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The development of therapeutic vaccines for treatment of established cancer has proven challenging. Cancer vaccines not only need to induce a robust tumor Ag-specific immune response but also need to overcome the tolerogenic and immunosuppressive microenvironments that exist within many solid cancers. ISCOMATRIX adjuvant (ISCOMATRIX) is able to induce both tumor Ag-specific cellular and Ab responses to protect mice against tumor challenge, but this is insufficient to result in regression of established solid tumors. In the current study, we have used B16-OVA melanoma, Panc-OVA pancreatic, and TRAMP-C1 prostate cancer mouse tumor models to test therapeutic efficacy of ISCOMATRIX vaccines combined with other immune modulators. The coadministration of an ISCOMATRIX vaccine with the TLR3 agonist, polyinosinic-polycytidylic acid, and TLR9 agonist, CpG, reduced tumor growth in all tumor models and the presence of ISCOMATRIX in the formulation was critical for the therapeutic efficacy of the vaccine. This vaccine combination induced a robust and multifunctional CD8+ T cell response. Therapeutic protection required IFN-γ and CD8+ T cells, whereas NK and CD4+ T cells were found to be redundant. ISCOMATRIX vaccines combined with TLR3 and TLR9 agonists represent a promising cancer immunotherapy strategy.  

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Abbreviations used in this article: ISCOMATRIX, ISCOMATRIX adjuvant; mu, murine; PAP, prosthetic acid phosphatase; Poly I:C, polyinosinic-polycytidylic acid; TAA, tumor-associated Ag; Tg, transgenic; TIL, tumor-infiltrating lymphocyte.
a therapeutic setting. The combination of ISCOMATRIX-OLA vaccine with polyinosinic-polycytidylic acid (Poly I:C) and CpG enhanced the magnitude and quality of OVA-specific CD8+ T cell responses resulting in significant tumor growth control and increased overall survival in two different tumor models. In addition, the administration of the self-mouse Ag prostatic acid phosphatase (PAP) expressed by TRAMP-C1 prostate cancer cells with ISCOMATRIX-Poly I:C and CpG increased the survival rate of mice in a therapeutic model of prostate cancer. Our data also demonstrate that ISCOMATRIX was critical for the combination vaccine to induce therapeutic protection.

Materials and Methods

Mice

Specific pathogen-free C57BL/6 (CD45.2), B6.SJL-J-Ptprc58/J (CD45.1) mice, IL-18 knockout (The Jackson Laboratory), IFN-γ−/− (The Jackson Laboratory), OT-I (243.2) transgenic mice (G. Belz, Walter and Eliza Hall Institute) that were src−/−, a 8 transgenic (C. A. Lew, Walter and Eliza Hall Institute) were bred at Bio21 Institute (Melbourne, VIC, Australia). In all cases, gender-matched mice at 6–8 wk of age were used. All animal studies were approved by the CSL Zoetics Animal Ethics Committee (Melbourne, VIC, Australia).

Ags and adjuvants

ISCOMATRIX (CSL Biotherapies, King of Prussia, PA; ISCOMATRIX is a registered trademark of ISCOTEC Ab) is a CSL-registered trademark of CSL was prepared as described previously and used at 3.8 ISCO units/dose (7). Poly I:C and CpG type A-1585; type B-1826; or type C-2395 (InvivoGen) were used at 5 μg/dose unless otherwise indicated. Low-endotoxin chicken OVA (Hyglos) was used at 30 μg/dose. The N-terminal 381-aa extracellular domain of murine (mu)PAP (GenBank accession numbers NP_997551 and AAP23171; http://www.ncbi.nlm.nih.gov/genbank) with an in-frame C-terminal 8 Histidine-tag was coexpressed for human expression, and purified by TALON (G. Belz, Walter and Eliza Hall Institute) from FS293F (FS293F) cells were cultured in FreeStyle 293 Expression Medium (Life Technologies). The cDNA was digested with NheI and XhoI and ligated into pcDNA3.1 (Life Technologies), and the nucleotide sequence confirmed using BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing and an Applied Biosystems 3130xl Genetic Analyzer. FreeStyle 293-F (FS293F) cells were cultured in FreeStyle 293 Expression Medium (Life Technologies) that was supplemented with Antibiotic-Antimycotic (Life Technologies) and maintained at 37°C with an atmosphere of 8% CO2. Transient transfections of expression plasmids encoding muPAP using FS293F cells were performed using 293fectin Transfection Reagent (Life Technologies), according to the manufacturer’s instructions in a Cellbag 10L (GE Healthcare Life Sciences) on a 20/50 Wave Bioreactor system (GE Healthcare). Cultures were supplemented at 4 h posttransfection with PlasmaClear (Life Technologies) to a final concentration of 0.1% v/v and 24 h posttransfection with LuciferTone Lumin (Millipore) to a final concentration of 0.5% v/v. The cell culture supernatants were harvested by centrifugation at 2500 rpm and passed through a 0.45-μm filter (Nalgene). The recombinant mouse PAP was purified on a Ni Sepharose 6 FF matrix (30 ml) (GE Lifesciences) packed into a XK26/20 column (GE Lifesciences) and pre-equilibrated with binding buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole [pH 8]). MuPAP-His was eluted with a 0–100% gradient of elution buffer (50 mM NaH2PO4, 300 mM NaCl, and 500 mM imidazole [pH 8]). Peak fractions were pooled, and the monomeric MuPAP-His was purified by size exclusion chromatography using an AKTAexplorer 100 (GE Lifesciences) HPLC with Superdex 200 (GE Lifesciences). The purified PAP was concentrated and tested for low-endotoxin content using the Limulus amebocyte lysate Kinetic-QCL assay (Lonza). The final product was stored long term at −80°C and used at 300 μg/dose in all experiments.

Immunization and tumor challenge protocols

For analysis of immune responses, mice were vaccinated on days 0 and 7, and T cell responses were analyzed on day 14. When indicated, mice were also boosted 3 mo later, and T cell recall responses were analyzed 1 wk after the last vaccination. For prophylactic tumor experiments, mice were vaccinated on days 0 and 7 and challenged with 1 × 10⁶ B16-OVA (A. Sutphin; BMR-Berounice, Brno, Czech Republic) tumor cells to determine therapeutic tumor experiments for analysis of recall responses as indicated. For therapeutic tumor experiments mice were injected with 1 × 10⁵ B16-OVA, 5 × 10⁶ PancOVA (M. Schnurr, University of Munich, Munich, Germany), or 3 × 10⁸ TRAMP-C1 (CRL-2730; American Type Culture Collection) on the right flank, s.c. Vaccinations were performed on days 2 and 9 or days 6 and 13, as indicated (s.c.) in 100 μl PBS. Tumor size was measured, and mice were culled when the tumor mass reached 100 mm2. The percentage of survival and tumor-free mice were also determined.

Analysis of CD8+ T cell response

Mice were primed and boosted on days 0 and 7, respectively, as described above. On day 14, splenocytes were cultured ex vivo for 4 h in the presence of brefeldin A (20 μg/ml with SININFEL peptide (OVA257-264) at 2 μg/ml; Mimotopes). Splenocytes were then stained with anti-CDS-FITC (17-12A2) and anti-CD8-PE (2.43) (both from BD Biosciences) washed, fixed, and permeabilized (Perm/Wash; BD Biosciences). Cells were then stained with anti–IFN-γ-APC (XM1G2.1; BD Biosciences). CD8+ T cells were analyzed by flow cytometry for the expression of IFN-γ using a FACS Calibur or FACS Canto instruments (BD Biosciences). Some cells were also costained with anti-human granzyme B-Alexa 647 (GB11; BD Biosciences) and anti–IFN-γ-APC (XM1G2.1; BD Biosciences).

The presence of splenic OVA–specific CD8+ T cells was analyzed on day 14 using PE-SININFEL-Kb tetramers (University of Melbourne, Melbourne, VIC, Australia), anti–CD8-APC, anti–CD3-ITC, and 7-ami-noacetoxyiminocyt D to exclude dead cells. In long-term experiments, analysis of OVA-specific CD8+ T cells in spleens was performed using PE-SININFEL-Kb tetramer 1 wk after the last boost. For degranulation assays, cells were incubated with anti–CD107a- and anti–CD107b-FITC (H4A3 and ABL-93; BD Biosciences) and Monensin (GolgiStop; BD Biosciences) for 4 h, followed by staining with anti–CD8-APC (2.43, BD Biosciences) and anti–CD3-PE (17A2; BD Biosciences).

In vivo proliferation of CD8+ T cells

Peripheral lymph nodes (inguinal, axillary, brachial, sacral, and superficial cervical) from OT-I TCR transgenic mice (CD45.2) were used to purify CD8+ T cells. Lymph node cells were incubated with Abs specific to CD11b (Mac-1, M170; Mac-3 (F4/80), Ter-119, Gr1 (RB6-8C5), MHC class II (M5/14); and CD4 (GK1.5), followed by goat anti-rat magnetic beads (Biomag, Qiajen, the Netherlands). The enriched CD8+ T cells (87–96% purity) were labeled with CFSE (Molecular Probes) as described previously (17). B6.SJL-J-Ptprc58/J (CD45.1) mice were inoculated with 5 × 10⁵ B16-OVA tumor cells on the flank as described previously. One day later, they were injected with 1 × 10⁶ CFSE-labeled OT-I cell, i.v. The following day, mice were primed as indicated. Fifty to 72 h postprime, tumor-draining lymph nodes were harvested and OT-I cell proliferation was determined as CFSE dilution in live CD45.2+CD8+ cells by flow cytometry.

Control of tumor growth by CD8+ T cells in vitro

C57BL/6 mice were primed and 7 d later boosted with OVA + ISCOMATRIX vaccines as previously indicated. One week later, spleens were harvested and CD8+ T cells purified using CD8 MicroBead Kit (Miltenyi Biotec). B16-OVA and CD8+ T cells were plated at the ratios indicated and incubated for 5 h. The percentage of live tumor cells at the end of incubation time was determined using Cell Titer 96 Nonradioactive Cell Proliferation Assay (Promega).

In vivo CD8+ and NK cell depletions

On day 0, C57BL/6 mice were inoculated with B16-OVA tumor cells as described previously. For depletion of CD8+ T cells, mice were injected with 100 μg rat anti–CD8α mAb (53-5-8; Walter and Eliza Hall Institute) or rat IgG1 isotype control, i.p., on days 0, 1, and 2 and then once per week until the end of the experiment. Prime and boost with indicated vaccines were performed on days 2 and 9. For NK cell depletion, mice were injected with 200 μg mouse anti-NK1.1 mAb (PK136; BioXCell) or isotype control on days −2, −1, and 0 and then every 3 d until the end of the experiment. Prime and boost with indicated vaccines were performed on days 3 and 10. Depletion of CD8+ T cells or NK cells was >97%.

Statistical analyses

Analysis of tumor growth was performed using the nonparametric Mann-Whitney U test to compare the mean values between two groups. Kaplan–Meier survival curves were analyzed using GraphPad Prism 6.0 for Windows software (GraphPad Software, San Diego, CA). Differences were considered significant when p < 0.05.
Results

ISCeMATRIX formulated with OVA confers prophylactic protection against B16-OVA tumor

We have previously shown that mice vaccinated with ISCOMATRIX-OVA in a day 0/day 7 prime-boost regimen, followed by challenge with B16-OVA tumor cells 7 d after the boost, rejected tumor growth. Furthermore, this effect was abrogated when mice were depleted of CD8+ T cells (9). We set out to probe more deeply the longevity of the vaccine-induced immune response over an extended period of follow-up. In the current study, mice were challenged with B16-OVA tumor cells 3 mo after the last vaccination. This setting would rely on the establishment of long-term memory CD8+ T cells to facilitate protection against tumor challenge. Indeed, the ISCOMATRIX-OVA vaccine conferred significant protection where 90% of vaccinated mice survived B16-OVA tumor challenge and remained tumor free for at least 4 mo (Fig. 1A). In the same experiment, we analyzed the ability of GK1.5 Tg mice, which are permanently depleted of CD4+ cells, to control B16-OVA tumor growth. Results demonstrate that CD4+ T cells were not required for the induction of prophylactic protection by the ISCOMATRIX-OVA vaccine. This result suggests that the ISCOMATRIX-OVA vaccine induces memory CD8+ T cells in the absence of CD4+ T cell help. To test this hypothesis further, we analyzed the presence of OVA-specific CD8+ T cells in both wild-type and GK1.5 Tg mice 3 mo post-priming to determine the percentage of long-lived, OVA-specific CD8+ T cells. In addition, we investigated the ability of these CD8+ T cells to mount a recall response 1 wk after a boost with the ISCOMATRIX-OVA vaccine. As shown in Fig. 1B, similar percentages of OVA-specific CD8+ T cells were detected in wild-type and GK1.5 Tg mice, likely representing a population of memory CD8+ T cells. In addition, these recall responses were comparable in magnitude between the wild-type and GK1.5 Tg mice (Fig. 1B, 1C). Taken together, these data indicate that the ISCOMATRIX-OVA vaccine induced functional memory CD8+ T cells in the absence of CD4+ T cell help. Protection was abrogated in mice lacking IFN-γ or IL-18 (data not shown), which is in agreement with previous data showing that the IL-18R signaling is required for induction of functional NK and CD8+ T cell responses by ISCOMATRIX vaccines (17).

ISCeMATRIX-OVA vaccine in combination with TLR agonists provide therapeutic protection against B16-OVA melanoma and Panc-OVA pancreatic tumors

The efficacy of the ISCOMATRIX-OVA vaccine as a single-adjuvanted vaccine is limited in the therapeutic cancer setting of established tumors. It is likely that, in these settings, combinations of immune enhancers are required to trigger multiple activation pathways and elicit robust and multifunctional antitumor immune responses. In an effort to improve the therapeutic efficacy of the ISCOMATRIX-OVA vaccine, we established B16-OVA or Panc-OVA tumors and evaluated combinations of TLR3 and TLR9 agonists with ISCOMATRIX. Vaccines comprising ISCOMATRIX and different doses of Poly I:C or CpG-1826 induced detectable CD8+ T cell responses and the magnitude of this response was dependent of the TLR-agonist dose (Fig. 2A). In addition, all types of CpG (A, B, or C) induced enhanced T cell responses in combination with ISCOMATRIX irrespective of their mechanism of action (Fig. 2B). Despite the enhancement of the T cell response induced by ISCOMATRIX in combination with Poly I:C or CpG, such vaccines did not induce protection in the therapeutic cancer models (data not shown). However, the combination of ISCOMATRIX, Poly I:C, and CpG (multiadjuvanted vaccine) induced significant protection in mice bearing B16-OVA or Panc-OVA tumors (Fig. 3A, 3B, respectively). Results in Fig. 3A demonstrate that the multiadjuvanted vaccine induced significant regression of established tumors. The multiadjuvanted vaccine not only improved survival and reduced tumor growth but also induced...
complete tumor rejection in 60% of mice in the B16-OVA tumor model. Therapeutic efficacy was also observed when mice were vaccinated at six days after tumor engraftment (Supplemental Fig. 1). Similar results were obtained in the Panc-OVA tumor model. Consistently the multiadjuvanted vaccine induced significant tumor regression of established pancreatic tumor and reduced tumor growth, improved survival and induced complete responses in 20% of mice (Fig. 3B).

To further extend the significance of these studies, we investigated the therapeutic efficacy of the multiadjuvanted vaccine in a prostate cancer tumor model using TRAMP-C1 tumor cells and the self-Ag PAP. Results shown in Fig. 4 support that the multiadjuvanted vaccine is effective against less immunogenic tumors and enhanced the survival of mice bearing tumors. In addition, 40% of mice vaccinated with ISCOMATRIX-PAP-Poly I:C-CpG showed complete tumor rejection up to day 110.

These data strongly support the findings that a multiadjuvanted vaccine strategy combining a tumor Ag, ISCOMATRIX, with Poly I:C and CpG may represent a promising and efficacious strategy for the therapeutic treatment of established melanoma and pancreatic and prostate cancers.

**CD8**+ T cells but not NK cells are required for the induction of therapeutic protection by ISCOMATRIX-OVA-Poly I:C-CpG vaccine

Antitumor immunity in vivo can be facilitated by several mechanisms. These include Ab-dependent cytotoxic effects, such as...
complement activation or Ab-dependent cell cytotoxicity mediated by NK cells or Ab-dependent cell phagocytosis by myeloid cells, and direct cell–mediated cytotoxicity by NK cells or Ag-specific CD8+ T cells. To address whether NK cells and/or CD8+ T cells were required for the therapeutic protection conferred by the multiadjuvanted vaccine, tumor growth was monitored in vaccinated mice depleted of either NK cells or CD8+ T cells. Results shown in Fig. 5A demonstrate that vaccination of mice depleted of CD8+ T cells was unable to induce the regression of established tumors with the animals requiring euthanasia at day 17. In contrast, depletion of NK cells did not significantly abrogate the ability of the multiadjuvanted vaccine to induce tumor regression (Fig. 5B). The depletion of NK cells had a slight effect on tumor growth in mice vaccinated with ISCOMATRIX-OVA, which suggests some involvement of NK cells in controlling tumor growth although not to biological significance. This is consistent with the fact that ISCOMATRIX enhances NK activation (17), and NK cells are important for elimination of tumor cells. This demonstrates that the mechanism by which the multiadjuvanted vaccine elicits regression of established B16-OVA tumors is via the induction of CD8+ T cell responses. It also demonstrates that the antitumor effects are not dependent on NK-mediated mechanisms.

ISCOMATRIX-OVA-Poly I:C-CpG vaccine induces multifunctional CD8+ T cells in mice bearing tumors

To further understand how the multiadjuvanted vaccine elicits its antitumor effects, we investigated the quantitative and qualitative features of the CD8+ T cell response induced in vaccinated mice using the OVA-transgenic T cell adoptive transfer system. As shown in Fig. 5A, striking differences were observed in the proliferative capacity of CFSE-labeled, OVA-specific transgenic T cells and vaccinated with various vaccine formulations. In addition, CD8+ T cells from mice vaccinated with the multiadjuvanted vaccine also showed higher tumor cell killing in vitro (Fig. 7A). The presence of ISCOMATRIX in the vaccine formulation was critical for the induction of significant numbers of CD8+CD107+ T cells capable...
to degranulate and express the effector molecule granzyme B (Fig. 7B). Most importantly, the therapeutic effect of the multiadjuvanted vaccine was lost when ISCOMATRIX was not included in the vaccine formulation, and most importantly no tumor-free mice were observed in mice vaccinated with OVA-Poly I:C-CpG without ISCOMATRIX (Supplemental Fig. 2). Overall, these data demonstrate that the combination of ISCOMATRIX, Poly I:C, and CpG induces Ag-specific CD8+ T cell responses of higher magnitude and superior quality, both of which may contribute to efficient control and/or rejection of established tumors in vivo.

Finally, we examined whether the multiadjuvanted vaccine improved the migration of effector CD8+ T cells to the tumor site. For this purpose, the percentage of OVA-specific CD8+ T cells present within the tumor mass was analyzed in mice vaccinated with ISCOMATRIX-OVA or the multiadjuvanted vaccine. No differences were observed between either vaccine formulations (Supplemental Fig. 3), suggesting that the multiadjuvanted vaccine elicited improved antitumor efficacy against established tumors primarily via enhancing the antitumor effector functions of the Ag-specific CD8+ T cells rather than improving their frequency of homing into the tumor site.

We next examined whether IFN-γ was also required for the therapeutic efficacy observed in mice vaccinated with the multiadjuvanted vaccine. As shown in Fig. 8B, mice lacking IFN-γ and immunized with the multiadjuvanted vaccine were unable to control tumor growth and showed poor survival suggesting a key role for IFN-γ in the therapeutic effect of the multiadjuvanted vaccine.

**FIGURE 5.** Role of CD8+ T cells and NK cells in the therapeutic efficacy of ISCOMATRIX-OVA-Poly I:C-CpG vaccine. Wild-type (WT) mice were injected with 5 × 10^5 B16-OVA tumor cells, s.c., and treated twice with ISCOMATRIX-OVA or ISCOMATRIX-OVA-Poly I:C (5 μg)-CpG-1826 (5 μg) vaccines or saline (n = 10/group). The priming was given when tumors were palpable (day 2), and the boost was administered 7 d later as described in Materials and Methods. Mice were treated with anti-CD8 mAb or isotype control (A) or anti-NK1.1 mAb or isotype control (B) as indicated in Materials and Methods. Tumor size and percent survival were determined. Data from one of two independent experiments are shown. *p < 0.05.

**FIGURE 6.** Expansion of OT-I CD8+ T cells induced by ISCOMATRIX-OVA-Poly I:C-CpG vaccine in mice bearing B16-OVA tumors. CFSE-profile of purified naive OT-I CD8+ T cells (CD45.2) transferred into B6.SJL/J-PTPRCa (CD45.1) mice bearing B16-OVA tumors, followed by vaccination with ISCOMATRIX-OVA vaccine alone or in combination with Poly I:C 5 μg and CpG-1826 5 μg as described in Materials and Methods. (A) Representative FACS plots for CFSE proliferation of various groups. (B) Percentage of OVA-specific CD8+ T cells and IFN-γ+CD8+ T cells in mice bearing B16-OVA tumors, followed by vaccination with ISCOMATRIX-OVA-Poly I:C (5 μg)-CpG-1826 (5 μg) or OVA-Poly I:C (5 μg)-CpG-1826 (5 μg) vaccines (n = 5 mice/group). Data represent mean ± SEM from one of two independent experiments. *p < 0.05.
role for IFN-γ in the mechanism of action of the multiadjuvanted vaccine. Interestingly, the lack of efficacy in IFN-γ−/− mice was not due to a reduced Ag-specific CD8+ T cell response as the magnitude of OVA-specific CD8+ T cell responses were comparable between wild type and IFN-γ−/− mice (data not shown). Similarly, IFN-γ deficiency did not affect the ability of OVA-specific CD8+ T cells to migrate to the tumor site (Supplemental Fig. 4).

The main sources of IFN-γ production in vivo include NK cells and CD8+ or CD4+ T cells. The data indicate that NK cell–derived IFN-γ is not a major contributing source to the therapeutic effect elicited by the multiadjuvanted vaccine, because NK cell depletion did not abrogate its antitumor efficacy. To determine whether CD4+ T cells induced by the multiadjuvanted vaccine represented an important source of IFN-γ in facilitating the antitumor efficacy of the multiadjuvanted vaccine is unlikely mediated by CD4+ T cells.

Discussion

The aim of the current study was to evaluate the efficacy of ISCOMATRIX vaccines in both prophylactic and therapeutic mouse models of cancer. The results indicated that the ISCOMATRIX-OVA vaccine could elicit significant protective antitumor immunity with only two vaccinations when used in a prophylactic setting. However, the same vaccine showed limited efficacy when used to treat established tumors. It is well known that vaccine-mediated killing of tumor cells relies on the generation of tumor Ag-specific CD8+ T cells. The ISCOMATRIX-OVA vaccine certainly induced functional CD8+ memory T cells in wild-type mice as well as mice deficient in CD4+ T cells, demonstrating that CD8+ T cell memory induced by ISCOMATRIX was CD4+ T cell independent. In addition, there is evidence that CD4+ T cells are critical for CD8+ T cell memory differentiation (18), and one way of overcoming the requirement of CD4+ T cells is by coadministration of Ag with pattern recognition receptor ligands, such as TLR agonists (19, 20). Although ISCOMATRIX does not contain TLR ligands, it does induce CD8+ T cell memory differentiation in the absence of CD4+ T cells. This suggests that ISCOMATRIX and TLR agonists may share signaling pathways that are involved in the T helper–independent generation of memory CD8+ T cells.
Alternatively, there may be different pathways that can generate CD8+ T cell differentiation and TLR agonists, and ISCOMATRIX might differentially engage them.

Cancer still remains a major cause of death worldwide, and although significant progress in treatments has been made with drug and Ab therapies, the successful development of cancer vaccines lags behind with only a few prophylactic vaccines approved to protect against pathogen-induced cancers (e.g., human papillomavirus or hepatitis B virus) (1, 2) and one complex, therapeutic, cell-based vaccine for the treatment of hormone refractory prostate cancer (3). It is clear that treating established cancer needs to not only amplify antitumor immune responses but also to overcome suppressive mechanisms that facilitate tumor escape. This likely will require the engagement of multiple immune pathways emphasizing the need for combination therapies to achieve clinically meaningful outcomes.

In this context, it is not surprising that the ISCOMATRIX-OVA vaccine was not very effective at inducing therapeutic immunity to clear established tumors in our hands. To investigate whether a combination ISCOMATRIX vaccine would have improved therapeutic efficacy, we tested TLR ligands in combination with ISCOMATRIX in a therapeutic setting of B16-OVA tumor model. The data in the B16-OVA therapeutic model indicate that the magnitude of CD8+ T cell responses per se does not predict vaccine efficacy. For example, the magnitude of the CD8+ T cell response induced by ISCOMATRIX-OVA-Poly I:C or -CpG vaccines was comparable to that induced by the multiadjuvanted vaccine (ISCOMATRIX-OVA-Poly I:C-CpG), yet only the latter vaccine generated therapeutic regression of established B16-OVA melanoma. This observation suggests that the combination of ISCOMATRIX, Poly I:C, and CpG triggers several activation pathways inducing tumor-specific CD8+ CTL effectors capable of regression of established cancer. Consistently, the multiadjuvanted vaccine induced a high percentage of CD8+ T cells expressing IFN-γ, granzyme B, and markers of degranulation. Interestingly, the incorporation of Poly I:C and CpG to the ISCOMATRIX-OVA vaccine did not enhance the recruitment of cells into the tumor site, suggesting that the quality of the effector function imprinted on the activated CD8+ CTL is the most relevant immunological parameter associated with protective immunity rather than expansion and homing of tumor-specific CTL.

It is interesting that IFN-γ was critical in the efficacy of the multiadjuvanted vaccine. The role of IFN-γ in immunity against cancer is not novel, and many reports have shown that IFN-γ contributes to the control of tumor growth (21–24). There is no clear evidence showing that B16 or Panc tumor cells are directly killed by IFN-γ, so it is more likely that IFN-γ activates immune cells and/or enhances the expression of MHC class I molecules on the tumor cells making them more susceptible to recognition by tumor-specific CTL-mediated killing. Cell-specific depletion experiments suggest that the source of IFN-γ resides in non-NK cell and non-CD4+ T cell compartments.

It has been reported that the inoculation of Poly I:C and CpG intratumorally improves vaccine efficacy by reducing B16F10 tumor-driven immunosuppressive effects (25) In our case, the same TLR agonists coadministered with ISCOMATRIX bypassed the need for intratumoral injections without compromising vaccine efficacy. Our results also highlight that a multiadjuvanted ISCOMATRIX vaccine will therefore be more suitable in cases where intratumoral inoculations are not feasible such as in visceral metastatic disease. In addition, McCluskie et al. (26) has reported that the combination of ISCOMATRIX-OVA-CpG reduced the number of lung metastases in a mouse model of metastatic B16-OVA. This vaccine performed better than the ISCOMATRIX-OVA vaccine supporting the concept that multiadjuvanted vaccines may be required for therapeutic vaccination against cancer (26).

![FIGURE 8. Role of CD4+ T cells and IFN-γ in the therapeutic efficacy of the multiadjuvanted vaccine. Wild-type (WT) mice (A) or IFN-γ−/− mice (B) or GK1.5 Tg (CD4-deficient) mice (C) were injected with 5 × 10^5 B16-OVA tumor cells (s.c.) and vaccinated with ISCOMATRIX-OVA (n = 10 mice/group) or ISCOMATRIX-OVA-Poly I:C (5 μg)-CpG-1826 (5 μg) (n = 10 mice/group). Mice injected with PBS (n = 10 mice/group) were used as untreated controls. The priming was given when tumors were palpable (day 2), and the boost was administered 7 d later as described in Materials and Methods. Percentage of survival and tumor size were determined. Data from one of two experiments are shown. KO, knock-out.](http://www.jimmunol.org/cgi/content/figure/178/6/3598/fig8)
Finally, it is important to highlight that mice bearing tumors and vaccinated with OVA-Poly I:C-CpG without ISCOMATRIX generated a lower frequency of tumor-specific CD8+ T cell responses as compared with the multiadjuvanted vaccine. This is in contrast to a recent report by Perret and colleagues which found either CpG or Poly I:C to be best suited for use in peptide-based therapeutic cancer vaccines. Indeed they conclude that this combination promoted both CD4+ and CD8+ effector responses above that of Ag-specific T regulatory cells as compared with Quil A saponin or the TLR7/8 agonist, imiquimod (27). It should be noted that these studies were performed with naked saponin (as compared with the cage-like structure of ISCOMATRIX) and defined peptides (rather than whole protein) where the various advantages of Ag delivery and trafficking to APCs will not be represented. Furthermore, they did not compare the combination of saponin with Poly I:C and CpG to see whether this generated an even more robust antitumor response and what further impact this may have had on the T effector:T regulatory cell ratio. In the current study, the addition of ISCOMATRIX to OVA-Poly I:C-CpG vaccine dramatically enhanced the magnitude of the CD8+ T cell responses demonstrating that synergistic effects among ISCOMATRIX, Poly I:C, and CpG are key for the immunogenicity and antitumor efficacy of this multiadjuvanted vaccine.

In conclusion, our data demonstrate that ISCOMATRIX vaccine formulations can be developed for the prophylactic and therapeutic cancer settings but likely will differ in their complexity. Whereas single ISCOMATRIX-adjuvanted formulations provide prophylactic protection against emergence of solid tumors, therapeutic ISCOMATRIX vaccines will likely need to be combined with additional immune stimulators such as the TLR agonists, Poly I:C and CpG, to achieve significant efficacy. A vaccine comprising tumor Ags, ISCOMATRIX, and the TLR agonists Poly I:C and CpG represents a promising therapeutic approach in the treatment of cancer.

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Disclosures
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