Identification of Immune Factors Regulating Antitumor Immunity Using Polymeric Vaccines with Multiple Adjuvants

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Abstract

The innate cellular and molecular components required to mediate effective vaccination against weak tumor-associated antigens remain unclear. In this study, we used polymeric cancer vaccines incorporating different classes of adjuvants to induce tumor protection, to identify dendritic cell (DC) subsets and cytokines critical to this efficacy. Three-dimensional, porous polymer matrices loaded with tumor lysates and presenting distinct combinations of granulocyte macrophage colony-stimulating factor (GM-CSF) and various Toll-like receptor (TLR) agonists affected 70% to 90% prophylactic tumor protection in B16-F10 melanoma models. In aggressive, therapeutic B16 models, the vaccine systems incorporating GM-CSF in combination with P(lC) or CpG-ODN induced the complete regression of solid tumors (≤40 mm²), resulting in 33% long-term survival. Regression analysis revealed that the numbers of vaccine-resident CD8(+) DCs, plasmacytoid DCs (pDC), along with local interleukin (IL)-12, and granulocyte colony-stimulating factor (G-CSF) concentrations correlated strongly to vaccine efficacy regardless of adjuvant type. Furthermore, vaccine studies in Batf3−/− mice revealed that CD8(+) DCs are required to affect tumor protection, as vaccines in these mice were deficient in cytotoxic T lymphocytes priming and IL-12 induction in comparison with wild-type. These studies broadly demonstrate that three-dimensional polymeric vaccines provide a potent platform for prophylactic and therapeutic protection, and can be used as a tool to identify critical components of a desired immune response. Specifically, these results suggest that CD8(+) DCs, pDCs, IL-12, and G-CSF play important roles in priming effective antitumor responses with these vaccines. Cancer Res; 74(6); 1–12. ©2014 AACR.

Introduction

The generation of immunity requires collaboration between dendritic cells (DC) and T cells, as the priming of cytotoxic T lymphocytes (CTL) by DCs is a crucial event in the fight against infection and tumors (1). DCs regulate immune responses by recognizing, processing, and decoding pathogen-associated molecular patterns (PAMP) and antigenic molecules (2–6). PAMP recognition by intercellular or surface receptors on DCs signals the presence of infection and triggers signal transduction pathways, ultimately resulting in DC activation (4–6). Generally, activated DCs are characterized by enhanced expression of MHC, costimulatory molecules, and proinflammatory cytokines, which enable DCs to translate pathogenic signals to naive T cells and trigger adaptive immune responses (2–8). Recent studies have demonstrated that DCs may act as a network of distinct subsets, each performing specialized functions to stimulate and polarize T-cell responses to coordinate immune regulation (9–15). Antigen processing and presentation to T cells is predominantly attributed to the conventional DC subset (cDC), consisting of both CD8(−) DCs and CD8(+) DCs. CD8(+) DCs are especially adept at cross-presentation of exogenous antigen, interleukin (IL)-12 production and induction of CTL responses (16–20). The plasmacytoid DC (pDC) subset has the capacity to produce significant amounts of type I IFNs in response to microbial nucleic acids, particularly during viral infection, to facilitate T-cell activation, growth, and survival for disease clearance (13–15, 21). Moreover, the processes mediated by pDC and CD8(+) DC subsets have been associated with priming Th1 effector cells for the control of infection and tumors. A balanced distribution of activated DC subsets is associated with the control of autoimmune disease and tumors, indicating that these cells may cooperate during the generation of protective immunity (9, 10, 12, 13).

Currently, cancer vaccines are designed to introduce antigen in combination with adjuvants to activate DCs either ex vivo

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that regulate the trafficking and activation of DCs in vivo by precisely controlling the presentation of granulocyte macrophage colony-stimulating factor (GM-CSF) and CpG-oligonucleotide (CpG-ODN) adjuvants (25, 26). When applied as cancer vaccines, these matrices led to CTL-mediated eradication of melanoma tumors (26). Here, these matrices were modified to present three different classes of TLR agonists, CpG-ODN, monophosphoryl lipid A (MLPA), and polyinosinic-polycytidylic acid [P(IIc)], all in combination with GM-CSF. We hypothesized that poly(D,L-lactide-co-glycolide) (PLG) matrices presenting various combinations of adjuvants would modulate the distribution of DC subsets at the vaccine site, and influence the development of immunity to antigens loaded within these vaccine systems. This approach would then allow us to identify critical cellular and molecular hallmarks for effective vaccination or immunotherapy. Therefore, we first quantified the ability of various adjuvants to generate activated DC subsets and cytokines in vivo. The impact of DC induction on T cell–mediated immunity and cancer vaccine efficacy with models of B16-F10 melanoma was next assessed. These studies demonstrated that antitumor efficacy requires CD8(+) DCs and is strongly correlated with pDC numbers. Survival outcomes were also correlated to an array of inflammatory cytokines, which revealed a strong relationship between IL-12 and granulocyte colony-stimulating factor (G-CSF) production and antitumor efficacy. Altogether, these studies demonstrate that one can recruit and use various DC subsets for in situ vaccination, and provide important cellular and molecular insights into cancer vaccine design.

Materials and Methods

Cell culture

B16-F10 melanoma cells were obtained from American Type Culture Collection (catalog: ATCC CRL-6475) in 2010 and 2012. Upon receipt, the cells were cultured to passage three, aliquoted, and frozen in liquid nitrogen. For tumor experiments, B16-F10 cells were thawed and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Inc.), containing 10% FBS (Life Technologies, Inc.), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C in a humidified 5% CO2/95% air atmosphere and early-passage cells (between 4 and 9) were used for experiments. The cells have not been reauthenticated since receipt.

Mice

C57BL/6 mice (6–8-week-old female; The Jackson Laboratory), B6.129S2–Cd8atm1Mak/J (CD8 T cell knockout; 6–8-week-old female; The Jackson Laboratory), B6.129P2(SJL)-Myd88tm1.1Defr/J (MyD88 knockout; 6–8-week-old female; The Jackson Laboratory), C57BL/6j–Ticam1Lps2/J (TRIF knockout; 6–8-week-old female; The Jackson Laboratory), and Batf3−/− knockout mice (B6.129S-C–Batf3tm1.1Kmm/J; 6–8-week-old female; The Jackson Laboratory) were cared for in accordance with the American Association for the Accreditation of Laboratory Animal Care International regulations. B6.129S(C–Batf3tm1.1Kmm/J) and B6.129S2–Cd8atm1Mak/J congenic mice were backcrossed with C57BL/6 mice for at least 11 and 13 generations, respectively. Experiments were all approved by the Harvard University Institutional Animal Care and Use Committee.

Matrix fabrication

A 85:15, 120-kD copolymer of PLG (Alkermes) was used in a gas-foaming process to form porous PLG matrices (27). In brief, PLG microspheres encapsulating GM-CSF were first made using standard double emulsion (28). The double emulsion process was also used to fabricate PLG microspheres containing MPLA (Avanti Polar Lipids) as an adjuvant (29). PLG microspheres were then mixed with 150 µg of the porogen, sucrose (sieved to a particle size between 250 and 425 µm), and compression molded. The resulting disc was allowed to equilibrate within a high-pressure CO2 environment, and a rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure (27). The sucrose was leached from the scaffolds by immersion in water, yielding scaffolds that were 80% to 90% porous.

To incorporate tumor lysates into PLG scaffolds, biopsies of B16-F10 tumors that had grown subcutaneously in the backs of C57BL/6 mice (The Jackson Laboratory) were digested in collagenase (250 U/mL; Worthington) and suspended at a concentration equivalent to 10⁷ cells/mL after filtration through 40-µm cell strainers. The tumor cell suspension was subjected to four cycles of rapid freeze in liquid nitrogen and thawed (37°C) and then centrifuged at 400 rpm for 10 minutes. The supernatant (1 mL) containing tumor lysates was collected, incubated with the PLG microspheres, and lyophilized. The resulting mixture was used to make PLG scaffold-based cancer vaccines.

To incorporate CpG-ODNs and P(I:C) into PLG scaffolds, CpG-ODN 1826, 5′–tcc atg acg ttc ctg acg tt–3′ (Invivogen) or P(I:C) (high molecular weight; Invivogen) was first condensed with poly(ethyleneimine; PEI; Mn ~60,000; Sigma-Aldrich) molecules by dropping CpG-ODN 1826 or P(I:C) solutions into scaffold pores.
a PEI solution, while vortexing the mixture (30). The charge ratio between PEI and CpG-ODN (NH$_3$$^+$/PO$_4$$^-$$^-$) was kept constant at 7 during condensation. The charge ratio between PEI and P(LEC) (NH$_3$$^+$/PO$_4$$^-$$^-$) was kept constant at 3 during condensation. The condensate solutions were then vortexed with 60 µL of 50% (w/v) succrose solution, lyophilized, and mixed with dry succrose to a final weight of 150 mg. The succrose containing PEI-CpG-ODN condensate was then mixed with blank, GM-CSF, and/or tumor lysate–loaded PLG microspheres to make PLG cancer vaccines.

**In vitro release studies**

To determine the efficiency of GM-CSF incorporation and the kinetics of GM-CSF release from PLG scaffolds, $^{125}$I-labeled hr-GM-CSF (PerkinElmer) was used as a tracer. Scaffolds were prepared with iodinated GM-CSF and placed in 3 mL of PBS in an incubator (37 °C). At various time points, the PBS release media was collected and replaced with fresh media. The amount of $^{125}$I-hr-GM-CSF released from the scaffolds was determined at each time point by counting the radioactivity of the removed media in a gamma counter and normalizing the result to the total $^{125}$I-GM-CSF incorporated into the scaffolds. The total amount of CpG-ODN incorporated into PLG scaffolds and released into PBS over time was determine by using an oligonucleotide assay (Invitrogen).

In addition, the MPLA incorporation and release was determined by dissolving the matrix in a 2:1 chloroform:methanol mixture. The solution was vigorously shaken for 10 minutes and evaporated under a stream of N$_2$. One milliliter of 3 N methanolic HCl, 1 mL of hexane, and 25 µL of a 10 mg/mL pentadecanoic acid internal standard stock solution were added to each reaction vessel. The vessels were capped tightly and heated at 50 °C for 45 minutes. The cell suspensions were then stained with primary antibodies (BD Pharmingen) and phycoerythrin (PE)-conjugated CD8 (B7, costimulatory molecule) stains were conducted for DC recruitment and activation analysis. To further delineate the presence of specific DC subsets, cells were also stained with APC-conjugated CD11c and PE-conjugated PDCA-1 (pDC marker) or APC-conjugated CD11c and PE-conjugated CD8 (CD8+ DCs). The DC subsets were classified as CD8(+)CD11c(+) DCs (CD8+ DCs) and CD11c(+) CD8(−)PDCA-1(+) (pDCs). To track the DC emigration from the implant site, FITC was incorporated into the scaffolds (25, 31). To assess T-cell infiltration, PE-Cy7-conjugated CD3 stains were performed in conjunction with APC-conjugated CD8a (CD8 T cells) and PE-conjugated FoxP3 [regulatory T cell (Treg)] and analyzed with flow cytometry. Cells were gated according to single-positive FITC, APC, and PE stainings and using isotype controls. The percentage of cells staining positive for each surface antigen was recorded.

**Tumor growth assays, protective cytokines, and Trp2 pentamer analysis**

PLG scaffolds with melanoma tumor lysates and GM-CSF in combination with CpG-ODN, MPLA, or P(LEC) were implanted subcutaneously into the lower left flank of C57BL/6/j mice. For prophylactic vaccinations, animals were challenged 14 days later with a subcutaneous injection of $10^5$ B16-F10 melanoma cells (ATCC) in the back of the neck. Animals were monitored for the onset of tumor growth (~1 mm$^3$) and sacrificed for humane reasons when tumors grew to 20 to 25 mm (longest diameter).

To assess the PLG vaccine efficacy in the therapeutic setting, C57/BL6/j mice were challenged with a subcutaneous injection of $5 \times 10^6$ B16-F10 melanoma cells (ATCC) in the back of the neck. At day 9 after tumor challenge, PLG vaccines loaded with 3,000 ng GM-CSF in combination with 100 µg of CpG-ODN, MPLA, or P(LEC), and tumor lysates were implanted subcutaneously into the lower left flank of C57BL/6/j mice. A subset of mice was vaccinated again 10 days after the initial vaccination (day 19).

To determine in vivo concentrations of inflammatory cytokines at the matrix implant site, adjacent tissue was excised and digested with tissue protein extraction reagent (Pierce). After centrifugation, the concentrations of cytokines in the supernatant were then analyzed using ELISA (R&D systems) and Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Bio-Rad), according to the manufacturer’s instructions. Local cytokine analysis at the vaccine site was performed in wild-type (WT) C57BL/6/j mice, Batf3−/− mice, and CD8 T-cell knockout mice.

To study the generation of TRP-2-specific CTLs, single-cell suspensions were prepared from the spleens of mice immunized with PLG vaccines [antigen + 3,000 ng GM-CSF + 100 µg (CpG or MPLA or P(LEC)) at various time points. These cells were initially stained with PE-H-2Kb/TRP2 pentamers (Sigma-Aldrich), and subsequently stained with FITC-anti-CD8 and PE-CY7 CD3 monoclonal antibody (mAb; BD Pharmingen) before being analyzed using flow cytometry.

**Tfh characterization**

On the indicated days, B16-F10 tumors were removed from mice, and digested in 1 mg/mL collagenase II (250 U/mL; Worthington) and 0.1 mg/mL DNase for 1 hour at 37°C.
Dissociated cells were filtered through a 40-μm filter, and directly stained with antibodies for phenotype characterization by fluorescence-activated cell sorting (FACS) analysis. APC-anti-CD8 and PE-Cy7-anti CD3 were used to identify T cells isolated from the B16F10 tumors. These tumor-infiltrating leukocytes (TIL) were also co-stained with FITC-anti-IFN-γ and PE-anti-CD107a. All antibodies were obtained from eBioscience.

**Statistical analysis**

All values in the present study were expressed as mean ± SD. Statistical significance of differences between the groups were analyzed by a Student t test and a P value of less than 0.05 was considered significant.

**Results**

**Controlled GM-CSF and TLR agonist presentation**

Macroporous, PLG matrices (Fig. 1A) were designed to quickly release GM-CSF (25). Approximately 60% of the protein was released by day 10 (Fig. 1B–D) to induce the recruitment of DCs or their precursors. GM-CSF–loaded PLG scaffolds were also modified to present TLR-activating, CpG-ODN, MPLA, and P(I:C) molecules, as danger signals. The in vitro release kinetics of GM-CSF were similar in all conditions, irrespective of which TLR agonist was included in the vaccine (Fig. 1B–D). TLR agonists were more stably associated with scaffolds than was GM-CSF, as only 20% to 30% of incorporated CpG-ODN, P(I:C), and MPLA were released over the first 10 days in vitro, followed by slow and sustained release of danger signals over the next 14 days. Presentation of the TLR agonists in this system was designed to provide a long-term, local signal to activate DCs. Importantly, the relatively high molecular weight and composition of the particular PLG chosen to fabricate scaffolds results in slow scaffold degradation (31), allowing for long-term analysis of the vaccine site and its regulation over DC activation and T-cell immunity.

**Controlled DC generation and activation in vivo**

To examine the ability of PLG matrices to recruit and activate DCs in vivo, matrices delivering GM-CSF in combination with
danger signals were implanted subcutaneously into the backs of C57BL/6j mice. The magnitude of DC infiltration and activation into the matrices was determined by FACs analysis of cell populations subsequently isolated from the polymeric material after 7 days. Control matrices delivering GM-CSF alone contained $2.4 \pm 0.2 \times 10^5$ CD11c$^+$ cells (Fig. 2A and Supplementary Fig. S1), with relatively low-expression levels of the activation markers, MHCII$^+$ (2.6% of total CD11c$^+$ cells) and CD86$^+$ (4.6% of CD11c$^+$ cells). Inclusion of TLR-activating danger signals into PLG matrices significantly enhanced DC generation and activation in situ. Presentation of CpG-ODN, MPLA, and P(I:C) enhanced the total number of recruited DCs by 2.5-, 1.9-, and 2.2-fold, respectively (Supplementary Fig. S1), as compared with GM-CSF delivery alone. Analysis of the activation state of matrix-resident DCs revealed that local TLR induction produced significant percentages of activated DCs, as CD11c$^+$ cells positive for MHCII$^+$ and CD86$^+$ comprised approximately 30%, 19%, and 28% of the total cells recruited to CpG-, MPLA-, and P(I:C)-loaded matrices, respectively. Matrices presenting TLR agonists mediated approximately 15- (MPLA), 20- [P(I:C)], to 23-fold (CpG-ODN) increases in the total number of activated DCs at the implant site, relative to control matrices devoid of this signaling (Supplementary Fig. S1).

Because lymph node homing of DCs is a critical factor in generating immunity, we investigated the ability of TLR agonists to promote DC emigration from PLG matrices to the draining lymph nodes. As used in previous reports, FITC was incorporated into PLG scaffolds as a model antigen, as DCs recruited to the scaffold will ingest this label (32). The label can be later used to identify these cells following their trafficking to the inguinal lymph nodes. At day 2, MPLA stimulation led to a significantly higher overall number of scaffold-derived DCs in the inguinal lymph nodes as compared with controls and scaffolds containing CpG-ODN or P(I:C) as a stimulant (Fig. 2B). However, at later time points, day 4 and 7 after implantation, the number of DCs induced to home to lymph node by MPLA subsided to control levels (Fig. 2B), whereas CpG-ODN or P(I:C) stimulation resulted in a significantly higher number of DCs. At day 7 after implantation, CpG- and P(I:C)-loaded matrices resulted in more than a 10-fold increase in lymph node DCs over control conditions and MPLA stimulation (Fig. 2B), and these results were consistent with the DC activation observed at the vaccine site (Supplementary Figs. S1 and S2). These data indicate that CpG-ODN or P(I:C) agonists, as presented from PLG matrices, were able to promote greater and sustained lymph node homing as compared with MPLA or control conditions.

Interestingly, stimulation of the cells that infiltrated PLG matrices with CpG-ODN, MPLA, or P(I:C) enriched the numbers of CD11c$^+$ pDCs and CD11c$^+$ cDCs (Fig. 3A) relative to controls. The danger signals increased the numbers of pDCs at the implant site by approximately 4-fold relative to control matrices, with an average pDC number of 140,000 cells residing in the scaffolds presenting any of the TLR agonists (Fig. 3A). CD86$^+$ DCs were also present at the implant site at approximately 5-fold higher levels with MPLA and P(I:C) presentation, and at a 9-fold higher number when using CpG-ODN as a stimulant. Strikingly, the local delivery of TLR-activating agents promoted the local production of IL-12 (200–400 ng/mL) at the implant site (Fig. 3B), with CpG and P(I:C) inducing the highest levels. The IL-12 concentration correlated with the increased numbers of activated DCs and DC subsets in these conditions (Fig. 3A). In addition, the concentrations of a panel of candidate inflammatory cytokines were assayed at the vaccine site (Supplementary Tables S1 and S2 and Supplementary Fig. S3). Elevated levels of IFN-α (Supplementary Fig. S3) resulted from CpG-ODN and P(I:C) presentation, whereas MPLA had no effect on IFN-α concentration. However, MPLA led to 4-fold higher levels of TNF-α (Supplementary Table S1 and Supplementary Fig. S4). Interestingly,
IL-12 concentrations at MPLA-loaded matrices were 2-fold lower than found in CpG-ODN- and P(I:C)-loaded matrices. TNF-α may inhibit monocyte- and DC-derived IFN-γ, IL-12, and T-cell priming (33) and the aforementioned cytokine profiles, suggesting that MPLA-loaded matrices might be less efficient at stimulating antitumor T-cell responses compared with matrices incorporating CpG-ODN and P(I:C).

Prophylactic vaccination and correlation to its efficacy

Because PLG matrices presenting TLR agonists generate distinct and activated DC populations in situ and potent cytokine production, the antitumor efficacy of these systems was tested in the poorly immunogenic, B16-F10 melanoma model. B16 tumor lysates were used as a source of tumor antigen in vaccine formulations. Prophylactic PLG-based vaccines presenting both B16-F10 tumor lysates and GM-CSF resulted in 10% of the vaccinated mice surviving, tumor-free (Fig. 4A), after an otherwise lethal cell challenge at day 14 after vaccination. Importantly, antigen-loaded matrices with GM-CSF in combination with TLR agonists produced significant, and long-term tumor protection. CpG-ODN, MPLA, and P(I:C) presentation from PLG vaccines resulted in 90%, 80%, and 70% survival.

Figure 3. CD8(+) and pDC subsets, and IL-12 concentrations at vaccine site. A and B, the total numbers of CD11c(−)CD8(+) DCs and pDCs at scaffold site at day 7 (A), and the local IL-12 concentration after implantation of GM-CSF-loaded scaffolds (Con) and scaffolds loaded with GM-CSF in combination with CpG-ODN (CpG), MPLA, and P(I:C) (B). Values represent mean and SDs (n = 6). *, P < 0.05; **, P < 0.01, as compared with GM-CSF–loaded matrices (Con).

Figure 4. Prophylactic vaccination and survival correlation to CD8(+) and pDC subsets and IL-12 concentrations at vaccine site. Mice were vaccinated with PLG vaccines 14 days before B16-F10 melanoma tumor challenge (10⁵ cells). A, a comparison of survival in untreated mice (control) and mice treated with GM-CSF–loaded PLG scaffolds (GM-CSF) or with PLG scaffolds loaded with GM-CSF in combination with CpG-ODN (CpG), P(I:C), or MPLA. Plots of the normalized magnitude of CD11c(−)CD8(+) DC infiltration (B), pDC infiltration (C) at the vaccine site, and local IL-12 concentration (D) versus the percentage of animals surviving at day 100 following B16-F10 melanoma tumor challenge [survival data taken from experimental conditions in A (red data points) and previously reported data with this system; ref. 26]. R values in B and C represent the linear correlation coefficient between y-axis variables (normalized by baseline levels in control scaffolds) and survival percentage.
survival rates (Fig. 5B). Regression analysis was subsequently performed to determine whether induction of long-term survival was related to pDC, CD8(+) (Fig. 4A and B), and cytokine levels (Supplementary Table S2) at the vaccine site; previously published data using various doses of CpG-ODN (26) were included in the analysis. Strikingly, animal survival rates were
strongly correlated with the numbers of pDCs, CD8(+) DCs, and with endogenous IL-12 and G-CSF generated by PLG vaccines (Fig. 4A–D and Supplementary Table S2). These findings suggest that the important parameters potentiating vaccine efficacy include cross-presentation by CD8(+) DCs, and cooperative mechanisms promoted by pDCs and the cytokines, IL-12 and G-CSF.

Moreover, TLR-MyD88 and TLR-TRIF are critical signaling pathways regulating cytokine production and immune responses (34), and a complete loss of prophylactic vaccine efficacy was observed when using vaccines in TRIF and MyD88 knockout mice, regardless of the TLR agonist (Supplementary Fig. S5). Although P(\text{I:C}) is thought to be TRIF-dependent (TLR3), CpG MyD88-dependent (TLR9), and MPLA (TLR4) can activate via TRIF and MyD88, it is likely that TLR agonists presented within tumor cell lysate and polymeric formulations require both pathways to promote the appropriate cytokine production and cellular regulation that actuates effective vaccination.

**Therapeutic vaccination and antitumor CTL activity**

As specific vaccine formulations containing various TLR agonists produced significant numbers of activated DCs and conferred prophylactic immunity, it was hypothesized that these would lead to superior therapeutic responses and CTL responses. To test this hypothesis, mice challenged with 5 × 10^5 B16-F10 melanoma cells were subsequently vaccinated at days 9 and 19, after tumors were established. All tumor-bearing mice implanted with control PLG matrices demonstrated rapid tumor growth and required euthanasia by day 24, as expected (Fig. 5A and B). PLG vaccines presenting MPLA as an adjuvant decreased the rate of tumor progression (Fig. 5A), and a slight increase in mean survival time (\sim 1.5-fold increase) over controls was found (Fig. 5A and B). Complete tumor regression (tumors ≤ 36 mm^3) and long-term survival of mice (33% survival) was achieved in the subset of mice vaccinated with PLG vaccines exploiting P(\text{I:C}) and CpG-ODN as an adjuvant.

To further characterize the therapeutic response, mice were vaccinated at 9 days after tumor challenge and FACS analysis was used to determine the induction of B16-F10 TILs. Strikingly, a one-time dose of vaccines loaded with TLR agonists produced significant numbers of tumor-infiltrating CD8(+) CTLs, as compared with control animals (Fig. 5C and D). CD8(+) T-cell infiltrates were further characterized for coexpression of IFN-\gamma and CD107a, a marker for cytotoxic-associated cell degranulation. These cell populations were markedly enhanced in vaccine-treated animals (Fig. 5C and D). Vaccines featuring CpG-ODN, P(\text{I:C}), and MPLA signaling resulted in approximately 6.1-, 3.1-, and 1.4-fold increases in IFN-\gamma(+) CD107a(+) TILs in comparison with controls. Moreover, CpG-loaded vaccines resulted in significantly higher numbers of activated TILs in comparison with their P(\text{I:C}) and MPLA counterparts (Fig. 5D).

The activation of systemic CTL responses was also monitored by staining splenocytes with MHC class I/TRP2 peptide pentamers to identify CTLs with specificity to tyrosinase-related protein (TRP)-2. This is a main antigenic target of melanoma vaccines in mice and humans. A significant expansion of TRP2-specific CTLs was observed in the spleens of mice vaccinated with CpG-ODN-, MPLA-, and P(\text{I:C})-loaded vaccines, in comparison with controls devoid of TLR agonists (Fig. 5E). Analysis of cells infiltrating the vaccines incorporating TLR agonists also revealed a significant, local CD3(+)CD8(+) T-cell response (Supplementary Fig. S6), likely in response to sustained tumor antigen presentation.

Vaccination of transgenic mice lacking CD8(+) T lymphocytes resulted in decreased levels of IL-12 and IFN-\gamma (Supplementary Figs. S7 and S8), in comparison with WT. The IL-12–IFN-\gamma pathway is a positive feedback mechanism, with each cytokine augmenting production of its counterpart (33, 35); these data suggest that the immune responses induced by this vaccine system may be amplified by cytokine-mediated cross-talk between DCs and primed CTLs.

To investigate whether the potent antitumor efficacy induced by P(\text{I:C}) and CpG could translate to other tumor settings, we tested these systems in the Lewis lung carcinoma (LLC) model. The P(\text{I:C}) and CpG agonists were used because these agonists induced the highest survival rates in B16 melanoma models. Similar to the results in the B16 prophylactic model, P(\text{I:C}) and CpG vaccines loaded with LLC tumor cell lysates prophylactically promoted 80% and 70% survival (Fig. 5G). Also, in therapeutic models, mice were challenged with 5 × 10^5 LLC cells and subsequently vaccinated at day 7. A single vaccination with either the P(\text{I:C}) or CpG vaccine formulations was able to attenuate the growth rate of LLC tumors by approximately 3-fold (Fig. 5F) and double median survival times.

**Vaccine efficacy is impaired in mice lacking CD8(+) DC**

Because PLG vaccines incorporating TLR agonists were capable of generating CD8(+) DC populations in situ, which correlated to potent antitumor CTL responses and survival (Figs. 4 and 5), we next examined whether these cells were required to confer antitumor immunity in vivo. Batf3(−/−) transgenic mice were used in these experiments, as they lack CD8(+) DCs, without exhibiting abnormalities in other hematopoietic cell types or tissue architecture (20). WT and Batf3(−/−) mice were vaccinated with CpG-ODN–loaded PLG vaccines and challenged with B16-F10 cells 14 days later. Vaccination of WT mice promoted complete protection against tumor growth and long-term survival (100% survival), as expected, but vaccinated Batf3(−/−) were not protected and tumor growth rates were similar to unvaccinated, WT animals (Fig. 6A). Moreover, vaccinated Batf3(−/−) failed to produce the local CTL responses observed in WT mice, and a 3-fold decrease in TRP2-specific CTLs in these mice coincided with higher ratios of FoxP3(+) Tregs cells at the vaccine site in this condition (Fig. 6B). These results indicate that a lack of CD8(+) DCs resulted in limited cytotoxicity and allowed regulatory pathways mediated by Tregs to potentially extinguish the vaccine-mediated immune response. Surprisingly, the vaccine produced significantly lower numbers of local pDCs and activated DCs in Batf3(−/−) mice (Supplementary Fig. S9) compared with WT mice, suggesting that CD8(+) DCs play a role in generating or maintaining these cells and this effect likely contributed to the loss of vaccine efficacy. The systemic
production of antitumor CTLs was also impaired in Batf3–/– mice, as a 3-fold reduction in Trp2-specific CTLs was measured in the spleens of these mice in comparison with WT controls (Fig. 6D). These results indicate that vaccine efficacy in this system is critically regulated by CD8 DCs, potentially via their ability to cross-present tumor antigens, to produce Th1 induction factors, such as IL-12, and to generate and interact with CTLs.

Because our regression analysis identified IL-12, G-CSF, and IL-6 as strong correlates to survival (Supplementary Table S2), we investigated whether the presence of CD8(+) DCs affected induction of these cytokines by PLG vaccines. WT mice were able to induce the production of the T-cell growth factor, IL-12, at the vaccine site at 5-fold higher levels than found in vaccinated Batf3–/– mice (Fig. 6C). The partial loss of IL-12 production in CD8(+) DC knockout mice suggests that these cells are important producers or mediators of this Th1-polarizing cytokine. Similarly, knocking out the CD8(+) DC population resulted in approximately 3-fold decreases in the levels of IL-6 and G-CSF at the vaccine site (Supplementary Figs. S10 and S11). These cytokines are associated with priming innate immunity and danger signal activation, including TLR signaling, suggesting that these mechanisms of DC stimulation are compromised in Batf3–/– mice.

Discussion

To address the limitations of current cancer vaccines, we used a PLG matrix controlling the presentation of tumor lysate, GM-CSF, and TLR agonists to create a vaccine node that recruits and activates multiple DC subsets in situ. This tool was used to investigate the contribution of DC subsets to vaccine efficacy and demonstrated that effective tumor cell killing required the participation of CD8(+) DCs, with strong correlations to pDC number and IL-12, G-CSF, and IL-6 concentrations. This study expands on previous reports, by demonstrating that these components were critical to vaccine efficacy, regardless of the type of stimulant incorporated within the scaffolds.

As described previously, GM-CSF delivery was used to promote DC recruitment to the vaccine site where these cells are subsequently activated by TLR-agonist presentation. We determined that vaccines were able to maintain elevated levels of GM-CSF for at least 14 days regardless of the TLR agonist incorporated into the system. Interestingly, GM-CSF concentrations at the vaccine site at day 14 were approximately 80- to 110-fold higher than the concentrations that would be expected solely from release from the vaccine, as determined by in vitro release kinetics. The absolute concentrations were also lower than control matrices with only GM-CSF (Supplementary Table S1). The assay is not able to distinguish between exogenous and endogenous GM-CSF and cellular infiltrates may differentially produce and consume GM-CSF at the vaccine site in response to local vaccine activity (Supplementary Table S1).

In this report, we now describe methods to incorporate different classes of TLR agonists into biomaterial vaccine
systems and control their presentation. Inclusion of TLR stimulation was required to significantly upregulate DC coexpression of MHCII and CD86 (Fig. 2), enhancing their capacity to propagate antigen-specific T-cell populations. Appropriate TLR signaling increased the generation of CD8(+) and pDC subsets at the vaccine site, and stimulated the production of IFNs and the potent T-cell growth factor, IL-12. Moreover, removal of TLR agonists from the system resulted in decreased numbers of Trp2-specific, cytotoxic CD8(+) T cells locally at the vaccine site and systemically in spleens and tumors, and this coincided with reduced survival in vaccine studies.

Interestingly, a recent report described the sequestration of tumor-specific, CD8(+) T cells at the site of vaccines with persistent antigen presentation (47). In that study, this resulted in dysfunctional immune responses and local T-cell apoptosis relative to relatively nonpersistent vaccine formulations (36). In contrast to those peptide-based vaccines, the system in this report was fabricated to present whole-tumor cell lysates within controlled inflammatory microenvironments to confer an immunostimulatory and tumor-mimicking microenvironment, which produced significant, antigen-specific T-cell infiltration at both the vaccine site and the tumor environment (Figs. 5C–E and 6B; Supplementary Fig. S6). For example, after 10 days of CpG-mediated vaccination, the tumor and the PLG vaccine site were similarly enriched with CD8(+) T cells (~5% of total cells; >10^6 antigen-specific T cells in tumors), with potent antitumor effects. A double dose of PLG vaccines presenting P(LC) or CpG-ODN induced potent tumor rejection in therapeutic models of B16-F10 melanoma, causing complete tumor regression in over a third of vaccinated animals and eradicating tumors reaching 35 mm^2 in size. The tumor sites in vaccinated animals demonstrated intense and activated CD8 (+), CTL infiltrates, as determined by CD107a and IFN-γ expression, likely affecting tumor-cell killing. Although the PLG vaccines were able to induce prophylactic and therapeutic survival in B16 models, unlike peptide in mineral oil formulations, the findings by Hailemichael and colleagues (36) suggest that we could further improve efficacy by using more degradable PLG formulations that may reduce the duration of antigen presentation to focus the induced T-cell response on tumors.

These systems outperformed the preclinical, historical results of current vaccine modalities extensively studied in the clinic, including irradiated tumor cell vaccines and DC-based vaccines (7, 8, 26, 37, 38). Also, vaccines containing CpG-ODN and P(LC) stimulation were able to confer efficacious antitumor responses in both prophylactic and therapeutic LLC models, indicating that these results are transferable to other tumor settings.

Interestingly, vaccines presenting MPLA signaling slowed tumor growth rates but did not cause tumors to completely regress. The current vaccine design is able to effectively exploit CpG-ODN and P(LC) signaling, as compared with MPLA, to promote higher average levels of cell surface activation markers on DCs and cytokine profiles that promote Th1 polarization and CTL responses. In addition, MPLA was a strong inducer of TNF-α, in comparison with CPG-ODN and P(LC) (Supplementary Fig. S2), and this may inhibit IL-12 and IFN pathways priming CTL responses (33, 35). It is important to note that these results do not definitively address the potency of TLR4 antagonism relative to TLR3 and TLR9. Other design variables, such as MPLA dose, and alternative TLR4 agonists should be tested in the future. However, the data do indicate that the induction of activated DCs and CTL is dependent on TLR stimulation and is consistent with survival rates.

Strikingly, all the vaccinated Batf3^-/- mice generated tumors in prophylactic models, whereas 90% of WT mice were protected and survived in the long-term. This finding expands upon our correlative measures by proving that CD8(+) DCs are, in fact, a critical component of cancer vaccine efficacy in these systems. Furthermore, cytokine analysis of the vaccine site of Batf3^-/- mice revealed that local IL-12 levels, and CTL responses were markedly reduced, suggesting that CD8(+) DCs are an important source or mediator of local production of IL-12 and Th1 polarization. CD8(+) DC participation and vaccination not only resulted in reduced cytotoxic, CD8(+) T-cell activity (at tumor site and spleen), it also allowed the progression of Treg activity. High FoxP3(+) Treg to CD8(+) T-cell ratios indicate unbalanced immunosuppression that could extinguish vaccine efficacy and promote tumor growth (39, 40). CD8(+) DCs and IL-12 can cause Treg inhibition or their conversion to IFN-γ-producing, effector T cells (29, 41, 42), and these mechanisms are potentially critical to the efficacy of these material-based cancer vaccines. Surprisingly, another cytokine, G-CSF, was found to be a strong correlate of vaccine efficacy (Supplementary Table S2) even though it has been associated with suppression of T-cell proliferation and cytolytic effectors (43–45). G-CSF, however, is a strong mobilizing factor for mononuclear cells and DCs, and its elevated endogenous production may combine with vaccines presenting TLR ligands to enhance DC differentiation and activation, and antitumor responses (46, 47).

An interesting aspect of this vaccine system is CTL homing to the vaccine site (Supplementary Fig. S3), likely due to long-term antigen presentation. Knocking out CD8(+) T cells also resulted in a significant reduction of local levels of IFN-γ and IL-12 (Supplementary Figs. S4 and S5). Others have shown that T cell–derived IFN-γ enhances DC expression of IL-12 and costimulatory molecules, creating a feedback loop that amplifies CTL-mediated responses to infection. In the current study, after vaccine priming, T cells that home back to the antigen-presenting vaccine site may be important vaccine components themselves, as they may sustain and amplify CTL responses via IFN-γ-mediated DC activation and IL-12 production.

These findings provide evidence that CD8(+) DCs, pDCs, and IL-12, or their equivalent functionally play key roles in material-based cancer vaccines. The relation between pDC, IL-12, G-CSF and vaccine efficacy are currently correlative and further studies should be undertaken to critically examine these relationships. Finally, the methods described here may be adapted to identify cellular and molecular components that may be used to design immunotherapeutic applications for other clinical indications, such as infectious and autoimmune disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
References


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Identification of Immune Factors Regulating Antitumor Immunity Using Polymeric Vaccines with Multiple Adjuvants

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