Dendritic Cell–Activating Vaccine Adjuvants Differ in the Ability to Elicit Antitumor Immunity Due to an Adjuvant-Specific Induction of Immunosuppressive Cells

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Abstract

Purpose: We questioned whether the vaccine adjuvant combination of TLR-7 ligand agonist, imiquimod, with granulocyte macrophage colony-stimulating factor (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 ovalbumin (OVA) and MMTVneu transgenic mice using peptide-based vaccines. Type I immunity, serum cytokines, myeloid-derived suppressive cells (MDSC), and regulatory T cells (Treg) 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 antitumor efficacy of adjuvant GM-CSF and imiquimod immunizing against murine insulin-like growth factor–binding protein-2 (IGFBP-2), a nonmutated 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 antitumor activity of the vaccine when GM-CSF was used as the sole adjuvant. While GM-CSF stimulated significant levels of antigen-specific T-helper cell (TH)1, imiquimod induced elevated serum interleukin (IL)-10. Both MDSC and Tregs 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 antitumor 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 immunosuppression may limit vaccine efficacy and should be evaluated in preclinical models especially when contemplating combination approaches. Clin Cancer Res; 18(11); 3122–31. ©2012 AACR.

Introduction

Numerous detailed analyses of immune-related prognostic biomarkers in patients with cancer have led to an improved understanding of the type of cellular response needed for immune-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 patients with cancer. 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 use 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 antiviral vaccine significantly enhanced the functional avidity of the T cells, rather than increasing their...
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 show that although both granulocyte macrophage colony-stimulating factor (GM-CSF) and imiquimod can induce DC activation and elicit CD4+ and CD8+ ovalbumin (OVA)-specific T cells, they differ significantly in their ability to induce antitumor immunity when used to immunize against a self-tumor antigen, insulin-like growth factor–binding protein-2 (IGFBP-2). This discrepancy is largely due to the elaboration of regulatory T cells and myeloid-derived suppressive cells by the use of imiquimod as an adjuvant. Moreover, the immunosuppressive effects of imiquimod negate the immunostimulatory 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.

Materials and Methods
Mice
The mouse strains used in this study include BALB/c, DO11.10 [strain name: C3H-Tg (DO11.10)10Dio/J], C57BL/6, OT-1 [strain name: C57BL/6-Tg (TcraTcrb) 1100Mj/J], and TgMMTVNeu [strain name: FVB/N-Tg (MMTVneu)202Mul/J]. These mice, obtained from Jackson Laboratory, were bred and maintained under standard pathogen-free conditions at the University of Washington (Seattle, WA). Female mice 6- to 8-week-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) or GM-CSF (PeproTech). All vaccines were prepared in 50 μL PBS and applied transdermally (t.d.) on the ear pinna (8). Imiquimod cream, approximately 80 mg, was administered t.d. at the vaccine site immediately after the antigen administration and daily for 3 consecutive days (9). Murine recombinant GM-CSF, 5 μg/mouse, was dissolved in sterile PBS and mixed with the antigens and applied t.d. in a total volume of 50 μL. 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).

Ovalbumin immunization
BALB/c mice received t.d. immunization with 0.5 μg ovalbumin (OVA) peptide 323–339 (p323, ISQAVHAAHAEINEAGR; Sigma Genosys), an I-Ad–restricted epitope (10). C57BL/6 mice received 0.5 μg of OVA peptide 257–264 (p257, SIINFEKL; Sigma Genosys), a H2-Kb–restricted epitope (11). GM-CSF and imiquimod were given adjuvants as described earlier 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 × 106 in 100 μL PBS) harvested from DO11.10 mice, in which CD8+ T cells express a TCR specific for OVA p323, were adoptively transferred into the tail vein of BALB/c mice (10). Splenocytes (10 × 106 in 100 μL 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 later. In some experiments, splenocytes from DO11.10 or OT-1 mice were prelabeled with 3 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 minutes at 37°C by a Vybrant CFDA SE Cell Tracer Kit (Molecular Probes) 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 4 days following the vaccination.

Insulin-like growth factor–binding protein-2 immunization
TgMMTVNeu mice were vaccinated t.d. with 50 μg of each insulin-like growth factor–binding protein-2 (IGFBP-2)
peptide 8–22 (p8, PALPLPPPLLPPP), 251–265 (p251, GPLEHLYSHLIHCND), and 291–305 (p291, PNTGKLIQ-GAPITHG; Genemed Synthesis Inc.) 3 times, 2 weeks apart (12). Imiquimod and GM-CSF were given on the day of the vaccination as described earlier. 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 (MMC) cells (1 × 10^6), which were established from a spontaneous tumor harvested from the TgMMTVneu mice, were inoculated on the middorsum of the experimental animals. Tumor growth was measured every 2 to 3 days. Serum was collected from the animals 2 days after the last IGFBP-2 vaccination for cytokine analysis. In separate experiments, spleens were harvested 2 weeks after last vaccination for IFN-γ ELISPOT analysis.

**Myeloid-derived suppressive cells and regulatory T-cell depletion**

CD11b^+^Gr-1^+^ myeloid-derived suppressive cells (MDSC) and CD4^+^CD25^+^Foxp3^+^ regulatory T cells (Treg) were depleted in some IGFBP-2 immunization experiments. An anti-CD25 monoclonal antibody (mAb; clone PC61, UCSC monoclonal antibody core) was used to deplete Tregs as previously published (13, 14), and an anti-Gr-1 mAb (clone RB6-8C5, UCSC 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 100 μg of either anti-CD25 or anti-Gr-1 mAb intraperitoneally (i.p.) for 3 days. Imiquimod was given at the same time. The antibodies were given significantly depleted Treg and MDSC cells as compared with isotype controls (Supplementary Fig. S1). In the depletion assay conducted, 100 μg 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 to 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 preincubated with anti-mouse CD16/CD32 Ab to block nonspecific 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). DO11.10 TCR PE (Caltag) and OVA p257 H2-K^d^ tetramer-PE (Beckman Coulter) were also used. The antibody-stained cells were incubated for 30 minutes in the dark and washed twice with fluorescence-activated cell-sorting (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. The stained cells were acquired with FACSCanto flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star). Results are reported as total numbers or cells or percentage of a cell population as indicated.

**IFN-γ ELISPOT**

IFN-γ responses to IGFBP-2 peptides were examined by a standard ELISPOT assay as previously described with modification (12). Briefly, the splenocytes harvested after 3 IGFBP-2 vaccinations from TgMMTVneu mice were placed at 200 μL/2 × 10^5 cells in media with 10 μg/mL of IGFBP-2 peptides. Cells incubated without antigen were used as negative controls. On day 5, 10 μg/mL of IL-2 (Hoffmann-La Roche) was added into the wells. On day 8, the cells were restimulated with the peptides loaded on irradiated autologous antigen-presenting cells using splenocytes. The restimulated cells were transferred into an anti-mouse IFN-γ mAb (AN18; Mabtech) coated 96-well nitrocellulose plate (Millipore Corp.) on the following day and incubated for 20 hours. On day 10, anti-mouse IFN-γ biotinylated mAb (R4-6A2; Mabtech) was added into each well at 5 μg/mL and incubated for 2 hours at room temperature. The IFN-γ secreting spots were then detected with streptavidin-HRP (Mabtech) and AEC substrate (BD Pharmingen) and the resultant spots were counted with an ELISPOT reader (Cell Technology). Results are reported as absolute spots per well.

**Serum cytokine analysis**

The sera were analyzed by a multiplex cytokine assay kit (Millipore) according to the manufacturer’s protocol. The cytokines assessed included: IL-12 (p70), IL-1-α, IL-1β, IFN-γ, IL-2, TNF-α, IL-5, IL-10, and IL-17. The levels of serum cytokines were evaluated on a Luminex instrument (Qiagen) and data were analyzed with the instrument software and reported in pg/mL.

**Statistical analysis**

Statistical analysis was conducted with GraphPad Prism (GraphPad Software). Data were analyzed using Student t test or Mann–Whitney U 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 DCs**

As DCs 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 DLNs. After t.d. immunization with OVA and either GM-CSF or imiquimod, the total number of CD11c^+^ DC per vaccine DLNs (×10^5) was significantly increased in both adjuvant-treated mice.
compared with the PBS control: PBS $1.9 \pm 0.3$ (mean ± SE) versus GM-CSF $3.6 \pm 0.2$, $P = 0.002$; versus imiquimod $4.3 \pm 0.4$, $P = 0.001$ (Fig. 1A). The total number of mature DC (CD11c$^+$CD86$^+$) per vaccine DLNs ($\times 10^5$) was also significantly increased in both GM-CSF and imiquimod-treated mice; PBS $0.7 \pm 0.1$ versus GM-CSF $1.5 \pm 0.2$, $P = 0.018$; versus imiquimod $2.2 \pm 0.2$; $P = 0.000$ (Fig. 1B). Representative dot plots are shown in Fig. 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 DLNs.

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 $0.3 \pm 0$, GM-CSF $0.7 \pm 0.1$ ($P = 0.034$); versus imiquimod $0.7 \pm 0.1$ ($P = 0.003$). Both adjuvants also increased CD11c$^+$CD8$^+$ DC: PBS $0.4 \pm 0$, GM-CSF $0.8 \pm 0.1$ ($P = 0.048$); versus imiquimod $1.2 \pm 0.2$ ($P = 0.030$). 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

DCs 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 with p323 administered with PBS; PBS $0.6 \pm 0.1$ versus GM-CSF $2.2 \pm 0.5$, $P = 0.000$; versus imiquimod $1.2 \pm 0.2$, $P = 0.006$; 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 $0.5 \pm 0.1$ versus GM-CSF $1.2 \pm 0.2$, $P = 0.001$; versus imiquimod $1.2 \pm 0.2$; $P = 0.013$; but neither adjuvant was superior ($P = 0.791$; Fig. 2B). The percentage proliferation of OVA p323–specific DO11.10 CD4$^+$ T cells in spleen was similarly increased in GM-CSF–treated ($P = 0.033$) and imiquimod ($P = 0.009$)–treated mice compared with 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 Fig. 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 DLNs compared with PBS control ($\times 10^5$); mean ± SE: PBS $0.8 \pm 0.2$ versus GM-CSF $1.9 \pm 0.2$, $P = 0.003$; versus imiquimod $1.8 \pm 0.4$; $P = 0.026$ (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 with PBS ($\times 10^5$); mean ± SE: PBS $0.4 \pm 0.1$ versus GM-CSF $1.0 \pm 0.3$, $P = 0.018$; versus imiquimod $2.0 \pm 0.4$, $P = 0.001$, with imiquimod being more potent ($P = 0.033$; Fig. 2D). The percentage proliferation of OVA p257–specific OT-1 CD8$^+$ T cells in spleen was similarly increased in GM-CSF–treated ($P = 0.021$) and imiquimod ($P = 0.004$)–treated mice compared with controls ($P = 0.013$).

Figure 1. GM-CSF and imiquimod, as vaccine adjuvants, equally induce the mobilization and maturation of DCs. 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 with control. ns, not significant. C, representative dot plots of CD11c$^+$ (x-axis) CD86$^+$ (y-axis) DC in DLNs of mice treated with PBS, GM-CSF, and imiquimod as adjuvants, respectively.
0.057 between GM-CSF and imiquimod). Representative histograms of CFSE-labeled OT-1 CD8\(^+\) T cells are shown in Fig. 2F.

**GM-CSF and imiquimod differ in the ability to stimulate antitumor 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 antitumor response in TgMMTVneu mice and are highly homologous between mouse and man (12). As shown in Fig. 3, vaccines administered with GM-CSF significantly inhibited the tumor growth compared to the control group.
growth of tumor compared with peptides administered with PBS; peptides in PBS, 682 ± 20 mm³ versus in GM-CSF 244 ± 33 mm³, \( P = 0.000 \) (Fig. 3A). In contrast, the use of imiquimod as a vaccine adjuvant resulted in no antitumor effect; PBS 682 ± 20 mm³ versus imiquimod 613 ± 37 mm³, \( P = 0.141 \) (Fig. 3B). Note, neither GM-CSF nor imiquimod when administered at the doses used for vaccine adjuvants, but without IGFBP-2 peptides, showed antitumor activity as compared with control.

In separate experiments, the splenocytes of nontumor-bearing mice vaccinated with PBS or with the IGFBP-2 peptides with PBS, GM-CSF, or imiquimod as adjuvants were collected 2 weeks after the last vaccine and analyzed for T-cell responses. GM-CSF as an adjuvant, but not imiquimod, induced IGFBP-2–specific IFN-γ secretion (mean ± SD of spots per \( 2 \times 10^5 \) cells); peptides + PBS, 3 ± 3; versus peptides + GM-CSF, 64 ± 27, \( P = 0.018 \); versus 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-1α (\( P = 0.530 \)), IL-1β (\( P = 0.733 \)), IFN-γ (\( P = 0.349 \)), IL-2 (\( P = 0.199 \)), TNF-α (\( P = 0.112 \)), 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 ± SE of pg/mL): peptides + PBS: 27 ± 5; versus peptides + GM-CSF, 22 ± 5, \( P = 0.901 \); versus 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 immunosuppression, we examined the effect of the combination of GM-CSF and imiquimod as adjuvants for IGFBP-2 peptide immunization. As shown in Fig. 3E, the addition of imiquimod to GM-CSF appeared to negate the antitumor effect of the latter when it was administered as a single adjuvant (mean ± SE of tumor size): peptides + GM-CSF 305 ± 26 mm\(^3\) versus PBS + peptides, 673 ± 36 mm\(^3\); \( P = 0.001 \); versus peptides + GM-CSF and imiquimod 910 ± 36 mm\(^3\), \( P = 0.002 \).

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

IL-10 is secreted by cells associated with immunosuppression 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 with PBS (mean ± SE of percentage 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 with other groups; imiquimod, 0.64 ± 0.03 (\( P = 0.000 \)); GM-CSF + imiquimod, 0.71 ± 0.04 (\( P = 0.000 \); Fig. 4A). Similarly, Foxp3\(^+\) Tregs (CD4\(^+\)Foxp3\(^+\)) as a percentage 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, Tregs 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).

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

To evaluate whether the elevated Tregs or MDSC induced by imiquimod were responsible for inhibiting the antitumor 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 ± SE of tumor size): imiquimod only, 222 ± 6 mm³ versus imiquimod + PC61, 165 ± 17 mm³, P = 0.012; versus imiquimod + RB6-8C5, 152 ± 10 mm³, P = 0.000 (Fig. 5B and C). The percentage of tumor growth in mice treated with PC61 and RB6-8C5 was 73% and 67% ± 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 ± 34 mm³ versus peptides + imiquimod + PC61, 77 ± 7 mm³, P = 0.001; versus peptides + imiquimod + RB6-8C5, 87 ± 4 mm³, P = 0.004 (Fig. 5B and C). The percentage of tumor growth in mice treated with PC61 in peptides + imiquimod group was 34% ± 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% ± 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 antitumor activity as that in peptides + GM-CSF-treated mice, P = 0.241 and 0.134, respectively (Fig. 5B–D).

**Discussion**

We show that both GM-CSF and imiquimod induce DC migration to vaccine DLN 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 2 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 antitumor immunity. This discrepancy was largely due to the elaboration of Treg and MDSC by the use of imiquimod as an adjuvant. Moreover, the immunosuppressive effects of imiquimod negated the immunostimulatory 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, 75 µg, s.c. over 3 days as a vaccine adjuvant (19). Indeed, murine experiments have shown that the administration of GM-CSF i.p., 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 µg/m²/d s.c. × 14 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 patients with advanced stage melanoma (21). As an adjuvant given at a 100 µg dose admixed with an i.d. peptide vaccine, GM-CSF has been reported to induce significant levels of type I immunity in patients with breast cancer (22). However, when administered s.c. 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 immunosuppressive 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 nontumor-bearing mice. In tumor-bearing mice, the increased vaccine-induced inflammatory response could drive proliferation of existing immunosuppressive 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 2 published trials of cancer vaccines using 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 showed the development of peptide-specific IFN-γ secreting T cells in 7 of 9 patients immunized. The T-cell responses were low level, a maximum of 0.3% of all IFN-γ 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 delayed-type hypersensitivity 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 II cytokines were markedly elevated. Treating mice with an IL-10 directed antibody markedly increased the efficacy of imiquimod treatment (9). A recent preclinical vaccine study comparing the effects of a TLR-9 (ODN1826) and the TLR-7 agonist imiquimod as an adjuvant with an adenovirus-based melanoma-directed vaccine showed 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 2 models; OVA transgenic mice and a self-tumor antigen model with IGFBP-2. In the OVA model, transgenic T cells are transplanted into nontransgenic 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-γ (28). In the TgMMTVneu model, vaccine experiments evaluating the immunogenicity of each agent were carried out in nontumor-bearing mice and mice never received a tumor implant. The inability of imiquimod to induce a measurable level of IGFBP-2–specific IFN-γ 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-γ by both T cells and natural killer cells (29). When Treg and MDSC were depleted during initial immunizations, but depletions stopped before the introduction of tumor, the antitumor 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 immunosuppressive 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 before the application of adjuvant approaches in human clinical trials.

Disclosure of Potential Conflicts of Interest
M.L. Disis has commercial research grants from GlaxoSmithKline and Hemasphere; has ownership interest (including patents) in University of Washington, Epigenomics; and is the consultant/advisory board member for Roche, BiMS, Ventixx, and Immunovaccine. No potential conflicts of interest were disclosed by the other authors.

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