Adjuvants that improve the ratio of antigen-specific effector to regulatory T cells

enhance tumor immunity

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Running Title: Adjuvants modulate anti-tumor Teff:Treg ratios

Keywords: Adjuvant, effector T cell, Treg, tumor, vaccine.

Financial Support

R. Perret received fellowships from the New Zealand Foundation for Research Science and Technology and the Emma Muschamp Foundation.

P. Romero was supported by grants from the Swiss National Science Foundation (310030-130812 and CRSII3_141879) and the Medic Foundation.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

Manuscript details: text 5,173 words and abstract 210 words, 6 figures and 6 supplementary figures.
Abstract

Antitumor immunity is strongly influenced by the balance of tumor antigen-specific effector and regulatory T cells. 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 T regulatory cells (Treg) were induced following subcutaneous vaccination with either OVA or melanoma-derived peptides, with a restricted expansion of effector T cells. Addition of the adjuvants CpG-ODN or Poly(I:C) preferentially amplified effector T cells over Tregs, dramatically increasing the antigen-specific T effector: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 effector T cell:Treg ratios. Following therapeutic vaccination of tumor-bearing mice, high ratios of tumor-specific effector T cells:Tregs in draining lymph nodes were associated with enhanced CD8+ T cell infiltration at the tumor site and a durable rejection of tumors. Vaccine formulations of peptide+CpG-ODN or Poly(I:C) induced selective production of pro-inflammatory Type I cytokines early after vaccination. This environment promoted CD8+ and CD4+ effector T cell expansion over that of antigen-specific Tregs, tipping the effector T cell 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.
**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 anti-tumor immune responses in patients with advanced melanoma (1, 2) but clinical benefits have not lived up to 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. Tregs play a major role in the control of anti-tumor immunity (5-7) and existing cancer vaccines activate and expand Tregs, resulting in suppression of anti-tumor 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 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 APC maturation and production of inflammatory cytokines, favouring type I effector T cell responses and restricting Treg expansion (13-15). Conflicting data shows that TLR agonists CpG-ODN, LPS, Zymozan (TLR-6), Poly(I:C) (TLR-3), Imiquimod and R-848 (TLR-7/8)
can expand both effector T cells 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 colorectal carcinoma patients 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 DCs (19). In a clinical study of melanoma patients immunised with NY-ESO-1 protein in ISCOMATRIX, vaccination increased the frequency of NY-ESO-1-specific Tregs in PBMCs and tumor tissue (20). We have shown that therapeutic vaccination of melanoma patients 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 anti-tumor 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 Poly(I:C) preferentially induce strong proliferation of antigen-specific
effector T cells, while minimising antigen-specific Treg expansion. High Teff:Treg ratios were linked to strong proinflammatory cytokine production in the lymph nodes early after immunisation 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 SPF Unit. C57BL/6, CD45.1 congenic (B6.SJL-PtprcaPep3b/BoyJArc), OT-I mice and OT-II mice were obtained from Harlan laboratories (The Netherlands) (22, 23). Pmel and Trp-1 mice were obtained from Jackson Laboratories (USA) (24, 25). Foxp3-eGFP reporter mice were purchased from EMMA (EM:01945) (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 6-14 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) (27). The B16 and EG7 lymphoma cell lines were obtained from the American Type Culture Collection (ATCC® CRL-6475 and CRL-2113) (28, 29). Tumor cell lines were maintained in complete IMDM medium supplemented with G418, at 1mg/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 labelled with Vα2 and Vβ5.1/5.2 antibodies; Trp-1 cells with CD4 and Vβ14 antibodies; and Pmel cells with H-2Db/hgp10025-33 tetramers. Naïve CD45.1 recipient mice received 1 x 10^5 or 1 x 10^6 OT-I cells and 3 x
$10^6$ or $1 \times 10^6$ OT-II cells in 200 μl DMEM i.v., as indicated. Alternatively, mice received $1 \times 10^5$ Pmel and $1 \times 10^5$ Trp-1 cells in 200 μl DMEM i.v.

**Immunisations**

Mice were immunised with 10 μg OVA<sub>257-264</sub> and 10 μg OVA<sub>323-339</sub> peptides or with 10 μg hgp100<sub>25-33</sub> and 10 μg Trp-1<sub>106-130</sub> peptides in 100 μl PBS s.c. 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 Poly(I:C), Imiquimod and Quil A or 5 μg LPS and Flagellin from *S. typhimurium*, all +/- emulsification in 50 μl IFA (30, 31). Peptides were manufactured by the Protein and Peptide Chemistry Facility (PPCF) of the UNIL (Lausanne, Switzerland). Adjuvants were sourced from Invivogen except CpG-ODN (Coley Pharmaceuticals) and the Quil A saponin mix from *Q. saponaria* (generously provided by Brenntag Nordic A/S, Ballerup, Denmark). Incomplete Freund’s Adjuvant (IFA) was purchased from Sigma–Aldrich, Inc.

**Flow Cytometry**

Draining lymph nodes (inguinal) and spleens were harvested on day 7 after immunisation. Cell suspensions were incubated with appropriate concentrations of antibody in PBS containing 2% FBS. The anti-FcγRII mAb 2.4G2 was used to inhibit non-specific 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 FACS-Canto flow cytometers (Becton-Dickinson) and analysed using FlowJo software (Tree Star). 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 1μM specific MHC-I and MHC-II peptides for 4 hrs in the presence of CD107a-specific antibodies. 1 μM Golgiplug® and Golgistop® were added after 1 hr of incubation. Cells were harvested, surface labelled, fixed and permeabilised using the Fix/Perm kit. Intracellular cytokines were detected using anti-IFN-γ and anti-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 labelled with 10 μM CellTracker Orange (CTO, Molecular Probes), or incubated for 2 hrs with 10 nM or 100 nM OVA or hgp100 peptide, and then labeled with 0.02 μM or 0.2 μM CFSE (Molecular Probes) respectively. Labeled cells were mixed at equal ratios, and ~2 x 10^6 cells of each population were injected i.v. At 6 hrs or 24 hrs after target cell administration blood was collected for FACS analysis. % specific killing = 100 – [100 x (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 $2 \times 10^5$ B16.OVA tumor cells s.c. in the left flank. One week later, once tumors were palpable, mice were immunised as described above. After 7 days, dLNs and tumors were excised and tumors were weighed and digested in Collagenase I and DNase I (Roche). CD45+ cells were purified by positive selection using magnetic MACS beads and the AutoMACS automatic cell separator (Miltenyi Biotech).

**Tumor protection**

Prophylactic setting: mice received OT-I and OT-II T cells i.v. and were immunised the next day with the indicated OVA vaccine formulations. $2 \times 10^5$ B16.OVA melanoma cells were injected s.c. in the left flank one week later, tumor growth was monitored every 2–3 days as described (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 i.v. and were challenged the next day with $5 \times 10^6$ EG7.OVA tumor cells s.c. in the left flank, and examined every 2–3 days to monitor tumor growth. Ten days later, mice with well-established tumors were immunised as above. Self-antigen model: mice received Pmel and Trp-1 cells i.v. and were immunised the next day with the indicated hgp100/Trp-1 vaccine formulations and boosted one week later. $1 \times 10^5$ B16.F10 melanoma cells were injected s.c. at the time of boosting.

**Cytokine multiplex assay & IFN-β ELISA**

C57BL/6 mice were immunised s.c. at the base of the tail with peptides +/- adjuvants as described above. The draining (inguinal) LNs were harvested 12 or 24 hours later. Total LN cell suspensions were incubated at 37 °C in IMDM supplemented with 5 % FBS. Supernatant samples were collected and frozen at 1 hr for analysis of type I
interferon and at 1, 6 and 12 hrs for cytokine multiplex analysis. Cytokine production was measured with the mouse IFN-β ELISA kit and 10-plex Luminex panel (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’s 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 post-tests, 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 effector and regulatory T cell 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 immunised with OVA$_{257-264}$ and OVA$_{323-339}$ peptides +/- adjuvants. After 7 days, antigen-specific effector T cells and Tregs were examined by flow cytometry in inguinal draining LNs and spleen. We initially vaccinated mice with OVA peptide alone, OVA+CpG +/-IFA to determine if this system could faithfully reproduce the effect observed in the blood of melanoma patients vaccinated with MelanA+CpG+IFA (21). This was indeed the case, with CpG +/-IFA significantly increasing the OVA-specific Teff:Treg ratio in vaccine-draining LNs and spleen (Supplementary Fig S1a&b). 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 if the antigen-specific Teff:Treg ratios were similarly regulated by other adjuvants. Antigen-specific T cells were identified using the gating strategy shown in Figure 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 immunisation, with OVA+CpG and OVA+Poly(I:C) inducing significantly greater expansion than other vaccine formulations (Figure 1b). Antigen-specific CD4+ effector T cells decreased
expression of the resting marker CD62L (Supplementary Fig S2b) and expanded to a
similar extent in response to all adjuvants except Imiquimod and Quil A (Figure 1c).
Absolute OT-II Tregs generally increased following vaccination regardless of the
adjuvant (Figure 1d). All adjuvants, with the notable exception of Quil A, reduced the
proportion of Tregs among OT-II cells (Figure 1e). Ratios of OVA-specific effector T
cells 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 to mice vaccinated with OVA alone (Figure 1f). CpG, Pam3Cys and
Flagellin enhanced antigen-specific CD4+ Teff:Treg ratios (Figure 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
immunological 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 tumour setting was examined by
immunising mice bearing palpable B16.OVA melanomas. One week following
vaccination, mice were sacrificed and tumor size as well as tumor infiltrating
lymphocytes were examined. The degree of OT-I tumor-infiltration was significantly
greater in mice immunised with OVA+CpG and OVA+Poly(I:C) compared to other
groups (Figure 2a, left panel) and was inversely related to tumor size (Figure 2a, right
panel). OT-II Teffs and Tregs infiltrated tumors poorly in all of the experimental
groups (Figure 2b). Due to 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-draining LNs of tumor-bearing mice resembled that of tumor-free mice (Supplementary Fig S4). LN Teff:Treg ratios correlated with the degree of antigen-specific CD8+ T cell infiltration and early tumor control (Figure 2c vs. Figure 2a). To investigate longer-lasting tumor protection, mice were immunised one week before tumor challenge, followed by a booster immunisation 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 immunised with OVA+Imiquimod or OVA+Quil A (Figure 2d). Cytotoxic function of OT-I cells was tested in vivo one 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 (Figure 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 to controls. Three and two out of five mice in these respective groups completely rejected their tumors (Figure 3a & b). The overall survival of these two
groups of mice was consequently enhanced (Figure 3b, right panel), 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 (Figure 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 hgp100 and Trp-1 peptides. A week later antigen-specific T cell responses were measured in dLNs and spleen. Vaccination with hgp100/Trp-1+Poly(I:C) or CpG induced significantly greater expansion of Pmel T cells than peptide alone (Figure 4a, left panel). Effector potential was assessed by restimulating splenocytes with specific peptides for 4 hrs. 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 externalisation of CD107a (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 (Figure 4a, right panel). This corresponded with the greatly enhanced Teff:Treg ratios observed in the hgp100/Trp-1+Poly(I:C) or CpG groups (Figure 4c). Trp-1 Teff expansion was significantly enhanced in response to immunisation with self-peptides+Poly(I:C), CpG or Quil A (Figure 4b, left panel). Similar proportions of Trp-1 Teffs in all vaccinated groups displayed at least one effector function (Supplementary Fig S5b), while 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 (Figure 4b, right panel). Self-peptide immunisation with Poly(I:C), CpG or Imiquimod reduced the number of Tregs among Trp-1 T cells, while Quil A increased their number (Figure 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 (Figure 4e). Importantly, the higher antigen-specific Teff:Treg ratios observed with hgp100/Trp-1+Poly(I:C) or CpG immunisation resulted in significantly greater in vivo killing of hgp100-pulsed targets (Figure 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 effector T cells 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 immunised with gp100+Trp-1 peptides +/- adjuvant one week before tumor challenge, followed by a booster immunisation on the day of tumor graft. Mice vaccinated with hgp100/Trp-1+CpG or Poly(I:C) developed tumors much more slowly than controls (Figures 5a), leading to a significant improvement in survival time (Figure 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 (Figure 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 I cytokines**
There is abundant evidence that certain TLR ligands induce maturation of APCs and proinflammatory cytokine production, differentially polarising T cells during priming. Thus, draining LNs were harvested 12 or 24 hrs after immunisation 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 extracted 12 hrs after vaccination and incubated \textit{in vitro} for 1 hr (Figure 6a). Inflammatory mediators IL-12, IFN-γ and IL-6 were detected in 1, 6 and 12 hr culture supernatants of dLNs from mice vaccinated with OVA+CpG or OVA+Poly(I:C) 12 hrs earlier (Figure 6b, left panels). OVA+Quil A and OVA+Imiquimod induced less or no IL-12, IFN-γ and IL-6 at the same time-points. Lymph nodes extracted from mice vaccinated with OVA+CpG 24 hrs earlier continued to produce a small but significant amount of IL-12 detected in the culture supernatant, while the levels of the other analytes returned to baseline in all groups (Figure 6b, right panels). Variable levels of TNF-α and IL-2 production were detected but no significant difference was apparent between groups (Supplementary Fig S6). No production of GM-CSF, IL-1β, 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 characterise their antigen-specific effects on anti-tumor 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 physiological setting. We were able to determine a hierarchy amongst the different adjuvants tested with regards to their ability to modulate antigen-specific effector and regulatory T cell 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 melanoma patients vaccinated with Melan-A peptide and CpG-ODN (21).

We found that immunisation with peptide and adjuvants CpG-ODN and Poly(I:C) preferentially promoted the expansion of antigen-specific CD8+ and CD4+ effector T cells over that of antigen-specific Tregs, resulting in increased Teff:Treg ratios. Additionally, 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, while 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 anti-viral 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 is emerging on the effector T cell and antigen-specific Treg relationships in autoimmunity and transplantation. Immunotherapies that enhance antigen-specific Treg expansion and suppressive function delayed multi-cytokine-producing effector T cell activation, resulting in disease control (41, 42). To date, the relationship between antigen-specific effector T cells and Tregs has not been examined in detail in cancer, although some in vitro and observational reports exist (19-21). 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 1c). Thus, the behaviour 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 effector T cells versus Tregs with defined antigen-specificities in order to create the most effective vaccines.
We found that the ratios of antigen-specific Teffs:Tregs in tumor-draining LNs were similar to those observed in vaccine-draining LNs of tumor-free mice. High Teff:Treg ratios induced by CpG-ODN and Poly(I:C) in the lymphoid tissues correlated with increased OT-I 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 ourselves (data not shown) and others (43, 44). That antigen-specific T cells should play an important role in the LNs 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 non-specifically 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 anti-tumor 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 regulatory T cell 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 DCs, inducing expression of costimulatory molecules such as CD40 and modifying their T cell priming potential (51). Type I interferons are necessary for the maturation of DCs 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). DCs are also matured by stimulation with the TLR ligands LPS and CpG. They up-regulate 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 DC maturing/Type I polarising cytokine milieu consisting of IFN-β, IL-12, IFN-γ and IL-6 was induced in draining LNs early after vaccination with peptide+CpG or Poly(I:C). These adjuvants induced the highest antigen-specific Teff:Treg ratios and the best anti-tumor outcomes, providing clues concerning 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 provides 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 effector T cells and avoid Treg expansion. This study also highlights the importance of studying antigen-specific effector and regulatory T cell 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 effector T cell 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.

Authors' Contributions

Conception and design: R. Perret, P. Romero

Development of methodology: R. Perret, S. Sierro

Acquisition of data: R. Perret, N. Botelho, S. Corgnac, S. Sierro

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Acknowledgments

The authors would like to thank Aurélie Hanoteau for assistance with experiments and the staff of the SPF mouse facility of the University of Lausanne for animal husbandry and care.

Grant Support

This work was supported by grants from the New Zealand Foundation for Research Science and Technology and the Emma Muschamp Foundation to R. Perret and from the Swiss National Science Foundation (310030-130812 and CRSII3_141879) and the Medic Foundation to P. Romero.
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Figure legends

**Figure 1.** CpG and Poly(I:C) induce 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 i.v. One day later, mice were immunised s.c. with OVA$_{257-264}$ and OVA$_{323-339}$ peptides alone or in combination with Pam3Cys, Poly I:C, LPS, flagellin, Imiquimod, CpG or Quil A. Draining LNs were harvested 7 days later and antigen-specific T cells were analysed. **(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)** Absolute number and **(E)** frequency 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 3–9 mice/group are shown.

**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 i.v. as in Figure 1 and were challenged with B16.OVA tumor cells s.c. in the left flank. Seven days later, mice were immunised with OVA$_{257-264}$ and OVA$_{323-339}$ peptides +/- adjuvant. Tumors and dLNs were excised 7 days later and lymphocyte populations were analysed by flow cytometry (A-C). Alternatively, mice were vaccinated one 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 i.v. one week after vaccination, in the absence of tumors. Surviving target cell frequencies were detected in blood 6 hrs 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 in the tumor/vaccine draining LNs. (D) Mean tumor sizes (left) and Kaplan-Meyer survival curves (right). (E) Specific killing of 10 nM peptide-pulsed targets at 6 hrs. A, B C & E show 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.

Figure 3. Therapeutic vaccination with adjuvants that induce high antigen-specific Teff:Treg ratios confers durable rejection of established tumors.

Mice received 1 x 10^5 OT-I and 1 x 10^6 OT-II T cells i.v. Three days later, mice were challenged with EG7 tumor cells s.c. After 10 days, once tumors were well established, mice were immunised as above and tumor growth was monitored over time. (A) Growth curves of EG7 tumors are shown for each individual mouse in the different groups. (B) Mean tumor sizes (left) and survival times (right) are shown for groups of 5 mice in one of two independent experiments.

Figure 4. Tumor/self antigen-specific Teff:Treg balance and effector function are most strongly enhanced by vaccination with peptide and Poly(I:C) or CpG.

CD45.1 mice received 1 x 10^5 Pmel and 1 x 10^5 Trp-1 T cells i.v. One day later, mice were immunised s.c. with hgp100_25-33 and Trp-1_106-130 peptides +/- adjuvant. Spleens were harvested 7 days later and lymphocytes analysed (A-E). To assess in vivo killing, splenocytes were loaded with hgp100-peptide and injected i.v. one week after vaccination and surviving target cell frequencies were detected in blood 24 hrs later (F). (A) Absolute number of Pmel effector cells (CD8+CD45.2+, left) and frequency of Pmel cells producing IFN-γ, IL-2 and CD107a (right). (B) Absolute number of
Trp-1 effector cells (CD4+CD45.2+Foxp3-, left) and frequency of Trp-1 cells producing IFN-γ and IL-2 (right). (C) Absolute number of Trp-1 Tregs (CD4+CD45.2+Foxp3+). (D) Ratio of Pmel Teffs:Trp-1 Tregs (left) and ratio of Trp-1 Teffs:Trp-1 Tregs (right). (E) Specific killing of 10 and 100 nM peptide-pulsed targets at 24 hrs. Graphs show data from groups of 3 mice in one of two independent experiments.

**Figure 5. Vaccination with tumor/self-peptides and Poly(I:C) or CpG confers protection against tumor challenge**

Mice received Pmel and Trp-1 T cells i.v. as in Figure 4 and were vaccinated one day later with hgp10025-33 and Trp-1106-130 peptides +/- adjuvant. Seven days later, mice were challenged with B16 tumor cells s.c. and at the same time received a second vaccine dose. Tumor growth and survival were monitored over time. (A) Growth curves of B16 tumors are shown for individual mice in the different groups. (B) Survival following tumor challenge. (C) Vaccine-induced depigmentation (left axis) vs. survival (right axis) at day 20. Graphs show data for groups of 5 mice in one of two independent experiments.

**Figure 6. CpG and Poly(I:C) induce strong type I polarisation early after immunisation with peptide and adjuvant.**

Mice were immunised s.c. with OVA257-264 and OVA323-339 peptides +/- adjuvant. Draining LNs were collected 12 or 24 hours later and homogenised and incubated at 37 °C. Supernatants were collected from the LN cultures after 1, 6 and 12 hours and analysed by ELISA and Luminex. (A) IFN-β production measured by ELISA 1 hr after LN extraction. (B) Cytokine production measured by Luminex after LN
extraction and in vitro incubation of cell suspensions. Graphs show data from 3 samples per group in one of three independent experiments. Dotted lines show the detection limit.
Figure 2

A

B

C

D

E

Author Manuscript Published OnlineFirst on September 18, 2013; DOI: 10.1158/0008-5472.CAN-13-0875
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A

B

Time after tumor challenge (days)

Tumor size (mm^2)

Therapeutic vaccination

PBS

Peptides alone

Peptides + QuA

Peptides + iniquimod

Peptides + Poly(C)

Peptides + CpG

% survival

Vaccine
Figure 4
Figure 6

A

LN at 12hr

![Graph showing IFN-β levels after LN culture for 1 hour with different treatments.]

- PBS
- OVA alone
- OVA+QuilA
- OVA+Imiquimod
- OVA+Poly(I:C)
- OVA+CpG

B

LN at 12hr

- IL-12p70 (pg/ml)
- IFN-g (pg/ml)
- IL-6 (pg/ml)

Duration of LN culture (hours)

- 1
- 6
- 12

LN at 24hr

- IL-12p70 (pg/ml)
- IFN-g (pg/ml)
- IL-6 (pg/ml)

Duration of LN culture (hours)

- 12
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Cancer Res Published OnlineFirst September 18, 2013.

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