Published in final edited form as:

Clin Cancer Res. 2012 June 1; 18(11): 3122–3131. doi:10.1158/1078-0432.CCR-12-0113.

Dendritic cell activating vaccine adjuvants differ in the ability to elicit anti-tumor immunity due to an adjuvant specific induction of immune suppressive cells

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Abstract

Purpose—We questioned whether the vaccine adjuvant combination of TLR7 ligand agonist, imiquimod, with GM-CSF would result in enhanced dendritic cell recruitment and activation with increased antigen-specific immunity as compared with either adjuvant used alone.

Experimental Design—The adjuvant effects of GM-CSF and imiquimod were studied in OVA and MMTVneu transgenic mice using peptide-based vaccines. Type I immunity, serum cytokines, MDSC, and Treg cells levels were examined.

Results—Both GM-CSF and imiquimod equally induced local accumulation and activation of dendritic cells. Both adjuvants effectively enhanced OVA specific T-cell responses. We further evaluated the anti-tumor efficacy of adjuvant GM-CSF and imiquimod immunizing against murine IGFBP-2, a non-mutated oncoprotein overexpressed in the tumors of MMTVneu transgenic mice. Tumor growth was significantly inhibited in the mice receiving IGFBP-2 peptides with GM-CSF ($p=0.000$), but not in imiquimod-vaccine treated groups ($p=0.141$). Moreover, the addition of imiquimod to GM-CSF negated the anti-tumor activity of the vaccine when GM-CSF was used as the sole adjuvant. While GM-CSF stimulated significant levels of antigen specific Th1, imiquimod induced elevated serum IL-10. Both MDSC and Treg cells were increased in the imiquimod-treated but not GM-CSF-treated groups ($p=0.000$ and 0.006 respectively). Depleting MDSC and Treg in animals immunized with imiquimod and IGFBP-2 peptides restored anti-tumor activity to the levels observed with vaccination using GM-CSF as the sole adjuvant.

Conclusion—Adjuvants may induce regulatory responses in the context of a self-antigen vaccine. Adjuvant triggered immune suppression may limit vaccine efficacy and should be evaluated in pre-clinical models especially when contemplating combination approaches.

Keywords

vaccine adjuvant; IGFBP-2; GM-CSF; imiquimod; myeloid derived suppressor cells; T regulatory cells

INTRODUCTION

Numerous detailed analyses of immune related prognostic biomarkers in cancer patients have led to an improved understanding of the type of cellular response needed for immune-

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mediated destruction of cancer (1). A Type I T cell gene signature that promotes both the generation and tumor infiltration of cytotoxic T cells has been shown to be associated with an improved clinical outcome in a variety of tumor types (2, 3). Vaccines are being designed to induce such T cell responses in cancer patients. Vaccine adjuvants have the potential to manipulate the phenotype of the immunized cellular response to one that will establish a Type I immune microenvironment and support tumor specific cytotoxic T cells.

Most tumor antigens are self proteins, thus, there is a need to employ vaccine adjuvants that can stimulate the efficient presentation of weakly immunogenic proteins in a manner that will allow effective activation of T cells. Substantial progress has been made in identifying adjuvants that recruit and/or activate appropriate antigen presenting cells for eliciting tumor specific immunity (4). Many of these adjuvants are currently being evaluated in vaccine clinical trials (4). It is now conceivable to create combination adjuvant systems to orchestrate T cell priming during active immunization. Indeed, a recent investigation reported that the use of a cocktail of toll-like receptor (TLR) ligand agonists as an adjuvant for an anti-viral vaccine significantly enhanced the functional avidity of the T cells, rather than increasing their number, markedly improving the anti-viral response (5). Generating immunity against self, however, is also associated with self regulation. Thus, the more potent the adjuvant approach in inducing inflammation against self antigens, the greater the risk that mechanisms of self tolerance will limit the Type I response.

Granulocyte macrophage colony stimulating factor (GM-CSF) has been used as a vaccine adjuvant in cancer patients and has been shown to effectively recruit dendritic cells to the immunization site (6). Moreover, it has recently been shown that GM-CSF is required for the local accumulation of CD103⁺ dermal dendritic cells which can induce T cells to secrete both IFN-gamma as well as IL-17 (7). We questioned whether the combination of a TLR7-ligand agonist, imiquimod, also being studied as a single adjuvant in vaccine trials, with GM-CSF would result in both enhanced recruitment and activation of DC. In theory, large numbers of activated DC could prime highly functional tumor specific T cells to robust levels enhancing the clinical efficacy of therapeutic cancer vaccines. Such a strategy could be directly deployed to human clinical trials.

MATERIALS AND METHODS

Mice

The mouse strains used in this study include Balb/c, DO11.10 (strain name C.Cg-Tg (DO11.10)10Dlo/J), C57BL/6, OT-I (strain name C57BL/6-Tg (Tcr α Tcr β)1100Mjb/J) and TgMMTVneu (strain name FVB/N-Tg(MMTVneu)202Mul/J). These mice, obtained from Jackson Laboratory (Bar Harbor, ME), were bred and maintained under standard pathogen-free conditions at the University of Washington. Female mice 6-8 weeks old were used for study. All protocols were approved by the University's Institutional Animal Care and Use Committee.

Vaccine adjuvants

Vaccines were administered alone (in PBS) or with imiquimod (5% topical cream, 3M Pharmaceuticals, St. Paul, MN) or GM-CSF (PeproTech, Rocky Hill, NJ). All vaccines were prepared in 50ul PBS and applied transdermally (t.d) on the ear pinna (8). Imiquimod cream, approximately 80mg, was administered t.d. at the vaccine site immediately after the antigen administration and daily for 3 consecutive days (9). Murine recombinant GM-CSF, 5ug per mouse, was dissolved in sterile PBS and mixed with the antigens and applied t.d in a total volume of 50ul. In some experiments, the GM-CSF was given daily for 3 consecutive days. The dose of GM-CSF used had previously been shown to induce DC activation and antigen

specific T cell responses (6). Before vaccination, the ears were sterilized with 70% ethanol prep pads under mild anesthesia with ketamine (130 mg/kg) and xylazine (8.8 mg/kg).

OVA immunization

Balb/c mice received t.d immunization with 0.5ug OVA peptide 323-339 (p323, ISQAVHAAHAEINEAGR, Sigma Genosys, Woodlands, TX), an I-A^d restricted epitope (10). C57BL/6 mice received 0.5ug of OVA peptide 257-264 (p257, SIINFELK; Sigma Genosys), a H2-Kb restricted epitope (11). GM-CSF and imiquimod were given as adjuvants as described above with a control of PBS alone. An adoptive transfer model was used to monitor the induction of OVA peptide specific CD4⁺ and CD8⁺ T cell responses *in vivo*. Splenocytes (10×10^6 in 100ul PBS) harvested from DO11.10 mice, in which CD4⁺ T cells express a TCR specific for OVA p323, were adoptively transferred into the tail vein of Balb/c mice (10). Splenocytes (10×10^6 in 100ul PBS) harvested from OT-1 mice, in which CD8⁺ T cells express a TCR specific for OVA p257, were adoptively transferred into the tail vein of C57BL/6 mice (11). After the transfer, Balb/c mice received OVA p323 vaccination and C57BL/6 mice received p257 vaccination with imiquimod or GM-CSF as adjuvant or PBS. Both GM-CSF and imiquimod were given daily for 3 consecutive days. Six days after the immunization, the vaccine draining lymph nodes (DLN) and splenocytes from recipient mice were harvested for assessment of OVA specific CD4⁺ and CD8⁺ T cell responses as described below. In some experiments, splenocytes from DO11.10 or OT-1 mice were pre-labeled with 3uM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 minutes at 37°C using a Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes, Eugene, OR) before the adoptive transfer. The labeled cells were washed twice with PBS before the adoptive transfer. The spleens from recipient mice were collected for measuring proliferation of OVA specific CD4⁺ and CD8⁺ T cells four days following the vaccination.

Insulin-like growth factor-binding protein-2 (IGFBP-2) Immunization

TgMMTVneu mice were vaccinated t.d. with 50ug of each IGFBP-2 peptide 8-22 (p8, PALPLPPPPLLPLLP), 251-265 (p251, GPLEHLYSLHIPNCD) and 291-305 (p291, PNTGKLIQGAPTIRG) (Genemed Synthesis Inc, San Antonio, TX) 3 times, two weeks apart (12). Imiquimod and GM-CSF were given on the day of the vaccination as described above. Imiquimod was given daily for 3 consecutive days. In some experiments, both GM-CSF and imiquimod were given as a combined adjuvant. IGFBP-2 peptides in PBS and PBS only were used as controls. Two weeks after the last vaccination, mouse mammary carcinoma cells (MMC, 1×10^6), which were established from a spontaneous tumor harvested from the TgMMTVneu mice, were inoculated on the mid-dorsum of the experimental animals. Tumor growth was measured every 2-3 days. Serum was collected from the animals two days after the last IGFBP-2 vaccination for cytokine analysis. In separate experiments, spleens were harvested two weeks after last vaccination for IFN- γ ELISPOT analysis.

Myeloid derived suppressive cells (MDSC) and T regulatory cells (Treg) depletion

CD11b⁺Gr-1⁺ MDSC and CD4⁺CD25⁺Foxp3⁺ Treg cells were depleted in some IGFBP-2 immunization experiments. An anti-CD25 mAb (clone PC61, UCSF monoclonal antibody core, San Francisco, CA) was used to deplete Treg cells as previously published (13, 14), and an anti-Gr-1 mAb (clone RB6-8C5, UCSF monoclonal antibody core) was used to deplete MDSC (15, 16). A rat IgG1 (clone HRPN) and a rat IgG2b (clone LTF-2) mAb were used as isotype controls to the anti-CD25 and Gr-1 mAb respectively. In preliminary experiments, MMTVneu mice were injected with 100ug of either anti-CD25 or anti-Gr-1 mAb intraperitoneally (i.p) for three days. Imiquimod was given at the same time. The antibodies given significantly depleted Treg and MDSC cells as compare with isotype controls (Supplemental Figure 1). In the depletion assay performed, one hundred

micrograms of either anti-CD25 or Gr-1 mAb was injected i.p on the days that the experimental mice were vaccinated with IGFBP-2 peptides and imiquimod or imiquimod only and daily for 3 consecutive days. The antibodies were then given 2-3 times per week between vaccinations and after last vaccination until MMC tumor cells were implanted on Day 40 (Fig. 5A).

Flow cytometry

Splenocytes or vaccine DLN cells were pre-incubated with anti mouse CD16/CD32 Ab to block non-specific binding followed by antibody staining. The antibodies used included CD11b PE-Cy7, CD86 PE, Gr-1-FITC, CD3 FITC, CD4 PerCP, CD205 PE, and CD8 PerCP (purchased from BD Bioscience or eBioscience, San Diego, CA). DO11.10 TCR PE (Caltag, Burlingame, CA) and OVA p257 H2-K^b tetramer-PE (Beckman Coulter, Fullerton, CA) were also used. The antibody-stained cells were incubated for 30 minutes in the dark and washed twice with FACS buffer (PBS/1%FBS) and fixed in 1% paraformaldehyde before analysis. For Treg analysis, splenocytes were stained with CD3 FITC/ CD4 PE-Cy5 for 30 minutes. After washing with FACS buffer, the cells were stained with FOXP3 PE according to BioLegend's FOXP3 staining protocol (San Diego, CA). The stained cells were acquired with FACS Canto flow cytometer (BD Bioscience) and analyzed using FlowJo software (Tree Star, OR). Results are reported as total numbers or cells or percentage of a cell population as indicated.

IFN-gamma ELISPOT

IFN-gamma responses to IGFBP-2 peptides were examined using a standard ELISPOT assay as previously described with modification (12). Briefly, the splenocytes harvested after three IGFBP-2 vaccinations from TgMMTVneu mice were placed at 200ul/2×10⁵ cells in media with 10ug/ml of IGFBP-2 peptides. Cells incubated without antigen were used as negative controls. On day 5, 10u/ml of IL-2 (Hoffmann-La Roche, Nutley, NJ) was added into the wells. On Day 8, the cells were re-stimulated with the peptides loaded on irradiated autologous antigen presenting cells using splenocytes. The re-stimulated cells were transferred into an anti-mouse IFN-gamma mAb (AN18, Mabtech, Nacka Strand, Sweden) coated 96-well nitrocellulose plate (Millipore Corp, Bedford, MA) on the following day and incubated for 20 hours. On day 10, anti-mouse IFN-gamma biotinylated mAb (R4-6A2, Mabtech) was added into each well at 5ug/ml and incubated for 2 hours at room temperature. The IFN-gamma secreting spots were then detected using Streptavidin-HRP (Mabtech) and AEC substrate (BD Pharmingen) and the resultant spots were counted using an ELISPOT reader (Cell Technology, Columbia, MD). Results are reported as absolute spots/well.

Serum cytokine analysis

The sera were analyzed using a multiplex cytokine assay kit (Millipore, Pittsburgh, PA) according to the manufacturer's protocol. The cytokines assessed included: IL-12 (p70), IL-1-alpha, IL-1-beta, IFN-gamma, IL-2, TNF-alpha, IL-5, IL-10, and IL-17. The levels of serum cytokines were evaluated on a Luminex instrument (Qiagen, Germantown, MD) and data analyzed with the instrument software and reported in pg/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed using Student's *t* test or Mann Whitney test when Gaussian distribution could not be assumed. A value of *p*<0.05 was considered statistically significant.

RESULTS

GM-CSF and imiquimod, as vaccine adjuvants, equally induce the mobilization and maturation of dendritic cells

As DC are uniquely suited to efficiently process and present tumor antigens (17, 18), we assessed whether the adjuvants increased the number or impacted the activation status of DC trafficking to vaccine DLN. After t.d. immunization with ovalbumin and either GM-CSF or imiquimod, the total number of CD11c⁺ DC per vaccine DLN ($\times 10^5$) was significantly increased in both adjuvant treated mice compared to the PBS control; PBS 1.9 ± 0.3 (mean $\pm$ SE) vs. GM-CSF 3.6 ± 0.2 , $p=0.002$; vs. imiquimod 4.3 ± 0.4 , $p=0.001$ (Fig. 1A). The total number of mature DC (CD11c⁺/CD86⁺) per vaccine DLN ($\times 10^5$) was also significantly increased in both GM-CSF and imiquimod treated mice; PBS 0.7 ± 0.1 vs. GM-CSF 1.5 ± 0.2 , $p=0.018$; vs. imiquimod 2.2 ± 0.2 ; $p=0.000$ (Fig. 1B). Representative dot plots are shown in Figure 1C. There was no significant difference between GM-CSF and imiquimod in recruiting DC ($p=0.170$) or inducing the maturation of DC ($p=0.052$) in vaccine DLN.

In some experiments, we also assessed the numbers of CD11c⁺CD205⁺ DC per DLN ($\times 10^5$). We found that both adjuvants significantly increased the numbers of CD205⁺ DC: PBS 0.3 ± 0 , GM-CSF 0.7 ± 0.1 ($p=0.034$); vs. imiquimod 0.7 ± 0.1 ($p=0.003$). Both adjuvants also increased CD11c⁺CD8⁺ DC: PBS 0.4 ± 0 , GM-CSF 0.8 ± 0.1 ($p=0.048$); vs. imiquimod 1.2 ± 0.2 ($p=0.030$). There was no significant difference of CD11c⁺CD205⁺ ($p=0.775$) and CD11c⁺CD8⁺ DC ($p=0.231$) between GM-CSF and imiquimod groups.

Both GM-CSF and imiquimod are effective adjuvants in stimulating antigen specific T cell immunity after OVA peptide-based immunization

DC appeared to be both recruited and activated with either adjuvant, we therefore evaluated the efficacy of GM-CSF and imiquimod in stimulating CD4⁺ and CD8⁺ antigen-specific T cell responses with OVA-specific peptide vaccines. Both GM-CSF and imiquimod increased the total number of OVA p323-specific DO11.10 CD4⁺ T cells ($\times 10^5$) locally in lymph nodes draining the immunization site compared to p323 administered with PBS; PBS 0.6 ± 0.1 vs. GM-CSF 2.2 ± 0.5 , $p=0.000$; vs. imiquimod 1.2 ± 0.2 ; $p=0.006$; with GM-CSF being more potent than imiquimod ($p=0.024$) (Fig. 2A). While immunization in the absence of an adjuvant or with PBS alone did not result in detectable expansion of DO11.10 CD4⁺ T cells in the spleen, as a measure of systemic immunity, both GM-CSF and imiquimod significantly increased the percentage of splenic DO11.10 CD4⁺ T cells ($\times 10^5$); PBS 0.5 ± 0.1 vs. GM-CSF 1.2 ± 0.2 , $p=0.001$; vs. imiquimod 1.2 ± 0.2 ; $p=0.013$; but neither adjuvant was superior ($p=0.791$) (Fig. 2B). The % proliferation of OVA p323 specific DO11.10 CD4⁺ T cells in spleen was similarly increased in GM-CSF ($p=0.033$) and imiquimod ($p=0.009$) treated mice compared to those that received PBS and OVA p323 ($p=0.578$ between GM-CSF and imiquimod). Representative histograms of CFSE-labeled DO11.10 CD4⁺ cells are shown in Figure 2E.

Both GM-CSF and imiquimod, when used as adjuvants with an OVA p257 peptide vaccine, elicited a similar increase in OVA p257-specific OT-1 CD8⁺ T cells in local DLN compared to PBS control ($\times 10^5$); mean $\pm$ SE: PBS 0.8 ± 0.2 vs. GM-CSF 1.9 ± 0.2 , $p=0.003$; vs. imiquimod 1.8 ± 0.4 ; $p=0.026$ (Fig. 2C), $p=0.259$ between GM-CSF and imiquimod. The frequencies of OT-1 CD8⁺ T cells in spleen were significantly higher in animals that received either GM-CSF or imiquimod as an adjuvant compared to PBS ($\times 10^5$); mean $\pm$ SE: PBS 0.4 ± 0.1 vs. GM-CSF 1.0 ± 0.3 , $p=0.018$; vs. imiquimod 2.0 ± 0.4 ; $p=0.001$, with imiquimod being more potent ($p=0.053$) (Fig. 2D). The % proliferation of OVA p257 specific OT-1 CD8⁺ T cells in spleen was similarly increased in GM-CSF ($p=0.021$) and imiquimod ($p=0.004$) treated mice compared to controls ($p=0.057$ between GM-CSF and

imiquimod). Representative histograms of CFSE-labeled OT-1 CD8⁺ T cells are shown in Figure 2F.

GM-CSF and imiquimod differ in the ability to stimulate anti-tumor immunity when administered with an IGFBP-2 peptide vaccine

As both adjuvants were effective in inducing antigen specific CD4⁺ and CD8⁺ T cells in the OVA model, we questioned whether these DC stimulating adjuvants would impact tumor growth when used with peptide vaccination against a self-tumor antigen. TgMMTVneu mice express IGFBP-2 which has been shown to be a human tumor antigen (12). Peptides derived from IGFBP-2 have been identified that elicit T cells which mediate an anti-tumor response in TgMMTVneu mice and are highly homologous between mouse and man (12). As shown in Figure 3, vaccines administered with GM-CSF significantly inhibited the growth of tumor compared to peptides administered with PBS; peptides in PBS, $682 \pm 20\text{mm}^3$ vs. in GM-CSF $244 \pm 33\text{mm}^3$, $p=0.000$ (Fig. 3A). In contrast, the use of imiquimod as a vaccine adjuvant resulted in no anti-tumor effect; PBS $682 \pm 20\text{mm}^3$ vs. imiquimod $613 \pm 37\text{mm}^3$, $p=0.141$ (Fig. 3B). Note, neither GM-CSF nor imiquimod when administered at the doses used for vaccine adjuvants, but without IGFBP-2 peptides, demonstrated anti-tumor activity as compared to control.

In separate experiments, the splenocytes of non-tumor bearing mice vaccinated with PBS or with the IGFBP-2 peptides with PBS, GM-CSF or imiquimod as adjuvants were collected two weeks after the last vaccine and analyzed for T cell responses. GM-CSF as an adjuvant, but not imiquimod, induced IGFBP-2 specific IFN-gamma secretion (mean $\pm$ SD of spots/ 2×10^5 cells): peptides+PBS: 3 ± 3 ; vs. peptides+GM-CSF, 64 ± 27 , $p=0.018$; vs. peptides+imiquimod, 6 ± 5 ; $p=0.607$ (Fig. 3C). To determine the potential mechanism of vaccine failure, we evaluated serum cytokine levels in the experimental groups. There were no significant differences in the levels of IL-12 ($p=0.962$), IL-1-alpha ($p=0.530$), IL-1-beta ($p=0.733$), IFN-gamma ($p=0.349$), IL-2 ($p=0.199$), TNF-alpha ($p=0.112$), IL-5 ($p=0.221$) and IL-17 ($p=0.327$) between the GM-CSF and imiquimod treated groups. However, there was a significant difference in serum IL-10 levels. Imiquimod as an adjuvant, but not GM-CSF, increased serum levels of IL-10 (mean $\pm$ SE of pg/ml): peptides+PBS: 27 ± 5 ; vs. peptides+GM-CSF, 22 ± 5 , $p=0.901$; vs. peptides+imiquimod, 73 ± 10 ; $p=0.000$ (Fig. 3D).

To explore whether the lack of efficacy of imiquimod as an adjuvant was due to active immune suppression, we examined the effect of the combination of GM-CSF and imiquimod as adjuvants for IGFBP-2 peptide immunization. As shown in Figure 3E, the addition of imiquimod to GM-CSF appeared to negate the anti-tumor effect of the latter when it was administered as a single adjuvant (mean $\pm$ SE of tumor size): peptides+GM-CSF $305 \pm 26\text{mm}^3$ vs. PBS+peptides: $673 \pm 36\text{mm}^3$, $p=0.001$; vs. peptides+GM-CSF and imiquimod $910 \pm 36\text{mm}^3$, $p=0.002$.

Imiquimod, as a vaccine adjuvant, increases systemic levels of MDSC and Treg cells in IGFBP-2 immunized mice

IL-10 is secreted by cells associated with immune suppression such as MDSC and Treg. We questioned whether imiquimod induced elevated levels of these cells. We found that the percentage of MDSC (CD11b⁺Gr-1⁺) in splenic cells in GM-CSF-treated mice was not increased as compared to PBS (mean $\pm$ SE of % of splenic cells): PBS, 0.31 ± 0.05 ; GM-CSF, 0.25 ± 0.03 ($p=0.395$). However, in imiquimod and GM-CSF+imiquimod treated mice, MDSC were significantly increased compared to other groups; imiquimod, 0.64 ± 0.03 ($p=0.000$), GM-CSF+imiquimod 0.71 ± 0.04 ; ($p=0.000$) (Fig. 4A). Similarly, Foxp3⁺ Treg cells (CD4⁺Foxp3⁺) as a % of all CD4⁺ cells were not increased in GM-CSF-treated mice; PBS, 7.37 ± 0.22 ; GM-CSF, 6.96 ± 0.11 ($p=0.162$). In contrast, in imiquimod and GM-CSF

+ imiquimod treated mice, Treg were significantly increased over levels in all other groups; Imiquimod, 8.64 ± 0.23 ($p=0.006$); GM-CSF+Imiquimod 8.66 ± 0.25 ; ($p=0.008$) (Fig. 4B). Representative dot plots for MDSC (Fig. 4C) and Treg (Fig. 4D) are shown for all experimental groups.

Selective depletion of MDSC or Treg during vaccination with imiquimod as an adjuvant restored the anti-tumor effect of IGFBP-2 immunization

To evaluate whether the elevated Treg cells or MDSC induced by imiquimod were responsible for inhibiting the anti-tumor effect of IGFBP-2 vaccination, we depleted these specific cell populations during vaccine priming and boosting until MMC tumor cells were implanted on Day 40 (Fig. 5A). As shown previously (Fig. 3B), there was no difference in tumor growth among peptides alone, imiquimod alone, and peptides+imiquimod. The administration of PC61 (anti-CD25) or RB6-8C5 (anti-Gr-1) in mice treated with imiquimod only (no vaccine) inhibited the growth of tumor (mean $\pm$ SE of tumor size): imiquimod only; $222 \pm 6\text{mm}^3$ vs. imiquimod+PC61; $165 \pm 17\text{mm}^3$, $p=0.012$; vs. imiquimod+RB6-8C5; $152 \pm 10\text{mm}^3$, $p=0.000$ (Fig. 5B, C). The percentage of tumor growth in mice treated with PC61 and RB6-8C5 was $73 \pm 7\%$ and $67 \pm 4\%$ of the control groups respectively (Fig. 5D). The administration of PC61 or RB6-8C5 in mice treated with peptides+imiquimod further inhibited the tumor growth: peptides+imiquimod $250 \pm 34\text{mm}^3$, vs. peptides+imiquimod +PC61 $77 \pm 7\text{mm}^3$, $p=0.001$; vs. peptides+imiquimod+RB6-8C5 $87 \pm 4\text{mm}^3$, $p=0.004$ (Fig. 5B,C). The percentage of tumor growth in mice treated with PC61 in peptides+imiquimod group was $34 \pm 7\%$ of the control groups, which is significantly lower than that in mice treated with imiquimod only+PC61, $p=0.001$. Similarly, the percentage of tumor growth in mice treated with RB6-8C5 in the peptide+imiquimod group was $38 \pm 2\%$ of the control groups, which is significantly lower than that in mice treated with imiquimod only +RB6-8C5, $p=0.001$ (Fig. 5D). Furthermore, the tumor inhibition after PC61 and RB6-8C5 depletion in mice treated with peptides+imiquimod achieved similar levels of anti-tumor activity as that in peptides+GM-CSF treated mice, $p=0.241$ and 0.134 respectively (Fig. 5B,C,D).

DISCUSSION

We demonstrate that both GM-CSF and imiquimod induce DC migration to vaccine draining lymph nodes as well as stimulate DC activation. Both adjuvants could elicit CD4⁺ and CD8⁺ OVA specific T cells after vaccination when individually used as adjuvants. No conclusion could be drawn concerning the superiority of one adjuvant over another as the CD4 and CD8 OVA responses were assessed in two different strains of mice. However, the dose and route used for each adjuvant in each strain resulted in near equivalent ability to stimulate antigen specific immunity. When the same regimens were used to immunize against the naturally expressed self tumor antigen, IGFBP-2, the adjuvants differed significantly in the ability to induce anti-tumor immunity. This discrepancy was largely due to the elaboration of Treg and MDSC by the use of imiquimod as an adjuvant. Moreover, the immune suppressive effects of imiquimod negated the immune stimulatory activity of GM-CSF when the adjuvants were used in combination.

GM-CSF has long been exploited as a vaccine adjuvant in cancer clinical trials. The efficacy of the adjuvant in enhancing therapeutic immunity is controversial. Studies have shown that MDSC are present in the peripheral blood of patients with advanced stage melanoma and can be stimulated to proliferate by the use of low dose GM-CSF, 75ug, sq over 3 days as a vaccine adjuvant (19). Indeed, murine experiments have shown that the administration of GM-CSF ip, daily over several days, will expand Tregs to a level that suppresses the progression of autoimmune myasthenia gravis (20). In contrast, GM-CSF administered at higher doses over a longer period of time ($125 \text{ ug/m}^2/\text{d sq X14 days per cycle}$) was shown to

increase levels of circulating DC without an enhancement of MDSC levels. Elevated levels of DC were associated with improved clinical outcome in advanced stage melanoma patients (21). As an adjuvant given at a 100ug dose admixed with an id peptide vaccine, GM-CSF has been reported to induce significant levels of Type I immunity in breast cancer patients (22). However, when administered sq with incomplete Freund's adjuvant, GM-CSF is less effective when used with a peptide vaccine targeting melanoma antigens (23). There are many confounding factors in the comparison of these studies; the dose of GM-CSF used, the exposure (given once, over many days, or in a depot), the vaccine antigens, and finally the disease burden of the patients. It may be that immunization in individuals with larger disease burdens resulted in expansion of existing immune suppressive cell populations that were already found in high numbers in the chronically inflamed tumor bed. For this reason, we evaluated the adjuvant effects of the agents in non-tumor bearing mice. In tumor bearing mice, potentially, the increased vaccine induced inflammatory response could drive proliferation of existing immune suppressive populations even further (24).

The self regulatory effects of the TLR-7 agonist, imiquimod, when used in humans, is less well known. There have been two published trials of cancer vaccines employing imiquimod as a vaccine adjuvant. Both studies immunized patients with advanced stage melanoma and both vaccinated against NY-ESO as well as other melanoma antigens (25, 26). One study demonstrated the development of peptide specific IFN-gamma secreting T cells in 7 of the 9 patients immunized. The T cell responses were low level, a maximum of 0.3% of all IFN-gamma secreting T cells and responses persisted after the end of vaccination in only a minority of patients immunized (25). The use of imiquimod as a vaccine adjuvant was not as successful in eliciting either antibodies or T cells as other adjuvant approaches used by these investigators (25). The second study combined imiquimod with Flt-3 ligand in a subset of patients in an attempt to increase DC and activate them (26). Flt-3 ligand was marginally effective as a vaccine adjuvant and adding imiquimod to the regimen increased the number of patients who developed DTH responses. Quantitative data were not available to assess the magnitude or character of the immune response (26). In animal models, imiquimod has been shown to induce regulatory cells. In a mouse model of breast cancer using imiquimod as a topical treatment, animals developed high levels of serum IL-10 which was associated with disease relapse after successful therapy (9). In this study, Tregs were not elevated; however, T cells secreting type 2 cytokines were markedly elevated. Treating mice with an IL-10 directed antibody markedly increased the efficacy of imiquimod treatment (9). A recent pre-clinical vaccine study comparing the effects of a TLR-9 (ODN1826) and the TLR-7 agonist imiquimod as an adjuvant with an adeno-virus based melanoma directed vaccine demonstrated that each agent used alone was effective in stimulating Type 1 immunity (27). However, when the adjuvants were combined, no immunity was elicited, serum IL-10 levels increased, and MDSC were found in higher numbers (27).

The studies described here initiated immunizations in two models; OVA transgenic mice and a self tumor antigen model with IGFBP-2. In the OVA model, transgenic T cells are transplanted into non-transgenic mice, so that the immune response elicited with each adjuvant is not impacted by issues of tolerance. Both adjuvants were near equally effective in stimulating OVA specific CD4⁺ and CD8⁺ T-cells. The superiority of imiquimod in eliciting CD8⁺ T cells is most likely due to the known effects of the agent on upregulating class I MHC and inducing secretion of high levels of IFN-gamma (28). In the TgMMTVneu model, vaccine experiments evaluating the immunogenicity of each agent were performed in non-tumor bearing mice and mice never received a tumor implant. The inability of imiquimod to induce a measureable level of IGFBP-2 specific IFN-gamma secreting T cells indicates that regulatory mechanisms were operative in the initial priming of a self-immune response. It has recently been shown that MDSCs directly inhibit the development of Type I T cells and block the secretion of IFN-gamma by both T cells and NK cells (29). When Treg

and MDSC were depleted during initial immunizations, but depletions stopped prior to the introduction of tumor, the anti-tumor response induced by imiquimod was identical to that seen with GM-CSF. This observation implies that imiquimod administration, in the presence of a self antigen vaccine, results in a potent immune suppressive regulatory response inhibiting priming of a type I T cell response, even in animals without tumor. Both Treg and MDSC elaborated via imiquimod administration were operative in dampening vaccine induced immunity.

The development of adjuvants to stimulate cellular immunity has been an area of intense investigation for the last several years. It is acknowledged that for cancer vaccines to be effective, adjuvant systems that will promote the generation of Type I immunity are needed. Finding a potent mix of adjuvants has begun in earnest in the study of infectious disease vaccines (4). Immunization against self tumor antigens may introduce self regulatory responses that will not allow direct application of successes seen with adjuvants in foreign antigen vaccines to cancer vaccine clinical trials. Moreover, adjuvants that effectively stimulate immunity when used as single agents may have different effects when used in combination. Studies, such as the one reported here, underscore the need for preclinical modeling of adjuvant combinations in therapeutically relevant systems prior to the application of adjuvant approaches in human clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Cancer Institute (K24CA85218, N01-CN-53300/WA#10 and U01 CA141539) and the Ovarian Cancer Research Fund.

ABBREVIATIONS

Ab	antibody
CFSE	carboxyfluorescein diacetate succinimidyl ester
DC	dendritic cells
DLN	vaccine draining lymph node
ELISPOT	enzyme-linked immunosorbent spot
GM-CSF	granulocyte macrophage colony stimulating factor
IFN	interferon
IGFBP-2	insulin like growth factor binding protein 2
IL	interleukin
MDSC	myeloid derived suppressive cells
MMC	mouse mammary carcinoma
OVA	ovalbumin
PBS	phosphate buffered saline
TLR	Toll-like receptor
Treg T	regulatory cells

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Translational Relevance

Successful cancer vaccines will depend on the generation of robust levels of tumor specific Type I T cells with active immunization. Dendritic cell (DC) activating vaccine adjuvants have the potential to elicit significant immunity to self-tumor antigens via enhanced antigen presentation and are available for use in clinical trials. We demonstrate that although both GM-CSF and imiquimod can induce DC activation and elicit CD4⁺ and CD8⁺ OVA specific T cells, they differ significantly in their ability to induce anti-tumor immunity when used to immunize against a self tumor antigen, IGFBP-2. This discrepancy is largely due to the elaboration of Treg and MDSC by the use of imiquimod as an adjuvant. Moreover, the immune suppressive effects of imiquimod negate the immune stimulatory activity of GM-CSF when the adjuvants are used in combination. These results may have significant implications in the future development of cancer vaccine adjuvants, especially combinations of existing agents.

Figure 1. GM-CSF and imiquimod, as vaccine adjuvants, equally induce the mobilization and maturation of dendritic cells

(A) Total number of CD11c⁺ DC per vaccine DLN (y-axis) per adjuvant group (x-axis). Bars represent the mean ($\pm$ SE), n=5/group. (B) Total number of CD11c⁺ CD86⁺ mature DC per vaccine DLN (y-axis) per adjuvant group (x-axis). n=5/group. * = p<0.05 and ** = p<0.005 compared to control. ns=not significant. (C) Representative dot plots of CD11c⁺ (X-axis) CD86⁺ (y-axis) DC in DLN of mice treated with PBS, GM-CSF, and imiquimod as adjuvants respectively.

Figure 2. Both GM-CSF and imiquimod are effective adjuvants in stimulating antigen specific T cell immunity after OVA peptide-based immunization

(A) Number ($\times 10^5$) of OVA p323 specific (DO11.10 TCR⁺) CD4⁺ T cells in DLN (y-axis) per experimental group (x-axis). Bars represent the mean ($\pm$ SE), $n=9$ /group; (B) Percentage of OVA p323 specific DO11.10 CD4⁺ T cells of all splenic T cells (y axis) per experimental group (x-axis), $n=9$ /group; (C) Number ($\times 10^5$) of OVA p257 specific (OVA p257 H2-K^b tetramer⁺) CD8⁺ T cells in DLN (y-axis) per experimental group (x-axis). Bars represent the mean ($\pm$ SE), $n=7$ /group. (D) Percentage of OVA p257 specific OT-1 CD8⁺ T cells of all splenic T cells (y axis) per experimental group (x-axis), $n=7$. Representative Flow histograms of CFSE labeled CD4⁺ DO11.10 cells (E) and CD8⁺ OT-1 cells (F); PBS, GM-CSF, and imiquimod used with OVA p323 (E) or OVA p257 vaccination (F). * indicates $p<0.05$ and ** indicates $p<0.005$ vs. PBS control. Results were reproduced in two independent experiments.

Figure 3. GM-CSF and imiquimod differ in the ability to stimulate anti-tumor immunity when administered with an IGFBP-2 peptide vaccine

(A) Growth of MMC tumor (mm³) (y-axis) in mice immunized with PBS control (●), GM-CSF (▲), and IGFBP-2 peptide vaccine with PBS (V) (▼) or GM-CSF (■), (x-axis). Data is shown as the mean $\pm$ SE, n=5/group. (B) Growth of MMC tumor (mm³) (y-axis) in mice immunized with PBS control (●), imiquimod (▲), and IGFBP-2 peptide vaccine with PBS (V) (▼) or imiquimod (■), (x-axis). Data represent mean $\pm$ SE, n=5/group. The experiments were repeated on two independent occasions with similar results. (C) IGFBP-2 specific IFN- γ secretion (y-axis); bars represent PBS alone or IGFBP-2 peptides in PBS, GM-CSF and imiquimod (x-axis). Shown are the mean ($\pm$ SE) of spots/2 $\times$ 10⁵ cells, n=5/group. (D) Columns represent the mean ($\pm$ SE) of IL-10 (pg/ml) (y-axis) from PBS alone or IGFBP-2 peptides in PBS, GM-CSF and imiquimod (x-axis), n=12/group. In all above graphs, * indicates p<0.05 and ** indicates p<0.005 in comparison with IGFBP-2 peptides with PBS group. (E) Growth of MMC tumor (mm³) (y-axis) in mice immunized with PBS control (●), and IGFBP-2 peptide vaccine with PBS (▼) GM-CSF (■), imiquimod (▲), and GM-CSF and Imiquimod (◆) (x-axis). Data represent mean $\pm$ SE, n=3/group.

Figure 4. Imiquimod, as a vaccine adjuvant, increases systemic levels of MDSC and Treg cells in IGFBP-2 immunized mice

(A) % of CD11b⁺Gr-1⁺ MDSC on spleen (y-axis) and (B) % of Foxp3⁺ cells in splenic CD4⁺ T cells (y-axis). Experimental groups on x-axis; Control: PBS alone; PBS+peptides, GM-CSF+peptides, imiquimod+peptides and GM-CSF+imiquimod+peptides, n=5/group. Bars represent the mean. * indicate p<0.05 and ** indicates p<0.005 vs. controls. Representative dot plots of MDSC (C) and Treg cells (D) from the different experimental groups.

Figure 5. Selective depletion of Treg or MDSC during vaccination with imiquimod as an adjuvant restored the anti-tumor effect of IGFBP-2 immunization

(A) Schema of IGFBP2 immunization and the administration of anti-CD25 and anti-Gr-1 mAb. (B) Growth of MMC tumor (mm³) (y-axis) in mice immunized with imiquimod (○), imiquimod+CD25 Ab (▽), and IGFBP-2 peptide vaccine with PBS (V) (■), imiquimod (▲), imiquimod+ CD25 Ab (▼), and GM-CSF (◆) (x-axis). (C) Growth of MMC tumor (mm³) (y-axis) in mice immunized with imiquimod (○), imiquimod+ Gr-1 Ab (▽), and IGFBP-2 peptide vaccine with PBS (■), imiquimod (▲), imiquimod+Gr-1 Ab (▼), and GM-CSF (◆) (x-axis). (D) The % of tumor growth (y-axis): bars represent controls and the experimental groups. Data are shown as the mean $\pm$ SE, n=5/group. * indicate p<0.05 and ** indicates p<0.005 vs. controls.