Adjuvants That Improve the Ratio of Antigen-Specific Effector to Regulatory T Cells Enhance Tumor Immunity

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Abstract

Antitumor immunity is strongly influenced by the balance of tumor antigen-specific effector T cells (Teff) and regulatory T cells (Treg). However, the impact that vaccine adjuvants have in regulating the balance of antigen-specific T-cell populations is not well understood. We found that antigen-specific Tregs were induced following subcutaneous vaccination with either OVA or melanoma-derived peptides, with a restricted expansion of Teffs. Addition of the adjuvants CpG-ODN or Poly(I:C) preferentially amplified Teffs over Tregs, dramatically increasing the antigen-specific Teff:Treg ratios and inducing polyfunctional effector cells. In contrast, two other adjuvants, imiquimod and Quil A saponin, favored an expansion of antigen-specific Tregs and failed to increase Teff:Treg ratios. Following therapeutic vaccination of tumor-bearing mice, high ratios of tumor-specific Teffs:Tregs in draining lymph nodes were associated with enhanced CD8⁺ T-cell infiltration at the tumor site and a durable regression of tumors. Vaccine formulations of peptide + CpG-ODN or Poly(I:C) induced selective production of proinflammatory type I cytokines early after vaccination. This environment promoted CD8⁺ and CD4⁺ Teff expansion over that of antigen-specific Tregs, tipping the Teff to Treg balance to favor effector cells. Our findings advance understanding of the influence of different adjuvants on T-cell populations, facilitating the rational design of more effective cancer vaccines. Cancer Res; 73(22): 6597–608. ©2013 AACR

Introduction

The development of therapeutic cancer vaccines is a challenging task, and generating clinically relevant, potent, and polyfunctional T-cell responses to tumor/self-antigens has proven difficult. Therapeutic peptide vaccines stimulate antitumor immune responses in patients with advanced melanoma (1, 2), but clinical benefits have not lived up to the expectations (3, 4). For therapeutic vaccines to be effective, they must circumvent regulatory mechanisms that limit the activation and expansion of CD8⁺ and CD4⁺ T cells. Regulatory T cells (Treg) play a major role in the control of antitumor immunity (5–7) and existing cancer vaccines activate and expand Tregs, resulting in suppression of antitumor responses (8–10). Thus, the need to enhance immunogenicity of peptide vaccines is paramount. Adjuvants provide a means to improve the generation of potent and durable T-cell immunity by cancer vaccines (11). A greater understanding of the role of adjuvants in modulating T-cell responses and in particular Tregs is therefore urgently needed. The rational development of new adjuvant formulations to augment T-cell responses is key in cancer vaccine development. Adjuvants that strongly stimulate T-cell responses are still not readily available and the major clinically licensed adjuvants Alum and incomplete Freund’s adjuvant (IFA) primarily promote antibody responses but are poor at inducing the cytotoxic CD8⁺ T-cell responses needed in the case of cancer (12).

Members of the Toll-like receptor (TLR)-ligand class of adjuvants, including CpG-ODN (TLR-9), LPS (TLR-4), and Pam3Cys (TLR-2), induce antigen-presenting cell (APC) maturation and production of inflammatory cytokines, favoring type I effector T cell (Teff) responses and restricting Treg expansion (13–15). Conflicting data show that TLR agonists CpG-ODN, LPS, Zymozan (TLR-6), Poly(I:C) [TLR3], Imiqui-mod and R-848 (TLR-7/8) can expand both Teffs and Tregs leading to suppression of effector responses (16–18). In the studies mentioned above, CD8⁺ T-cell responses were compared with those of polyclonal Tregs. Very few studies have investigated the effect of adjuvants on antigen-specific Tregs. An ex vivo study from patients with colorectal carcinoma identified shared antigen-specificities between tumor-specific Teffs and Tregs. These Tregs were shown to suppress proliferation of Teffs in an antigen-specific manner when cultured with tumor peptide-loaded dendritic cells (19). In a clinical study of patients with melanoma immunized with NY-ESO-1 protein in ISCOMATRIX, vaccination increased the frequency of NY-ESO-1–specific Tregs in peripheral blood mononuclear cells (PBMC) and tumor tissue (20). We have shown that
therapeutic vaccination of patients with melanoma with Melan-A peptide plus CpG-ODN in Montanide results in robust expansion of Melan-A–specific CD8+ T cells within PBMCs with a concomitant decrease in Melan-A–specific Tregs (1, 2, 21). Importantly, although we observed a decrease in vaccine-specific Tregs, the total polyclonal Treg population remained unchanged. These results suggest that antigen-specific Tregs are regulated differently than polyclonal Tregs following adjuvanted-peptide vaccination, and that adjuvant choice may be important in selectively controlling the specific Treg response.

We therefore set out to extend these clinical observations using mouse models of peptide vaccination to dissect the role of vaccine formulations in shaping the antitumor immune response. We developed models that allowed us to compare tumor-specific CD8, CD4, and Treg responses to peptide vaccination in various adjuvant formulations. Here, we show that vaccines containing TLR-9 ligand CpG-ODN or TLR-3 ligand PolyIC preferentially induce strong proliferation of antigen-specific Teffs, while minimizing antigen-specific Treg expansion. High Teff:Treg ratios were linked to strong proinflammatory cytokine production in the lymph nodes early after immunization and resulted in polyfunctional CTLs with enhanced tumor infiltration and protective function.

Materials and Methods

Mice

Mouse strains were maintained at the University of Lausanne (UNIL, Lausanne, Switzerland) SPF Unit. C57BL/6, CD45.1 congenic (B6.SJL-PtprcaPep3b/BoyJArc), OT-I mice, and OT-II mice were obtained from Harlan Laboratories (22, 23). Pmel and Trp-1 mice were obtained from The Jackson Laboratory (24, 25). Foxp3-eGFP reporter mice were purchased from EMMA (EMo1945; ref. 26). Foxp3-eGFP mice were crossed to TCR-transgenics to create OT-IIxFoxp3-eGFP (referred to as OT-II) and Trp-1xFoxp3-eGFP (referred to as Trp-1). Age- and sex-matched mice between 6 and 14 weeks of age were used for all experiments. This study was approved by the local Veterinary Authority and performed in accordance with Swiss ethical guidelines.

Cell lines

The B16.OVA melanoma cell line was obtained from B. Huard (University Medical Center, Geneva, Switzerland; ref. 27). The B16 and EG7 lymphoma cell lines were obtained from the American Type Culture Collection (CRL-6475 and CRL-2113; refs. 28, 29). Tumor cell lines were maintained in complete Iscove’s Modified Dulbecco’s Medium (IMDM) medium supplemented with G418, at 1 mg/mL for B16.OVA and 0.4 mg/mL for EG7.OVA.

Adoptive cell transfers

Antigen-specific CD8+ and CD4+ T cells (CD45.2) were isolated from spleens of TCR-transgenic mice. The frequency of transgenic T cells was determined by flow cytometry. OT-I and OT-II cells were labeled with Vα2 and Vβ5.1/5.2 antibodies. Trp-1 cells with CD4 and Vβ14 antibodies, and Pmel cells with H-2Dβ/hgp10025-33 tetramers. naïve CD45.1 recipient mice received 1 × 10^5 or 1 × 10^6 OT-I cells and 3 × 10^5 or 1 × 10^6 OT-II cells in 200 μL of Dulbecco’s Modified Eagle Medium (DMEM) intravenously, as indicated. Alternatively, mice received 1 × 10^5 Pmel and 1 × 10^5 Trp-1 cells in 200 μL of DMEM intravenously.

Immunizations

Mice were immunized with 10 μg OVA257-264 and 10 μg OVA323-339 peptides or with 10 μg hgp10025-33 and 10 μg Trp-106-130 peptides in 100 μL PBS subcutaneously at the base of the tail. Peptides were injected alone or in combination with the following adjuvants: 50 μg CpG-ODN 1826 (CpG), Pam3CSK4 (Pam3Cys), HMW PolyIC, imiquimod, and Quil A or 5 μg LPS and Flagellin from Salmonella typhimurium, all ± emulsion in 50 μL IFA (30, 31). Peptides were manufactured by the Protein and Peptide Chemistry Facility (PPCF) of the UNIL. Adjuvants were sourced from InvivoGen except CpG-ODN (Coley Pharmaceuticals) and the Quil A saponin mix from Quillaja saponaria (generously provided by Brenntag Nordic A/S). IFA was purchased from Sigma–Aldrich, Inc.

Flow cytometry

Draining lymph nodes (inguinal) and spleens were harvested on day 7 after immunization. Cell suspensions were incubated with appropriate concentrations of antibody in PBS containing 2% FBS. The anti-FcγRII monoclonal antibody 2.4G2 was used to inhibit nonspecific antibody binding. Antibodies were obtained from BD Pharmingen and ebioscience or grown in-house from B-cell hybridomas. Samples were acquired using LSR-II and FACSCanto flow cytometers (Becton-Dickinson) and analyzed using FlowJo software (TreeStar). Lymphocytes were gated on the basis of forward scatter and side scatter properties and LIVE/DEAD Aqua Cell Stain (Life Technologies) was used to exclude dead cells.

In vitro restimulation and intracellular cytokine staining

Cell suspensions were incubated in complete DMEM containing 1 μmol/L of specific MHC-I and MHC-II peptides for 4 hours in the presence of CD107a-specific antibodies. Of note, 1 μmol/L GolgiPlug and GolgiStop were added after 1 hour of incubation. Cells were harvested, surface labeled, fixed, and permeabilized using the Fix/Perm Kit. Intracellular cytokines were detected using anti-IFN-γ and anti-interleukin (IL)-2 antibodies. All reagents were purchased from BD Biosciences and fixation and staining was performed according to the manufacturer’s specifications.

In vivo cytotoxicity assay

Cytotoxicity was measured using the VITAL assay (32). Briefly, C57BL/6 splenocytes were left untreated and labeled with 10 μmol/L CellTracker Orange (CTO; Molecular Probes), or incubated for 2 hours with 10 or 100 nmol/L OVA or hgp100 peptide, and then labeled with 0.02 or 0.2 μmol/L carboxyfluorescein diacetate succinimidy ester (CFSE) (Molecular Probes), respectively. Labeled cells were mixed at equal ratios, and approximately 2 × 10^5 cells of each population were injected intravenously. At 6 or 24 hours after target cell administration, blood was collected for flow cytometric
analysis. Percentage specific killing = 100 – [100 × (exp number CFSE+ cells/exp number CTO+ cells)/(control number CFSE+ cells/control number CTO+ cells)].

Tumor-infiltrating lymphocyte analysis

Mice that had received OT-I and OT-II T cells were challenged the next day with 2 × 10^6 B16.OVA tumor cells subcutaneously in the left flank. One week later, once tumors were palpable, mice were immunized as described above. After 7 days, draining lymph nodes (dLN) and tumors were excised and were weighed and digested in collagenase I and DNase I (Roche). CD45+ cells were purified by positive selection using magnetic cell separation (MACS) beads and the AutoMACS automatic cell separator (Miltenyi Biotech).

Tumor protection

Prophylactic setting. Mice received OT-I and OT-II T cells intravenously and were immunized the next day with the indicated OVA vaccine formulations. A total of 2 × 10^6 B16. OVA melanoma cells were injected subcutaneously in the left flank 1 week later, tumor growth was monitored every 2 to 3 days as described previously (33). Ten days after tumor challenge, mice received a vaccine boost proximal to the tumor.

Therapeutic setting. Mice received OT-I and OT-II T cells intravenously and were challenged the next day with 5 × 10^6 E.G7.OVA tumor cells subcutaneously in the left flank, and examined every 2 to 3 days to monitor tumor growth. Ten days later, mice with well-established tumors were immunized as above.

Self-antigen model. Mice received Pmel and Trp-1 cells intravenously and were immunized the next day with the indicated hgp100/Trp-1 vaccine formulations and boosted 1 week later. A total of 1 × 10^6 B16.F10 melanoma cells were injected subcutaneously at the time of boosting.

Cytokine multiplex assay and IFN-β ELISA

C57BL/6 mice were immunized subcutaneously at the base of the tail with peptides ± adjuvants as described above. The dLN (inguinal) were harvested 12 or 24 hours later. Total lymph node cell suspensions were incubated at 37°C in IMDM supplemented with 5% FBS. Supernatant samples were collected and frozen at 1 hour for analysis of type I IFN and at 1, 6, and 12 hours for cytokine multiplex analysis. Cytokine production was measured with the mouse IFN-β ELISA Kit and 10-plex LumineX panel (granulocyte macrophage colony-stimulating factor [GM-CSF], IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70 and TNF-α), using the LumineX 200 System with xPONENT Software (all from Life Technologies) and Epoch ELISA plate-reader (BioTek).

Statistical calculations

Statistical differences between groups were calculated using the ANOVA and Dunnett multiple comparison tests, comparing all groups to the peptide alone group. Differences in survival were calculated using the log-rank test, comparing all groups to the untreated control. The two-way ANOVA and Bonferroni posttests, matched by adjuvant group, were used for cytokine multiplex analysis. Error bars indicate SEM. All tests were performed using GraphPad Prism software. (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results

Antigen-specific Teff and Treg responses to peptide vaccination can be specifically modulated by adjuvant choice

To perform an in-depth investigation of the vaccine-mediated regulation of antigen-specific T-cell populations, mice were given an adoptive transfer of OT-I and OT-II T cells and then immunized with OVA276-291 and OVA263-279 peptides ± adjuvants. After 7 days, antigen-specific Teffs and Tregs were examined by flow cytometry in inguinal dLNs and spleen. We initially vaccinated mice with OVA peptide alone, OVA+CPG ±IFA to determine whether this system could faithfully reproduce the effect observed in the blood of patients with melanoma vaccinated with MelanA+CPG+IFA (21). This was indeed the case, with Cpg ±IFA significantly increasing the OVA-specific Teff:Treg ratio in vaccine-dLNs and spleen (Supplementary Fig. S1A and S1B). Polyclonal T cells did not undergo the same regulation in response to adjuvanted vaccines, as evidenced by stable Teff:Treg ratios (Supplementary Fig. S1C).

We next set out to determine whether the antigen-specific Teff:Treg ratios were similarly regulated by other adjuvants. Antigen-specific T cells were identified using the gating strategy shown in Fig. 1A. We observed that antigen-specific CD8+ T cells uniformly upregulated the activation marker CD44 (Supplementary Fig. S2A) in response to all vaccine formulations. However, there was substantial variation in the absolute numbers of OT-I T cells following immunization, with OVA+CPG and OVA+Poly(I:C) inducing significantly greater expansion than other vaccine formulations (Fig. 1B). Antigen-specific CD4+ Teffs decreased expression of the resting marker CD69 (Supplementary Fig. S2B) and expanded to a similar extent in response to all adjuvants except imiquimod and Quil A (Fig. 1C). Absolute OT-II Tregs generally increased following vaccination regardless of the adjuvant (Fig. 1D). All adjuvants, with the notable exception of Quil A, reduced the proportion of Tregs among OT-II cells (Fig. 1E). Ratios of OVA-specific Teffs to Tregs were calculated from absolute Teff and Treg counts. OVA+CPG and OVA+Poly(I:C) significantly enhanced antigen-specific CD8+ Teff:Treg ratios compared with mice vaccinated with OVA alone (Fig. 1F). Cpg, Pam3Cys, and Flagellin enhanced antigen-specific CD4+ Teff:Treg ratios (Fig. 1G). IFA increased the immune response to some of the peptide±adjuvant vaccines but did not significantly impact antigen-specific Teff:Treg ratios (Supplementary Fig. S3). Cpg, Poly(I:C), imiquimod, and Quil A (from which QS21 is derived) were selected for further analysis in in vivo models of tumor protection due to their contrasting immunologic effects and particular clinical relevance for cancer vaccination.

Adjuvants promoting high Teff:Treg ratios enhance lymphocyte infiltration into B16.OVA tumors and delay tumor growth

The immune response to peptide vaccination in a tumor setting was examined by immunizing mice bearing palpable...
Figure 1. CpG and Poly(I:C) induced the highest OVA-specific Teff:Treg ratios among a panel of adjuvants. CD45.1 mice received $1 \times 10^6$ OT-I and $3 \times 10^6$ OT-II T cells intravenously. One day later, mice were immunized subcutaneously with OVA257-264 and OVA323-339 peptides alone or in combination with Pam3Cys, Poly(I:C), LPS, flagellin (FLA), imiquimod (Imi), CpG, or Quil A. Draining lymph nodes were harvested 7 days later and antigen-specific T cells were analyzed. A, gating strategy for identifying antigen-specific T-cell populations. B, absolute number of OT-I effector cells. C, absolute number of OT-II effector cells. D and E, absolute number (D) and frequency (E) of OT-II Tregs. F, ratios of OT-I Teffs:OT-II Tregs. G, ratios of OT-II Teffs:Tregs. Combined data from three independent experiments comprising a total of 3 to 9 mice/group are shown.
R16.0VA melanomas. One week following vaccination, mice were sacrificed and tumor size as well as tumor-infiltrating lymphocytes was examined. The degree of OT-I tumor infiltration was significantly greater in mice immunized with OVA+ CpG and OVA+ Poly(I:C) compared with other groups (Fig. 2A, left) and was inversely related to tumor size (Fig. 2A, right). OT-II Teffs and Tregs infiltrated tumors poorly in all of the experimental groups (Fig. 2B). Because of low numbers of infiltrating OT-II T cells, ratios of antigen-specific Teffs:Tregs could not be reliably calculated at the tumor site. Accumulation of OT-I, OT-II Teffs, and OT-II Tregs in vaccine-dLNs of tumor-bearing mice resembled that of tumor-free mice (Supplementary Fig. S4). Lymph node Teff:Treg ratios correlated with the degree of antigen-specific CD8^+ T-cell infiltration and early tumor control (Fig. 2C vs. Fig. 2A). To investigate long-lasting tumor protection, mice were immunized 1 week before tumor challenge, followed by a booster immunization 10 days after, and tumor growth was monitored over time. Mice vaccinated with OVA+ CpG were completely protected against tumor development, and mice vaccinated with OVA+ Poly(I:C) developed tumors much more slowly than controls, leading to a significant improvement in survival time. Tumors developed and grew rapidly in all mice immunized with OVA+ Imiquimod or OVA+ Quil A (Fig. 2D). Cytotoxic function of OT-I cells was tested in vivo 1 week after vaccination in the absence of tumors. The highest killing of specific targets was seen in the OVA+ CpG and Poly(I:C) groups, supporting the role of antigen-specific CTL in mediating tumor protection when present at high ratios relative to antigen-specific Tregs (Fig. 2E).

Therapeutic vaccination with OVA+ CpG or OVA+ Poly(I:C) induces the rejection of established tumors

We next wanted to determine whether high Teff:Treg inducing vaccines could induce the rejection of established tumors in a therapeutic setting. Mice with well-established 10-day EG7 tumors were vaccinated with OVA peptides ± adjuvant. Tumor growth was substantially delayed in the OVA+ CpG and OVA+ Poly(I:C) vaccinated groups compared with controls. Three and 2 of 5 mice in these respective groups completely rejected their tumors (Fig. 3A and B). The overall survival of these two groups of mice was consequently enhanced (Fig. 3B, right), with a significant proportion of the mice remaining tumor-free for at least 50 days. On the other hand, the tumor growth rate in the OVA alone, OVA+ Quil A, and OVA+ Imiquimod groups was similar to that of the untreated controls (Fig. 3A).

Poly(I:C) and CpG enhance the tumor/self-antigen-specific Teff:Treg balance

To ascertain whether the adjuvant effects on Teff:Treg ratios were translatable to a self-/tumor-antigen system, we repeated our experiments in a gp100 and Trp-1 melanoma antigen model. We transferred CD8^+ Pmel and CD4^+ Trp-1 T cells into CD45.1^-recipient mice and vaccinated them with gp100 and Trp-1 peptides. A week later antigen-specific T-cell responses were measured in dLNs and spleen. Vaccination with gp100/Trp-1+Poly(I:C) or CpG induced significantly greater expansion of Pmel T cells than peptide alone (Fig. 4A, left). Effector potential was assessed by restimulating splenocytes with specific peptides for 4 hours. A substantial proportion of Pmel Teffs in all adjuvanted vaccination groups acquired at least one effector characteristic: producing IL-2, IFN-γ, or releasing cytotoxic granules via externalization of CD107α (Supplementary Fig. S5A). Polyfunctional effectors, simultaneously producing IL-2, IFN-γ, and degranulating, were most frequent following vaccination with self-peptides+Poly(I:C) or CpG (Fig. 4A, right). This corresponded with the greatly enhanced Teff:Treg ratios observed in the gp100/Trp-1+Poly(I:C) or CpG groups (Fig. 4D). Trp-1 Teff expansion was significantly enhanced in response to immunization with self-peptides+Poly(I:C), CpG, or Quil A (Fig. 4B, left). Similar proportions of Trp-1 Teffs in all vaccinated groups displayed at least one effector function (Supplementary Fig. S5B), whereas significantly higher frequencies of Trp-1 Teffs in the hgp100/Trp-1+Poly(I:C) or CpG groups simultaneously produced IFN-γ and IL-2 (Fig. 4B, right). Self-peptide immunization with Poly(I:C), CpG, or imiquimod reduced the number of Tregs among Trp-1 T cells, whereas Quil A increased their number (Fig. 4C). The ratio of Trp-1 Teffs:Tregs was significantly increased for groups receiving Poly(I:C) and CpG, but not for Quil A or imiquimod (Fig. 4E). Importantly, the higher antigen-specific Teff:Treg ratios observed with hgp100/Trp-1+Poly(I:C) or CpG immunization resulted in significantly greater in vivo killing of hgp100-pulsed targets (Fig. 4F). Altogether, these data corroborate the results obtained in the OVA model.

Adjuvants promoting high Teff:Treg ratios confer protection against tumor growth in a self-/tumor-antigen system

We next set out to determine whether the polyfunctional Teffs generated by vaccination with tumor/self-peptide and CpG or Poly(I:C) could confer protection against B16 tumor challenge. Mice received Pmel and Trp-1 T cells and were immunized with gp100+Trp-1 peptides ± adjuvant 1 week before tumor challenge, followed by a booster immunization on the day of tumor graft. Mice vaccinated with hgp100/Trp-1+Poly(I:C) or CpG developed tumors much more slowly than controls (Fig. 5A), leading to a significant improvement in survival time (Fig. 5B). Depigmentation was observed in several groups of vaccinated mice and was most prevalent in the peptide+CpG and Poly(I:C) groups, correlating with enhanced survival (Fig. 5C) and confirming that vaccination with high Teff:Treg-inducing adjuvants in a natural tumor-antigen setting induces a functional and protective immune response.

High Teff:Treg promoting adjuvants induce early production of type 1 cytokines

There is abundant evidence that certain TLR ligands induce maturation of APCs and proinflammatory cytokine production, differentially polarizing T cells during priming. Thus, dLNs were harvested 12 or 24 hours after immunization and cultured in the absence of further stimulation to assess the cytokine milieu produced by APC. Significant levels of IFN-β were detected in supernatants from dLNs
Figure 2. Increased infiltration of OT-I cells at the tumor site and reduction in tumor growth correlate with the Teff:Treg ratios in the dLNs. Mice received OT-I and OT-II T cells intravenously as in Fig. 1 and were challenged with B16.OVA tumor cells subcutaneously in the left flank. Seven days later, mice were immunized with OVA257-264 and OVA323-339 peptides adjuvant. Tumors and dLNs were excised 7 days later and lymphocyte populations were analyzed by flow cytometry (A–C). Alternatively, mice were vaccinated 1 week before tumor challenge and boosted 10 days after tumor establishment. Tumor growth was monitored over time (D). To assess in vivo killing, splenocytes were loaded with OVA-peptide and injected intravenously 1 week after vaccination in the absence of tumors. Surviving target cell frequencies were detected in blood 6 hours later (E). A, absolute number of OT-I effector cells per milligram of tumor tissue (left) and total tumor weight in milligrams (right). B, number of OT-II effector cells (left) and OT-II Tregs (right) per milligram of tumor tissue. C, ratios of OT-I Teffs:OT-II Tregs (left) and OT-II Teffs:OT-II Tregs (right) in the tumor/vaccine dLNs. D, mean tumor sizes (left) and Kaplan–Meier survival curves (right). E, specific killing of 10 nmol/L peptide-pulsed targets at 6 hours. A, B, C, and E, the data for groups of 3 mice in one of two independent experiments. Data in D are from groups of 5 mice representing one of four independent experiments.
extracted 12 hours after vaccination and incubated \textit{in vitro} for 1 hour (Fig. 6A). Inflammatory mediators IL-12, IFN-$\gamma$, and IL-6 were detected in 1-, 6-, and 12-hour culture supernatants of dLNs from mice vaccinated with OVA$+$CpG or OVA$+$Poly(I:C) 12 hours earlier (Fig. 6B, left). OVA$+$Quil A and OVA$+$Imiquimod induced less or no IL-12, IFN-$\gamma$, and IL-6 at the same time points. Lymph nodes extracted from mice vaccinated with OVA$+$CpG 24 hours earlier continued to produce a small but significant amount of IL-12 detected in the culture supernatant, whereas the levels of the other analytes returned to baseline in all groups (Fig. 6B, right). Variable levels of TNF-$\alpha$ and IL-2 production were detected but no significant difference was apparent between groups (Supplementary Fig. S6). No production of GM-CSF, IL-1$\beta$, IL-4, or IL-10 was detected (data not shown).

**Discussion**

In this study, we performed a comprehensive analysis of a panel of adjuvants to better characterize their antigen-specific effects on antitumor immune responses following peptide vaccination. Our dual T-cell adoptive transfer models were ideal tools to closely examine the relationship between vaccine-induced antigen-specific Teff and Treg populations, which are extremely rare in a physiologic setting. We were able to determine a hierarchy among the different adjuvants tested with regards to their ability to modulate antigen-specific Teff and Treg responses. Similar results were obtained in the ovalbumin and melanoma peptide models, highlighting the robustness of this experimental system. It is also noteworthy that our results in both mouse models tested recapitulated our observations in patients with melanoma vaccinated with Melan-A peptide and CpG-ODN (21).

We found that immunization with peptide and adjuvants CpG-ODN and Poly(I:C) preferentially promoted the expansion of antigen-specific CD8$^+$ and CD4$^+$ Teffs over that of antigen-specific Tregs, resulting in increased Teff:Treg ratios. In addition, these adjuvants endowed antigen-specific effector cells with polyfunctional effector capacity. In contrast, we found that Quil A and imiquimod maintained or even decreased Teff:Treg ratios due to a greater accumulation of antigen-specific
Tregs, whereas effector cell expansion and acquisition of polyfunctionality were reduced. Polyfunctionality is a defining feature of long-lived T cells, and correlates with increased disease protection in both human antiviral vaccination and murine tumor immunotherapy studies (34–36). Furthermore, a recent report demonstrated that human papillomavirus E7 peptide+CpG-ODN or Poly(I:C) vaccine formulations expanded multi-cytokine–producing CD8+ effector memory T cells, the presence of which predicted therapeutic efficacy against cancer in mice (37). Our results are consistent with these studies and provide the additional dimension of control of antigen-specific Treg expansion by CpG-ODN or Poly(I:C) adjuvants.

Previous studies have linked total CD8+ T cell:CD4+ Treg ratios to both natural tumor progression (5, 38) and cancer immunotherapy outcomes in mice and humans (39, 40). However, little information exists on the role of antigen-specific Tregs on disease outcome, or how this population might be regulated by immunotherapy. Data are emerging on the Teff and antigen-specific Treg relationships in autoimmunity and transplantation. Immunotherapies that enhance antigen-specific Treg expansion and suppressive function delayed multi-cytokine–producing Teff activation, resulting in disease control (41, 42). To date, the relationship between antigen-specific Teffs and Tregs has not been examined in detail in cancer, although some in vitro and observational reports exist.
Our study extends the knowledge base gained from previous investigations by comparing a large panel of adjuvants for their ability to modulate the vaccine-specific Teff:Treg balance. Our results indicate that each adjuvant uniquely modulates antigen-specific Teff:Treg ratios, which in turn correlate with tumor control. Interestingly, we found that in contrast to antigen-specific responses, polyclonal Teff:Treg ratios are not affected by vaccination with specific peptide in adjuvant (Supplementary Fig. S1C). Thus, the behavior of polyclonal T-cell populations cannot be used to predict the responses of antigen-specific cells. This fact should be considered in rational vaccine design, which should focus on controlling the balance of Tefs versus Tregs with defined antigen specificities to create the most effective vaccines.

We found that the ratios of antigen-specific Tefs:Tregs in tumor-DLNs were similar to those observed in vaccine-DLNs of tumor-free mice. High TefTreg ratios induced by CpG-ODN and Poly(I:C) in the lymphoid tissues correlated with increased OT-1 T-cell activation, cytotoxic activity, and tumor infiltration, leading to significantly higher protection against tumor growth. Surprisingly, antigen-specific Tregs infiltrated melanomas very poorly and are therefore unlikely to play a major role at the tumor site. This is in sharp contrast to the profound infiltration of polyclonal Tregs into many tumors, including melanomas, which has been observed by us (data not shown) and others (43, 44). That antigen-specific T cells should play an important role in the lymph nodes rather than at the disease site is consistent with reports that antigen-engagement by Tregs is important for initial priming, allowing fine-regulation of the priming of effector cells of the same specificity (45). Once activated, Tregs can suppress nonspecifically, thus eliminating the need for antigen-specific Tregs at disease sites (45, 46). These findings outline the relevance of antigen-specific Teff:Treg ratios in the development of antitumor immunity. Measuring this balance in the periphery may consequently provide a more accurate method of predicting vaccine efficacy (39, 47).
The mechanisms involved in the control of effector versus Treg induction, whether polyclonal or antigen-specific, are not completely understood. However, the maturation state of APCs and the resulting proinflammatory cytokine environment at the site of T-cell priming is known to be important (48–50). There is ample evidence that vaccine adjuvants, and TLR agonists in particular, can mature dendritic cells, inducing expression of costimulatory molecules such as CD40 and modifying their T cell priming potential (51). Type I IFNs are necessary for the maturation of dendritic cells and the generation of CTL and Th1 responses. Vaccines containing Poly(I:C), CpG or the TLR-7/8 ligand R-848 have been shown to induce IFN-α/β-dependent production of type I cytokines (48, 49). Dendritic cells are also matured by stimulation with the TLR ligands LPS and CpG. They upregulate MHC-II, CD80 and CD86 expression and produce the inflammatory cytokines IL-12, IFN-γ and IL-6, which promote effector CTL and Th1 development as well as directly inhibiting Tregs (50, 52, 53). We found that a dendritic cell maturing/type I polarizing cytokine milieu consisting of IFN-β, IL-12, IFN-γ and IL-6 was induced in dLNs early after vaccination with peptide+CpG or Poly(I:C). These adjuvants induced the highest antigen-specific Teff:Treg ratios and the best antitumor outcomes, providing clues about the mechanism behind the adjuvant effects on vaccine-specific T cell balance. Antibody-blockade of type I IFNs, and/or IL-12, or experiments in KO mouse strains would be necessary to produce definitive evidence for the role of these cytokines, but the concordance of our results with the large body of existing data provide strong support for this preliminary mechanistic explanation.

In conclusion, we have shown that not all adjuvants are equal in their ability to modulate vaccine-specific immune responses. Our data support the use of the adjuvants CpG-ODN and Poly(I:C) in peptide vaccines containing both CD4 and CD8 epitopes, to enhance the activation of polyfunctional Teffs and avoid Treg expansion. This study also highlights the importance of studying antigen-specific Teff and Treg responses in the context of peptide vaccination, as these cannot necessarily be extrapolated from an evaluation of total polyclonal responses. Finally, we reveal a correlation between the choice of adjuvant in a peptide vaccine formulation, the antigen-specific CD8+ and CD4+ Teff:Treg ratios, the size and quality of the resulting Teff response, and the degree of tumor protection induced. This suggests that antigen-specific Teff:Treg ratios are a useful measure of vaccination outcome and have the potential to be a valuable predictive biomarker of objective clinical responses in cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

P. Romero is a consultant/advisory board member of Immatics Biotechnologies, DC Prime, Matwin, and Center for Human Immunology, Pasteur Institute (Paris, France). No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R. Perret, P. Romero
Development of methodology: R. Perret
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Adjuvants Modulate Antitumor Teff:Treg Ratios


Adjuvants That Improve the Ratio of Antigen-Specific Effector to Regulatory T Cells Enhance Tumor Immunity

Rachel Perret, Sophie R. Sierro, Natalia K. Botelho, et al.

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