normal mice. In the first of these two experiments, all 10 mice of each group had completely cleared WR201 by eight weeks. In a repetition of the experiment, we examined the infection level at seven weeks and found that WR201 persisted at low levels in a minority of spleens of all groups. Infection levels in all mice that remained infected at this time point were less than 10 CFU/organ. There was no statistically significant difference between the groups in terms of clearance or mean log CFU per spleen. These studies showed that, unlike male mice, female mice clear WR201 between 7 and 8 weeks after immunization and that perforin and Fas play no role in its clearance.

3.2. Immunization and Challenge of CD8 Knockout Mice. We performed two separate but identical experiments in which male CD8 knockout and normal C57BL/6 mice were immunized orally with WR201 or sham-immunized. Eight weeks later, all mice were challenged intranasally with virulent 16M. We harvested spleens, lungs, and livers from mice 1 day and 2, 4 and 8 weeks following challenge to assess intensity and frequency of infection. At one day after challenge, all mice had *Brucella* in the lungs, but not in the liver or spleen (Figure 1). The course of infection in CD8 knockout and normal mice was indistinguishable (Figure 1). Immunized mice showed amelioration of spleen and liver infection, while immunization had no effect on clearance of virulent *Brucella* from the lung. In no case were culture results from C57BL/6 mice significantly different than results from CD8 knockout mice (immunized or naive) (Table 1).

Using combined data from the individual experiments, immunization with WR201 protected both C57BL/6 and CD8 knockout mice ($P < 0.05$) from infection in the liver and spleen at 2 and 4 weeks after challenge, but CD8 knockout mice were not protected in the spleen at 2 weeks. With the data from the 2 experiments combined, there was no significant difference in protection in any organ at any time point between mice of the same immunization status, that is, naive C57BL/6 versus naive CD8 knockout mice or immunized C57BL/6 versus immunized CD8 knockout mice.

At eight weeks after challenge, using combined data from both experiments, spleens of both C57BL/6 and CD8 knockout mice were significantly protected (Table 1). There was no significant difference between mice of the same immunization status. While immunization led to significant protection of the liver of C57BL/6 mice ($P = 0.011$) but not CD8 knockout mice, the importance of this finding is uncertain, since most unimmunized animals of both strains had also cleared the infection from this organ by eight weeks.

As a further assessment of strain differences, we analyzed CFU per spleen at eight weeks after challenge for individual mice. In both immunized and nonimmunized groups, the highest splenic CFU among individual animals were found in CD8 knockout mice (not shown). Although this pattern

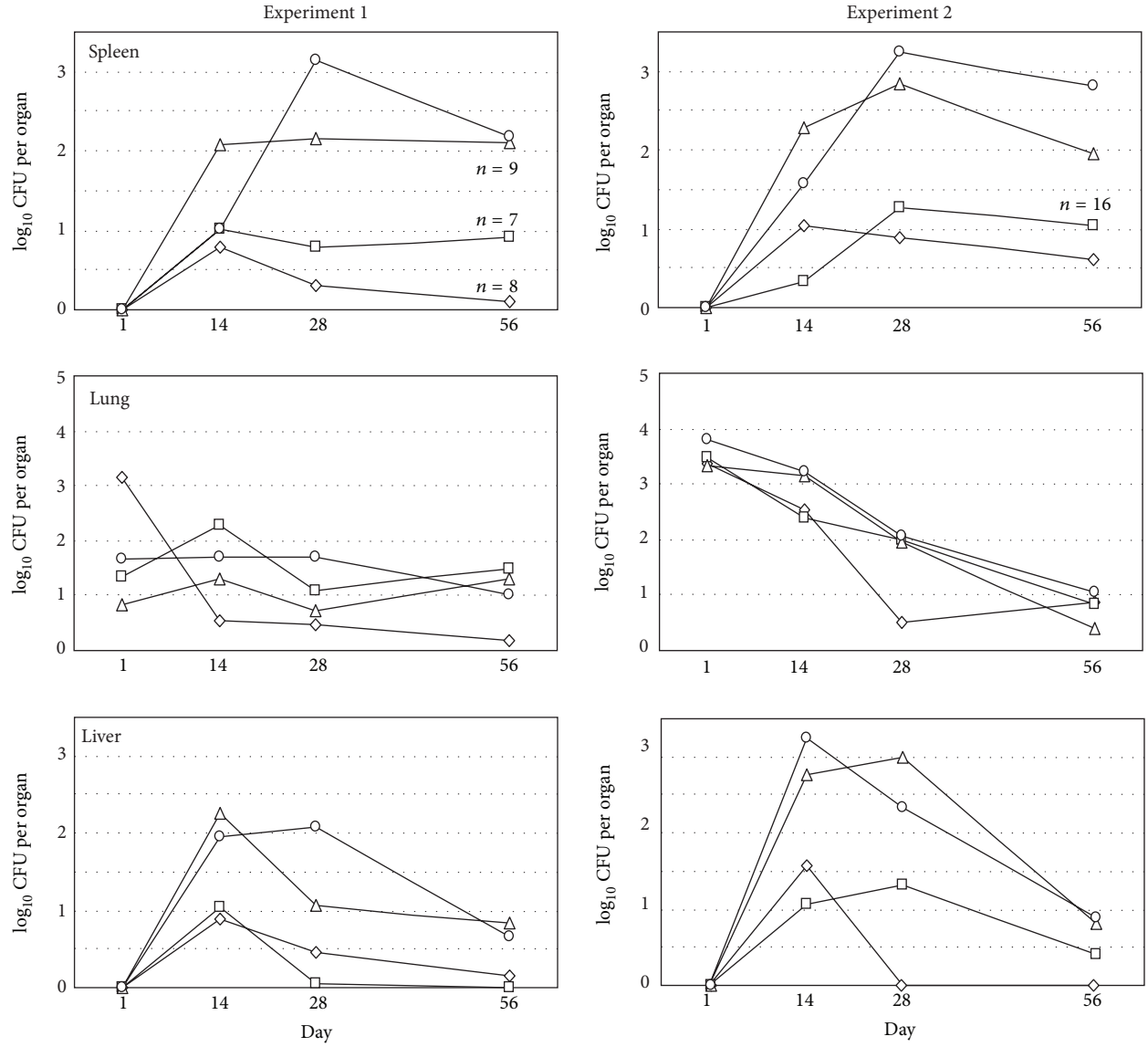

Figure 1: Immunization and challenge. Six-week-old male C57BL/6 and CD8 knockout mice were orally immunized with 10^{11} WR201 and eight weeks later challenged with 10^4 *B. melitensis* 16M. Data points represent mean CFU per organ (limit of detection = 2) for 4-5 mice (first three time points) and 10 mice (56-day time point) except where noted. The figure shows naive CD8 knockout mice (circles), naive C57BL/6 mice (triangles), immune CD8 knockout mice (squares), and immune C57BL/6 mice (diamonds). In no case did mice of the same immunization status have significantly different CFU levels at a given time point in a given organ.

seemed suggestive of reduced anti-*Brucella* activity in knockout mice, analysis by Mann-Whitney *U* test indicated that this trend was not significant.

3.3. Cellular and Humoral Immune Responses in Immunized Mice. To determine the ability of immune T cell subpopulations to respond to *Brucella* antigens, we collected spleen cells from female C57BL/6 mice immunized 8 weeks earlier with WR201. Cells were incubated with antigen or mitogen as described in methods. In both experiments, unseparated spleen cells and CD4 and CD8+ T cells from immunized mice produced more IFN-γ than cells from unimmunized mice (data not shown). Cell separation was highly effective. By flow cytometric analysis, T cell subpopulations were at least 82% pure and were contaminated with less than 1% of the other T cell subset. Adherent cells alone and all cells that were incubated without RFBL failed to produce measurable IFN-γ (not shown). All naive and immunized cells responded similarly to conA stimulation (not shown).

We measured anti-*Brucella* LPS IgG and IgG2a (shown, resp., in parentheses as mean calculated titer ± SEM) in CD8 knockout (7783 ± 2284, 62 ± 28, $n = 10$), perforin knockout (4421±2072, 41±20, $n = 10$), Fas mutant (5086±1388, 67±16, $n = 7$), and immunologically intact C57BL/6 (6893 ± 1055,

Table 1: Six-week-old male C57BL/6 and CD8 knockout (CD8 KO) mice were orally immunized with 10^{11} WR201 and eight weeks later challenged with 10^4 *B. melitensis* 16 M. Figures at 1 day and 2 weeks after challenge, when most or all animals were infected, are mean CFU ± SD. At 4 and 8 weeks, when many animals had cleared the infection and mean CFU counts became less meaningful, figures are shown as ratio of cleared/total. At day 1, $n = 5$ for C57BL/6 and $n = 4$ for CD8 KO; at 2 weeks $n = 10$ for all groups. CFU comparisons utilized Student's t-test while clearance comparisons utilized Fisher's exact test.

Organ	Time after challenge	C57BL/6 immunized	C57BL/6 naive	P value C57BL/6 immunized versus naive	CD8 KO immunized	CD8 KO naïve	P value CD8 KO immunized versus naive	P value immunized versus immunized	P value naïve versus naive
Liver	2 wks	1.53 ± 0.840	2.91 ± 0.646	<0.01	1.58 ± 0.887	2.88 ± 0.646	<0.05	>0.05	>0.05
	4 wks	9/10	1/10	<0.001	7/10	0/10	<0.01	>0.05	>0.05
	8 wks	17/18	11/19	<0.05	20/23	14/20	>0.05	>0.05	>0.05
Spleen	2 wks	1.29 ± 0.676	2.46 ± 1.22	<0.05	1.59 ± 0.227	1.62 ± 0.662	>0.05	>0.05	>0.05
	4 wks	6/10	1/10	<0.05	6/10	0/10	<0.01	>0.05	>0.05
	8 wks	12/18	1/19	<0.001	12/23	1/20	<0.001	>0.05	>0.05
Lung	1 day	3.38 ± 0.66	3.32 ± 0.18	>0.05	3.48 ± 0.21	3.81 ± 0.14	>0.05	>0.05	>0.05
	2 wks	2.2 ± 0.89	2.03 ± 1.17	>0.05	2.32 ± 0.82	3.08 ± 0.66	>0.05	>0.05	0.048
	4 wks	8/10	4/10	>0.05	4/8	1/10	>0.05	>0.05	>0.05
	8 wks	11/16	11/19	>0.05	10/23	10/20	>0.05	>0.05	>0.05

28±5.4, $n = 10$) mice immunized 7 or 8 weeks previously with WR201. These studies did not show any significant differences in antibody levels among the groups.

4. Discussion

Because we found persistence of attenuated brucellae in the spleens of some male CD8 knockout mice at the time point at which others were subsequently challenged, it must be acknowledged that the apparent protection may be due in part to persistent macrophage activation. This exception may reflect a different pattern of dissemination to the spleens in CD8 knockout mice compared to the control animals. Subsequent experiments should entail a longer lag period prior to challenge in order to clarify this question. Nonetheless, in challenge experiments, naive CD8 knockout mice showed very similar kinetics of virulent brucellae dissemination and clearance as compared to normal C57BL/6 mice. It is important to note that day 1 data primarily indicates the fidelity of inoculation and has no relevance for assessment of protection.

The CD8+ T cell has been identified as an important mediator or component of the immune response to brucellosis caused by *B. abortus* in prior studies [15, 16, 20–22, 24, 25, 30, 31]. Since the species are closely related genetically, it seemed plausible that this cell type would play a similar role in the response to *B. melitensis*. If so, CD8+ T cells would seem to be a useful target for vaccine development. CD8+ T cells may be preferentially stimulated by expressing costimulatory molecules and peptides with CD8+ T cell specific epitopes in nonprofessional phagocytes (i.e., cells not differentiated as macrophages) or by delivering antigens by viral vector [32]. Preferential stimulation of CD8+ T cells could be advantageous, because stimulation of CD4+ T cells results in a mixed T helper 1 and T helper 2 response, that is, simultaneous production of IFN-γ and IL-10 [18, 30]. The former response is important for anti-*Brucella* immunity, while the latter may play a counterregulatory or inhibitory role.

In preliminary studies [33], we were unable to detect cytotoxicity of immune CD8+ T cells for J774 macrophages infected with rough *B. melitensis* strain WRR51. The present studies were designed to determine whether CD8+ T cells play an essential role *in vivo*, by examining WR201-induced resistance to *B. melitensis*. Our results indicate that these cells are not crucial to protection in our model. In addition to the obvious difference (*B. melitensis* versus *B. abortus*), there are a number of other differences between the methods of our studies, which showed a minimal effect, and those used by others, who observed a strong effect [15, 16, 30]. First, there may be a fundamental difference in the immune response induced by immunization with the rough strain, RB51, and a smooth strain like WR201. Second, we used mice on a C57BL/6 background, while some other studies used animals on a BALB/c background. In some systems, the BALB/c mouse shows relative persistence of infection, which may indicate inherent differences in the immune response [18]. In limited studies from our group, C57BL/6 mice tended to be more resistant to intranasal challenge than BALB/c, although differences were not significant [29]. In addition, it is possible that one study that used MHC class I knockout mice as a surrogate for CD8-deficiency may have incorrectly attributed defective *Brucella* immunity to CD8+ T cells. Although these mice are CD8+ T cell deficient, they may have other defects (e.g., poor presentation of CD1-binding antigens) that may explain their impaired ability to control infection [34]. Third, our routes of immunization and challenge are different; we immunized orally and challenged intranasally, while other groups immunized and challenged i.v. [15, 30]. It is possible that challenge by a nonmucosal route, as in these previous studies, overestimates the impact of CD8+ cells. The relative importance of variables such as these could be assessed in

additional studies. Our results do not exclude a role for CD8+ T cells in naturally acquired brucellosis or in other experimental systems but indicate that they are not critical for WR201-mediated protection against intranasal *B. melitensis* challenge.

There are, however, some suggestions in these studies that CD8+ T cells may contribute to anti-*Brucella* effects in our model. In the studies in male mice, infection with WR201 was slightly, but significantly, more prolonged in CD8 knockout compared to normal animals. Similarly, there were trends toward higher intensity of infection and higher frequency of infection in spleens of both immunized and nonimmunized 16M-challenged CD8 knockout mice compared to corresponding normal mice. These differences are minor, however, and suggest a contribution of CD8+ T cells rather than a predominant effect. The near-identical infection of other organs between knockout and normal mice, however, supports the view that this contribution is of limited importance. The studies using perforin and Fas knockout mice also did not support a role for CD8+ T cell cytotoxicity as an important determinant of immunity to *Brucella*, in agreement with studies done on a *B. abortus* model [17, 35]. It is notable that we did not see differences between knockout and normal mice in our studies using female mice. While this variability may reflect inherent differences in immune responsiveness between the sexes, it may also reflect different levels of stress in these groups. Stress is experienced far more by group-housed male mice, to varying degrees by individual mice within a given group, and plays a critical role in their immune response [36]. It has been hypothesized that a key role for the CD8+ T cell is the suppression of production of T helper 2 type cytokines [30]. It may be that environmental factors, for example, stress in male mice, influence the overall trend of the T helper response and that, in certain circumstances, the CD8+ T cell plays a compensatory role.

It is possible that the role for the CD8+ T cell in murine infection with *B. abortus* is not applicable to infection with *B. melitensis* as in the present study [15, 16, 30, 31]. Perhaps the immune response to *B. abortus* relies more heavily on the CD8+ T cell. This may stem from the unusual property of *B. abortus* LPS to cross link MHC-II molecules, which could inhibit the CD4+ T cell response [37]. IFN-γ is essential for survival in the face of virulent *Brucella in vivo* [17] and mediates reduction in intracellular infection of cultured macrophages [38, 39]. Previous studies with CD4+ T cells, however, have shown that the CD4+ T cell is the major producer of IFN-γ in brucellosis [16, 18, 19]. We confirm these studies in *B. melitensis*-immunized mice and extend them by demonstrating that both CD4+ and CD8+ T cells produced IFN-γ in response to specific antigen, suggesting that both cell types should contribute to protective responses. These studies raise new questions about the importance of CD8+ T cells in defense against *Brucella* and suggest that the issue should be reexamined for *B. abortus*. They also suggest that a vaccine strategy aimed at sensitizing CD8+ T cells may have limited value, although this question also deserves further investigation.

Disclosure

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Departments of the Army, Navy, Air Force, or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition.

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