Paired Toll-like Receptor Agonists Enhance Vaccine Therapy through Induction of Interleukin-12

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Abstract

Minimal requirements for generating effective immunity include the delivery of antigenic (signal 1) and costimulatory (signal 2) signals to T lymphocytes. Recently, a class of third signals, often delivered by antigen-presenting dendritic cells, has been shown to greatly enhance immune responses, especially against tumors. Among signal 3 factors, interleukin (IL)-12 is particularly effective and can be conditionally induced by agonists of Toll-like transmembrane receptors (TLR). In this study, we assessed the therapeutic effect of adjuvant TLR agonist administration upon the capacity of dendritic cell (DC)-tumor electrofusion hybrids to eradicate established MCA205 sarcomas in syngeneic mice. Paired, but not solitary combinations of polyinosine:polycytadilic acid (P[I:C]; TLR3 agonist) and CpG DNA (ODN1826l; TLR9 agonist) stimulated IL-12 secretion from DCs *in vitro* and synergized with vaccination to achieve potent tumor rejection. Therapeutic effects, however, required coadministration of paired TLR agonists and DC-tumor fusion hybrids. The administration of TLR agonists alone or with fusion vaccine induced transient splenomegaly but without apparent toxicity. The therapeutic effects of this immunization regimen were significantly abrogated through the neutralization of IL-12p70, indicating that production of this third signal was essential to the observed tumor regression. These results show the profound functional consequences of TLR cooperativity and further highlight the critical role of IL-12 in antitumor immunity. [Cancer Res 2008;68(11):4045–9]

Introduction

Toll-like receptors (TLR) allow for the discrimination of self tissues from infectious nonself through molecular pattern recognition (1). It is this faculty of the innate immune system that sets in motion the adaptive arm of immunity and ensures appropriate and vigorous responses against microbial infection. Vaccination against tumor, on the other hand, seeks to generate immunity against self-derived tissues that usually do not express classic pathogen-associated molecular patterns. Therefore, combining TLR agonists with anticancer vaccines can potentially induce the immune system to respond against tumor antigens with a quality and intensity usually reserved for microbial infection.

Recent studies have shown that stimulating dendritic cells (DC) with select pairs of TLR agonists (e.g., ones that coordinately stimulate the My-D88- and Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling pathways) greatly enhances the synthesis of so-called DC1 polarization factors, including Delta Notch ligand, interleukin (IL)-23, and IL-12 p70 (2). IL-12 in particular has been shown to operate as a "third signal", which in addition to antigen (signal 1) and costimulation (signal 2), greatly enhances aspects of T-dependent immune responses (3–7), which may enhance antitumor immunity. Mechanisms by which IL-12 achieve this enhancement include Th1-biasing (8), augmentation of CTL avidity for tumor targets (7), and a BCL-3–mediated antiapoptotic effect, which preserves high viability during Ag-driven T-cell proliferation (6).

We therefore assessed the effect of parenteral TLR agonists as adjuvants for an extensively characterized DC-tumor electrofusion hybrid vaccine modality (9). Here, electrical fields are used to fuse and hybridize DCs with tumor cells. The resulting heterokaryons retain the superior antigen-presenting capacity of the DC and acquire the entire antigenic complement of the tumor partner, creating an immunogen rich in signals 1 and 2. Although this vaccine modality is exceptional for its capacity to bypass the constraints of exogenous processing to present endogenous tumor proteins in both MHC Class I– and II–restricted, and highly costimulatory contexts, vaccination has consistently benefited from parenteral codelivery of third signals for maximized therapeutic efficacy. Among tested parenteral third signals, IL-12 and OX40 ligating monoclonal antibody (mAb) have proved particularly effective (10, 11). Here, we show that fusion hybrid vaccination plus a single TLR agonist induces no detectable therapeutic antitumor immunity, whereas vaccination plus paired TLR agonists show powerful therapeutic responses against established lung metastases derived from the MCA205 sarcoma. This therapeutic effect is apparently mediated through a mechanism requiring host production of IL-12. This is the first demonstration, to our knowledge, that dual-administered TLR agonists can augment DC-based vaccination equivalently to recombinant signal 3 factors, leading to greatly enhanced antitumor immunity in a generally nontoxic manner.

Materials and Methods

Mice and tumors. Female C57BL/6N (B6) mice were purchased from the Biologic Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute. They were maintained in a specific pathogen-free environment and used for experiments at age 8 to 10 wk. All experiments using mice were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Lerner Research Institute and cared for according to institutional guidelines.

The 3-methylcholanthrene–induced MCA205 fibrosarcoma (12), syngeneic to B6 mice, was maintained *in vivo* via serial s.c. transplantation in

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doi:10.1158/0008-5472.CAN-07-6669

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syngeneic mice and was used for experiments within the 10th transplant generation. Single-cell suspensions were prepared from excised solid tumors via enzymatic digestion as described previously (13).

DC production. Bone marrow DCs were prepared as previously described (14) by a modification of Inaba and colleagues (15). Briefly, cells harvested from femora and tibiae were depleted of B and T cells by negative selection with mAb-coated magnetic beads (Dynal Biotech). These cells (0.5×10^6/mL) were cultured in flasks in complete medium supplemented with 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL IL-4 (Peprotech, Inc.). Complete medium consisted of RPMI 1640 with 10% heat-inactivated FCS, L-glutamine, and antibiotics as previously described (18). On day 6, nonadherent cells were harvested and further cultured (1.0×10^6/mL) with fresh medium for an additional 2 d.

Electrofusion. Procedures for electrofusion have been described previously (16–18). Briefly, tumor cells were irradiated (5,000 cGy) and labeled with the green fluorescent dye, carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes), before fusion. DCs and tumor cells at 1:1 ratio were then mixed and suspended in fusion medium. Fusion medium was composed of 5% glucose containing 0.1 mmol/L Ca (CH_3COO_2), 0.5 mmol/L Mg CH_3COO_2, and 0.3% bovine serum albumin (BSA). The pH of the fusion medium was adjusted to 7.2 to 7.4 with L-histidine. After brief centrifugation, the cells were resuspended in the same fusion medium without BSA at a concentration of 15×10^6 cells/mL. Electrofusion was carried out using a custom-made concentric chamber, connected to the ECM 2001 pulse generator (BTX Instrument; Genetronics). Fusion was accomplished by dielectropheresis with an alternating current pulse of 220 V/cm for 10 s immediately followed by a direct current pulse of 1,230 V/cm for 99 microseconds. The cell suspensions were then resuspended in conditioned medium and incubated overnight in a 37°C, 5% CO_2 incubator. The adherent cell fraction containing fusion hybrids was harvested and analyzed for fusion rates by staining with phycoerythrin (PE)-labeled mAbs against characteristic DC markers such as CD80, CD86, and I-A. Double-positive cells represented heterokaryons of DCs and MCA205 tumor cells.

Active immunotherapy. Pulmonary metastases were induced by i.v. inoculations of mice with 3×10^5 tumor cells suspended in 1.0 mL HBSS. Three days later, mice were vaccinated by the intranodal route as previously described (14). Routinely, 3 × 105 fusion cells in 10 μL HBSS were delivered to each of 2 superficial inguinal lymph nodes. Some mice were additionally supplied i.p. with 100 μg P[I:C], or 50 μg ODN-1826, or a combination of both in 0.5 mL HBSS on the day of vaccination and again 3 and 7 d later. For comparison, vaccinated mice were given either IL-12 (a gift from Wyeth, Cambridge, MA), 0.2 μg in 0.5 mL HBSS, i.p. for 4 consecutive d to provide a third signal or adjuvant. Some of the vaccinated mice were also treated with the neutralizing anti–IL-12 p70 mAb (R2-9A5) to block the *in vivo* activity of this cytokine. R2-9A5 was administered i.p. (0.45 mg) for 6 consecutive d starting the first day of vaccination with fusion hybrids and adjuvant.All mice were sacrificed on days 21 to 23, and metastatic nodules on the surface of the lung were enumerated after counterstain with India ink as previously described (16, 18).

Surface-staining fluorescence-activated cell sorting analysis. For direct immunofluorescence, PE-conjugated mAbs including CD11c, CD80, CD86, CD40, H-2K^b, I-A^b, intercellular adhesion molecule 1 (CD54), and OX-40L mAbs (BD PharMingen) were used for analyses of fusion products. At least 10,000 cells from each sample were analyzed using the FACS Calibur (BD).

Cytokine analysis. Culture supernatants from 24-h-stimulated DC cultures were subjected to ELISA analysis for IL-12 p70, via OPT-EIA kits (BD/PharMingen) according to manufacturer's recommended protocol. For intracellular fluorescence-activated cell sorting (FACS), lymph node cells harvested 7 d after vaccination were polyclonally expanded with anti-CD3 mAb and IL-2 as described previously (11). Cells were then harvested and cocultured with tumor targets at a 2:1 ratio in the presence of brefeldin A. After 24 h, cells were harvested, stained with anti-CD4 or anti-CD8, permeabilized and stained for intracellular IFN-γ (all stains from BD), and subjected to FACS analysis.

Results and Discussion

This laboratory has pioneered a unique tumor vaccination strategy wherein electric fields are used to fuse DC to tumor cells.

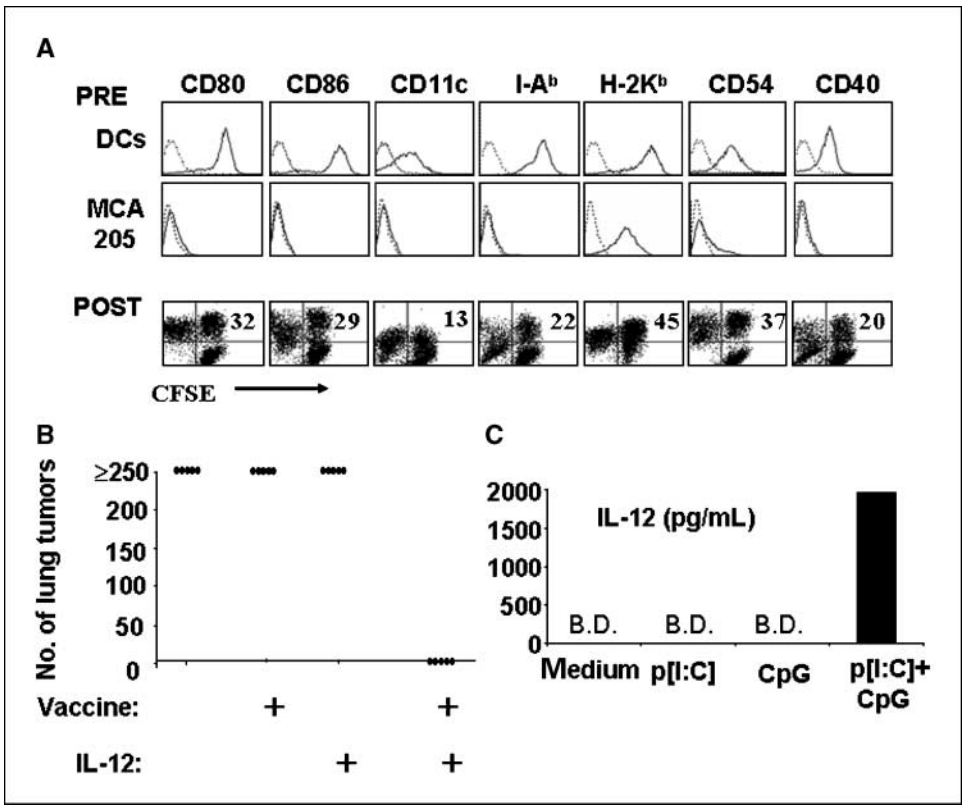

Figure 1. Electrofusion efficiently hybridizes MCA205 tumor cells and DCs to form antitumor vaccines that work in conjunction with IL-12 to suppress outgrowth of lung tumors. *A*, surface phenotype as determined by FACS of murine bone marrow–derived DCs and MCA205 tumor cells before fusion (*PRE*) and after fusion (*POST*). Tumor cells were labeled with CFSE before fusion. After 24 h, adherent cells were stained with PE-conjugated mAbs as indicated. Numbers in top right quadrants denote percentages of double-positive fusion hybrids. *B*, mice bearing 3-d MCA205 lung metastases were immunized intranodally with fusion hybrids or left unvaccinated. Some mice then received recombinant IL-12 adjuvant (0.2 μg/d; >4 consecutive d). On day 21 to 23, mice were sacrificed and lung surface metastases were enumerated. *C*, murine bone marrow–derived DCs were cultured in medium with GM-CSF and IL-4 and then treated with combinations of P[I:C] (50 μg/mL) or ODN1826 (1 μmol/L) or both. Culture supernatants were removed 24 h later and assayed for IL-12 p70 by ELISA. *BD,* below detection limit of 62 pg/mL.

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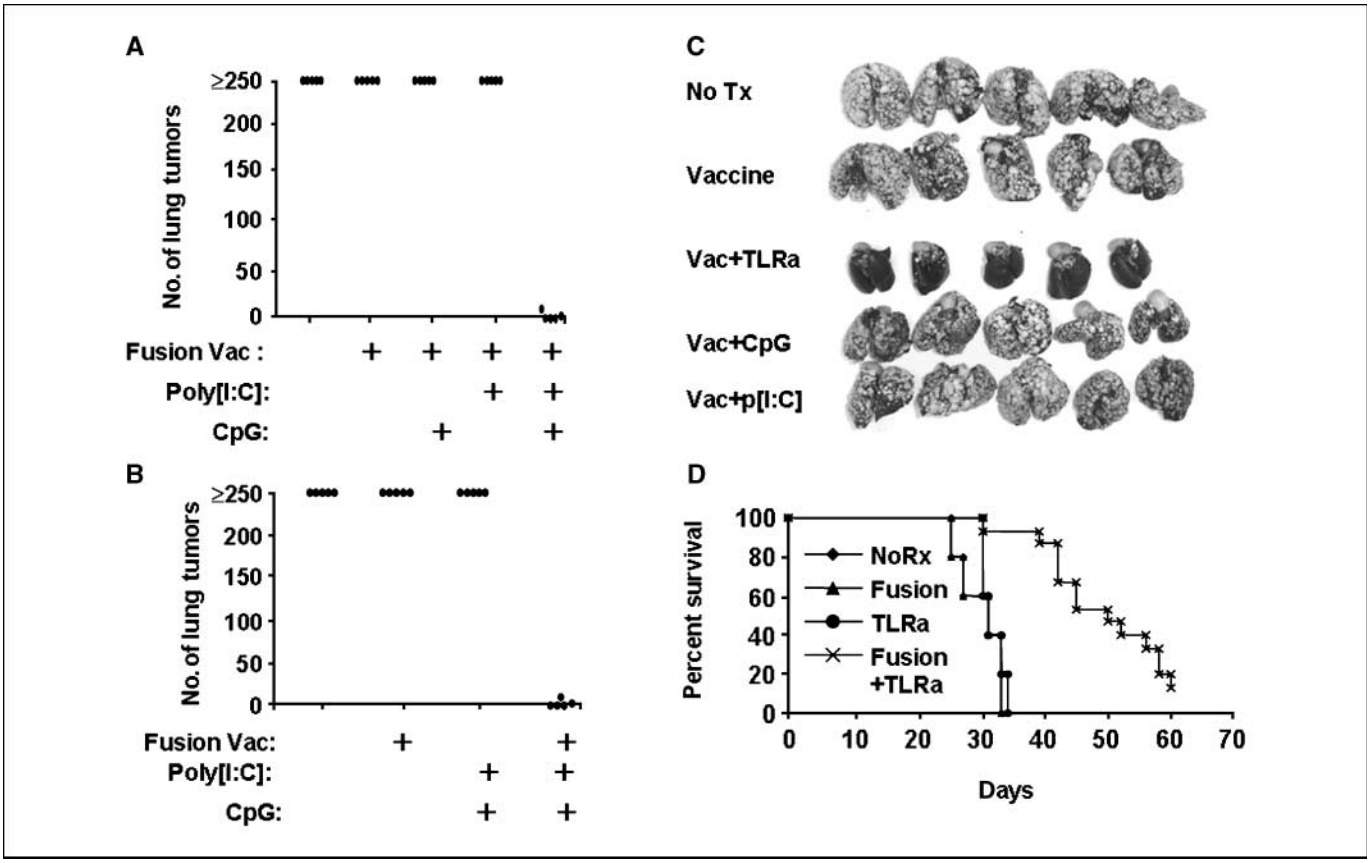

Figure 2. Therapeutic antitumor effect requires both vaccination and paired TLR agonist adjuvants. Mice were injected via the tail vein with MCA205 tumor cells to establish 3-d lung metastases. Some groups of mice were then immunized intranodally with DC-tumor fusion vaccines (*fusion vac*). On the day of vaccination, and days 3 and 7 postvaccination, combinations of P[I:C] (100 μg per mouse) and ODN 1826 (50 μg per mouse) were administered i.p. to both vaccinated and unvaccinated tumor-bearing mice. On days 21 to 23, mice were either sacrificed and lung surface metastatic nodules enumerated, or allowed to live until moribund or dead. *A*, vaccination plus dual, but not single TLR agonists, suppress lung metastases. *B*, paired TLR agonists must be used in conjunction with vaccination for therapeutic effect. *C*, photographic evidence from India ink–counterstained lungs that paired TLR agonist adjuvants (TLRa) work in conjunction with vaccination to suppress lung tumors while vaccination plus solitary agonist (CpG or P[I:C]) do not. *D*, treated mice were then observed over a 60-d period with mortality as an end point. *A* to *C,* representative of three separate experiments.

(Fig. 1*A, top*). These DC-tumor hybrids inherit the antigen-presenting phenotype of the DC (as evidenced by the continued expression of I-A and CD80 in the fused cells, which unfused tumor cells lack) and the full antigenic complement of the tumor cell. These DC-tumor fusion vaccines have been shown to be much more effective than DCs plus either tumor lysates or irradiated tumor (14). The very high fusion efficiency of this process was evidenced when tumor cells were labeled with the fluorescent dye, CFSE, before fusion with DC (Fig. 1*A, bottom*). The population of cells in the double-positive quadrant (up to 45%) of the two-dimensional dot-plots represented the fusion hybrids that possess the characteristic surface staining of the DC and the CFSE staining of the tumor fusion partner.

Mice were immunized by direct injection of fusion vaccine into the superficial inguinal lymph nodes (14) to circumvent the need for lymph node trafficking. It was found that for vaccines to be effective against established tumors, an adjuvant was required, such as exogeneously-supplied recombinant IL-12 p70 (Fig. 1*B*) or agonistic OX-40L mAb (11). This property of adjuvant-dependency prompted us to examine the potential of alternative adjuvants for improving antitumor responses. Recently, TLRs have been identified as primary sensors for molecular patterns that distinguish self tissues from infectious nonself (19). We therefore sought to determine whether TLR agonist stimulation would serve an adjuvant function for DC-tumor fusion vaccination. Based on preliminary profiles of TLR expression in mouse-derived conventional DCs (data not shown), we selected two TLR agonists, the double-stranded RNA analogue, P[I:C] (TLR 3 agonist; TRIF pathway) and the synthetic CpG DNA analogue ODN1826 (TLR9 agonist; MyD88 pathway). These TLR agonists were first used to stimulate cultured murine DCs *in vitro* to confirm synergistic induction of IL-12 p70 (Fig. 1*C*). Supernatants from 24-hour-stimulated cultures showed either low or no detectable IL-12 p70 in untreated DC, and in DC treated with single TLR agonists. On the other hand, combined treatment with P[I:C] and ODN1826 resulted in nanogram levels of IL-12 (Fig. 1*C*).

We next evaluated this combination of TLR agonists as an adjuvant in conjunction with DC-tumor fusion hybrid vaccination in the murine MCA205 sarcoma model. Here, mice with 3-day established pulmonary metastases were vaccinated intranodally with fusion vaccine (day 0) and then supplied by the i.p. route with P[I:C] (100 μg per mouse), or ODN1826 (50 μg per mouse), or a combination of both on days 0, 3, and 7. On day 21, mice were sacrificed and lung tumors were enumerated. It was found, as historically shown, that mice receiving no treatment displayed heavy tumor burdens, with >250 metastatic nodules on the surface of the lung (Fig. 2*A*). Likewise, immunization with fusion vaccine alone, or fusion vaccine plus single TLR agonist, had little or no

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significant effect on the outgrowth of tumor compared with untreated groups. However, mice receiving the fusion vaccine plus both TLR agonists proved to be either completely or mostly free of tumor (Fig. 2*A*). Further experiments revealed that dual TLR agonist treatment alone did not effect tumor outgrowth, but instead had to be combined with vaccination, indicating that the agonists were serving an adjuvant function (Fig. 2*B*). Results from a similar experiment are shown photographically in Fig. 2*C*, with surface tumors visible as white nodules on a black counterstained background.

We next sought to determine whether a single course of fusion vaccination plus dual TLR agonist treatment provided survival benefit (Fig. 2*D*). It was found that mice receiving either no treatment, vaccination alone, or paired TLR agonist alone all succumb to growing metastases with a median survival time of around 30 days. However, mice receiving both vaccination plus paired TLR agonists displayed statistically prolonged survival, with median survival time extended to ~45 days.

We then investigated mechanisms by which dual TLR agonist adjuvant enhanced vaccine therapeutic efficacy. Our original hypothesis was that induced secretion of IL-12 would be critical in this regard. We therefore designed experiments that would both directly test the necessity of IL-12 for tumor control as well as detect quantifiable changes in T-cell activity consistent with the known effects of IL-12. To achieve this, we first prepared lymph node cells from tumor-bearing mice that had received either no treatment, paired TLR adjuvant only, fusion vaccine only, or combined vaccine plus adjuvant. These cells were polyclonally expanded *in vitro* and then cocultured with either MCA205 tumors or, as a specificity control, MCA207 tumor cells, with IFN-γ production by $CD4^+$ or $CD8^+$ cells measured 24 hours later via intracellular FACS (Fig. 3*A*). We observed little evidence of IFN-γ–secreting $CD4^+$ or $CD8^+$ cells in untreated or adjuvant-only groups. However, specific IFN-γ–secreting cells were seen from mice receiving fusion vaccine, with numbers and specificity increasing when dual TLR agonist adjuvant was combined with vaccination. This is an expected finding if IL-12 is influencing vaccine efficacy because IL-12 enhances IFN-γ secretion by polarizing T cells toward the TH1 phenotype (8).

Finally, we more directly tested the role of IL-12 through *in vivo* neutralization. To accomplish this, either IL-12–neutralizing or normal rat IgG was given i.p. to fusion vaccine–treated, tumor-bearing mice that received either recombinant IL-12 or paired TLR agonists (two rather than the optimal three doses) as adjuvant. When mice were sacrificed and lung metastases were enumerated, it was apparent that IL-12–neutralizing but not control antibody inhibited the adjuvant properties of paired TLR agonists (Fig. 3*B*). This inhibition was quite strong, but nonetheless incomplete, suggesting either that induction of other factors, possibly Delta Notch Ligand or IL-23 (known to be up-regulated by paired TLR agonists; ref. 2) contribute to the adjuvant effect, or that IL-12 exerts some of its effects via close cell-cell interactions (such as across an immunosynapse between a DC and T cell) and, therefore, cannot be readily neutralized to completion. Nonetheless, these experiments show that IL-12 plays significant role in the adjuvant properties of paired TLR agonists for therapeutic vaccination against tumors.

Agonists for TLRs have found use as adjuvants or immune response modifiers in a variety of settings, both experimental and clinical for infections and malignancy, but relatively limited adjuvant effects have been observed with single TLR agonists. To

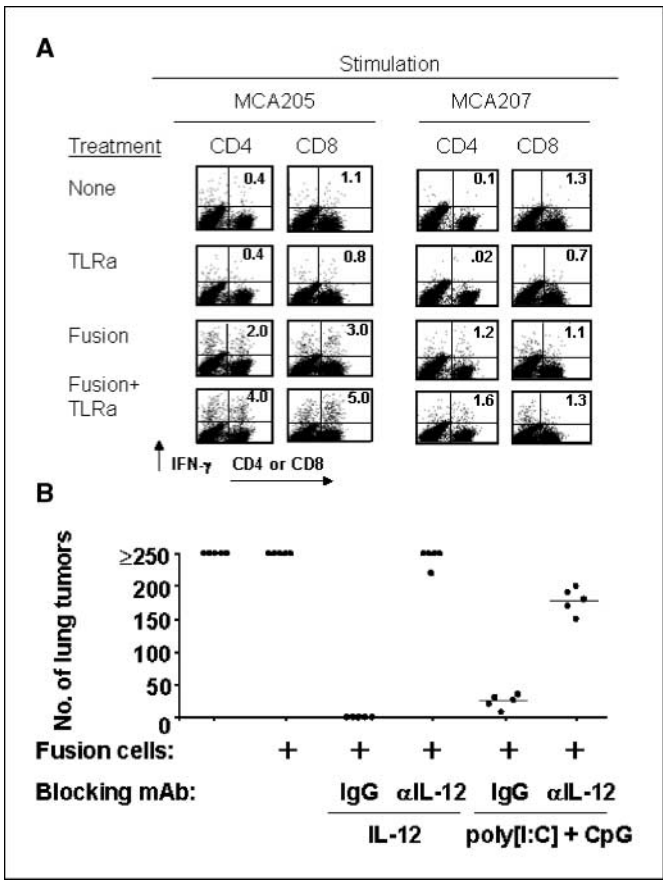

Figure 3. Vaccination plus paired TLR agonists enhance tumor-specific IFN-γ–secreting T cells and promote therapeutic effect through a mechanism mediated by IL-12. *A*, mice with 3-d MCA205 lung metastases were immunized or not with DC-tumor fusion vaccines. Some mice then received paired TLR agonist adjuvant (P[I:C], 100 μg/mL; ODN1826, 50 μg/mL) as described previously. On day 7, mice were sacrificed and lymph node cells were cultured for 5 d in the presence of anti-CD3 mAb (day 1) and 4 IU/mL IL-2 (day 3). Cells were then harvested and cocultured at a 2:1 ratio with MCA 205 or MCA207 targets and IFN-γ production by $CD4^+$ and $CD8^+$ cells determined by intracellular FACS. *B*, mice bearing 3-d MCA205 pulmonary metastases were likewise immunized intranodally with fusion vaccine. Some groups received either 0.45 mg IL-12–neutralizing mAb (R2-9A5) or a rat IgG control for 6 consecutive d. Select groups also received adjuvants consisting of either recombinant murine IL-12 (0.2 μg/d; >4 consecutive d), or a suboptimal dose schedule of P[I:C] and ODN1826 (100 and 50 μg per mouse, respectively on days 0 and 3). Mice were sacrificed on days 21 to 23, and surface lung metastases were enumerated.

our knowledge, this is the first example of *in vivo*, low toxicity synergy between paired TLR agonists (that collectively signal through both MyD88 and TRIF pathways) enhancing DC-based vaccines through apparent *in vivo* induction of IL-12.

For its part, IL-12 has steadily gained prominence as a major factor in controlling malignancies. We have shown that appropriately stimulated DCs can achieve two surges of IL12p70 production *ex vivo*, but strategies are required to ensure that the timing of surges is optimal to maximize T-cell sensitization. Towards this end, we have explored the feasibility of treating extracorporealized monocyte-derived DCs with the combination of lipopolysaccharide and IFN-γ just before their intranodal administration to patients. We recently used this strategy to treat early breast cancer (20). Intranodal vaccination with HER-2/neu peptide antigen-pulsed DCs that secreted large amounts of IL-12 elicited powerful immunity as well as apparent reduction of

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HER-2/neu expression in tumors from over half the vaccinated subjects (20).

Remarkably, however, the present murine studies show that DC1-polarization, or at least therapeutically significant host IL12p70 production, can also be safely triggered by parenteral administration of appropriately paired TLR agonists, potentially reducing the need to expose DCs to these stimuli before their administration. We observed no detectable toxicities at the studied TLR agonist doses (save for transient spleenic enlargement), despite the high therapeutic activity. The demonstration of TLR agonist synergy *in vivo* for a DC-based tumor vaccination regimen may have profound implications for the formulation and use of small synthetic TLR ligands as adjuvants or immune response modifiers. These studies supply further evidence that IL-12 is a critical factor for tumor control and argue that methods to elicit powerful third signals such as IL-12 should be a high priority for future cancer vaccination strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 12/19/2007; revised 2/20/2008; accepted 4/4/2008.

Grant support: NIH grant CA100163 (G.K. Koski), CA089511 (P.A. Cohen), CA103946 (S. Shu), and CA129815 (P.A. Cohen).

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Cancer Res 2008;68:4045-4049.

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