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*J Immunol* 2011; 186:5927-5937; Prepublished online 1 April 2011; doi: 10.4049/jimmunol.1003351

http://www.jimmunol.org/content/186/10/5927

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http://www.jimmunol.org/content/suppl/2011/04/01/jimmunol.1003351.DC1

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Prolonged Antitumor NK Cell Reactivity Elicited by CXCL10-Expressing Dendritic Cells Licensed by CD40L+ CD4+ Memory T Cells

Kanako Shimizu,*† Miki Asakura,* and Shin-ichiro Fujii*

Immunotherapy using dendritic cells (DCs) has the potential to activate both T cells and NK cells. We previously demonstrated the long-lasting antitumor responses by NK cells following immunization with bone marrow-derived DCs. In the current study, we demonstrate that long-term antitumor NK responses require endogenous DCs and a subset of effector memory CD4+ T (CD4+ TEM) cells. One month after DC immunization, injection of a tumor into DC-immunized mice leads to an increase in the expression of CXCL10 by endogenous DCs, thus directing NK cells into the white pulp where the endogenous DCs bridged CD4+ TEM cells and NK cells. In this interaction, CD4+ TEM cells express CD40L, which matures the endogenous DCs, and produce cytokines, such as IL-2, which activates NK cells. These findings suggest that DC vaccination can sustain long-term innate NK cell immunity but requires the participation of the adaptive immune system. The Journal of Immunology, 2011, 186: 5927–5937.

Cancer vaccines are being explored to treat patients with cancer and prevent cancer recurrence in patients in remission. Ideally, vaccine-induced protection should include an immune memory component. In this regard, CD8+ T cells of the adaptive immune system have been intensively studied in various infectious models (1–3). When memory T cells reencounter cognate Ag-expressing target cells, they respond with a robust secondary expansion, resulting in protective immunity. Recent evidence has uncovered a form of NK cell memory that is distinct from adaptive T cell immunity.

Innate lymphocytes such as NK and NKT cells are important sentinels of the immune system and alert the host to the presence of infectious organisms and malignantly transformed cells (4–8). Until recently, NK cell responses were thought to be short-lived and to lack memory. However, two recent studies have demonstrated Ag-specific prolonged responses by the NK cells of the innate immune system (9, 10). The first study used a contact hypersensitivity model (9). Adoptive transfer of liver Ly49C-I+ NK cells from hapten-sensitized mice resulted in a long-lived, hapten-specific NK cell-mediated recall response in recipient mice, lasting up to 4 wk. A second study evaluated memory NK cells that expressed higher levels of Ly49H (10). In the early response of mice to murine CMV, a murine CMV viral Ag, m157, binds to an activating receptor, Ly49H, on NK cells. These NK cells were shown to undergo a proliferation and expansion phase, followed by a contraction phase, and then, finally a maintenance phase, leading to long-term recognition of the infected cells. In addition, Cooper et al. (11) showed that IL-12–, IL-15–, and IL-18–induced NK cells were memory like and exhibited robust responses upon restimulation with cytokines (IL-12 plus IL-15) or via engagement of activating NK cell receptor Abs (anti-Ly49H or anti-NK1.1 mAbs). These three studies were conducted using Rag1−/− mice, and therefore, the memory NK cell response was independent of helper CD4+ T cells.

To demonstrate efficient cytotoxicity against tumors or virally infected cells, NK cells must first be activated. Dendritic cells (DCs) rapidly stimulate NK cells through the elaboration of cytokines, such as IL-2, IL-12, IL-15, IL-18, and type I IFN (12, 13). In addition to this short-term effect of DCs on NK cell activity, we and others have reported that DC vaccination generates a long-term NK cell-based resistance against tumor cells in an Ag-independent manner. This effect has been shown using a variety of tumor cell lines, such as B16 melanoma, Colo26, EL4, YAC-1, and J558 plasmacytoma (14–19). As we previously demonstrated, NK cells from DC-immunized mice also rapidly produce IFN-γ upon challenge with tumor cells (18). This activated NK cell response is different from the initial response of primary “resting” NK cells and can last for at least 3 mo (18). Interestingly, several months after NK cell depletion by anti-asialo-GM1 mAb, we were able to detect antitumor NK cell reactivation, indicating that this NK response is being continually sustained and not dependent on long-lived memory NK cells.

In this study, we will describe how NK cells can be sustained in an antitumor state following DC vaccination. CD4+ Th cells are known to improve CD8+ T cell effector function and memory (20–23). CD4+ T cell help can occur in both an Ag-dependent (24) and -independent manner (20). We as well as other groups found that...
long-term NK cell-mediated antitumor response induced by DC therapy depends on CD4+ T cells but not on specific Ags (14–16, 18, 19). Thus, the prolonged NK cell activity was lost in Rag1−/− or CD4−/− mice but not Ndx18−/− mice (18). In this study, we describe how CD4+ T cells maintain NK cells in an activated state, able to rapidly eradicate tumor cells after DC immunization. Such findings will help develop novel cancer therapies.

Materials and Methods

Mice and cell lines

Pathogen-free 6- to 8 wk-old C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan), and NOG (NOD/Shi-scid, IL-2Rγ−/− (NOG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and NOD/Shi-scid, IL-2Rγ−/− (NOG) mice were obtained from Central Institute for Experimental Animals (Kawasaki, Japan). B6 Rag1−/− mice were provided from The Institute of Physical and Chemical Research (RIKEN) Central Facility (Yokohama, Japan). IFNγ/γR−/− and MyD88−/− mice were provided by Dr. T. Kaisho (RIKEN), and CD11c-diphtheria toxin receptor (DTR)-GFP mice were provided by Dr. D. Litman (New York University, New York, NY). All the mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines. B16 melanoma lines and YAC-1 were purchased from the American Type Culture Collection (Manassas, VA) and the RIKEN Cell Bank, respectively. MC38 was provided by Dr. M.T. Lotze (University of Pittsburgh, Pittsburgh, PA).

Reagents

The following mAbs were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA): anti-mouse CD11c (1B1), -CD3 (145-2C11), -CD4 (GK1.5), -CD11c (HL3)-CD25 (PC61), -CD28 (37.51), -CD40L (Mr1), -CD44 (IM7), -CD62L (MEL14), -CD69 (H1.2.F3), -CD80 (16-10A1), -CD86 (GL1), -CD107a (1H4B3A), -CD11b (GR20), -B7-DC (T2S5), -IAb (Af6-120.1), -NK1.1 (PK136), -DNAM-1 (10E5) -MHC class I (28-14-8, 3-J1-2S), –I-A/E (MS/114.15.2), –IFN-γ (XM12.2), –IL-2 (JES6-6H4), –IL-12 (C15.6), and –TNF-α (MP6-XT22). For analysis, a FACSCalibur or FACSCan II instrument and CellQuest or FACS Diva (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software were used. For depletion or blocking in vivo, anti-NK1.1 Ab was prepared in our laboratory from a hybridoma (PK136; American Type Culture Collection). Anti-asialo-GM1 and anti-CXCL10 Abs were purchased from Wako Pure Chemistry Industries (Osaka, Japan) and R&D Systems (Minneapolis, MN), respectively.

Cell preparation

Bone marrow-derived DCs (BMDCs) were generated in the presence of GM-CSF and matured by LPS as described previously (25, 26). In some experiments, DCs were generated in mouse serum instead of FCS as reported previously (26). Mononuclear cells from the spleen, lung, and liver were isolated as described previously (27). In brief, splenocytes were obtained by pressing the spleen through a 70-μm cell strainer and erythrocytes were lysed with ACK lysis buffer (Invitrogen; Carlsbad, CA) followed by two washes in RPMI 1640 medium. For analysis of DCs in the spleen, lung, or liver, organs were digested with collagenase D (Roche, Penzberg, Germany). Lung and liver cells were floated on Percoll gradients (40/60%) (GE Healthcare, Uppsala, Sweden) and centrifuged for 20 min at 900 × g to float the mononuclear cells. In some experiments, splenic DCs were isolated using CD11c-magnetic beads (Miltenyi Biotec, Auburn, CA).

In vivo vaccination

Mice were immunized with 1 × 106 BMDCs i.v. at least 3 wk before immunological analysis and 4 wk before the tumor challenge. In some experiments, mice were treated with 100 μg polynucleosin:polycytidylic acid [poly(I:C)] (Invitrogen) and 25 μg LPS (Sigma-Aldrich, St. Louis, MO), 30 μg CpG-oligodeoxynucleotides (ODNs) (Hokkaido System Science, Hokkaido, Japan), or 1 × 106 BMDCs s.c.

Methylcholanthrene-induced sarcoma

Groups of 10–30 wild-type (WT) or Rag1−/− or C57BL/6 female mice were inoculated s.c. in the right hind flank with 0.1 ml peanut oil (Sigma-Aldrich) containing 100 μg methylcholanthrene (MCA) (Sigma-Aldrich) and monitored weekly for the development of fibrosarcomas. Tumors > 5 mm in diameter and demonstrating progressive growth over 3 wk were counted as positive. Three MCA-induced sarcomas (MCA1, MCA2, and MCA3) in Rag1−/− rats expressed a degree of 0.5 cm3 were excised aseptically. Tumors were cut into small pieces and treated with collagenase D at 37˚C for 1 h. Clumps were removed, and single cells were cultured in RPMI 1640 medium with 10% FCS. All tumor cell lines were kept in culture for at least 1 mo to minimize cellular contamination. One million sarcoma cells were injected s.c. into NOG, Rag1−/−, WT, or DC-immunized mice, where indicated. Tumor growth was monitored by measuring three perpendicular diameters. Tumor volume was calculated according to the formula V = L × W2 × 0.52, where V is the volume, L is the length, and W is the width.

Cytokine production

Spleen cells from naive WT or DC-immunized mice were cultured at 1 × 106/well in 96-well flat bottom wells plate for 48 h. The supernatants were collected, and IFN-γ production was measured by ELISA kit (R&D Systems, Minneapolis, MN). A total of 1 × 106 CD4+ T subsets (1 × 105), splenic DCs (1 × 106), allogeneic or syngeneic DCs (1 × 106) were cultured with or without BMDCs and then preincubated with anti-CD16/32 Ab to block nonspecific binding of Abs to FcγR, washed, and incubated with mAbs to surface markers. Cells were then permeabilized in Cytofix-Cytoperm Plus (BD Biosciences) and stained with anti-IFN-γ, –IL-2, –IL-12, –IFN-γ, –IL-12, –TNF-α, or –CD40L mAbs.

Syngeneic and allogeneic MLR

CD4+ T cells were isolated from the spleens of naive or DC-immunized mice using MACS Beads (Miltenyi Biotec) and FACSVantage (BD Biosciences). Splenic DCs were isolated using CD11c-MACS beads from collagenase-treated spleens of naive B6 or BALB/c mice. A total of 1 × 106 CD4+ T cells from naive or immunized mice were cocultured with graded doses of syngeneic DCs or allogeneic splenic DCs in 5% mouse serum for 4 d. For the last 16 h of culture, [3H]Thymidine was added, and cell proliferation was measured.

Cytotoxicity assay

A pooled spleen cell from naive WT, DC-immunized WT, or DC-immunized MHC class II−/− mice were depleted CD19+ cells using anti-CD19 magnetic beads (Miltenyi Biotec) and then cultured 5 × 107/well in culture medium in 96-well round plate for 2 d. CD3−NK1.1+ NK cells were sorted by FACS Aria and used as effector cells. 51Cr release assays were performed for evaluation of cytotoxic activity of NK cells. Briefly, target cells were labeled with Na2CrO4 (0.1 Ci/106 cells) for 1 h at 37˚C. The cells were washed twice with culture media before being added in triplicate to effector cells at the indicated E:T ratios. Four hours after incubation, supernatants were sampled, and the chromium release was determined by a scintillation counter. The percent-specific lysis was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Immunofluorescence microscopy

Spleens were embedded in OCT compound (Sakura Fintech, Tokyo, Japan) and snap-frozen in liquid nitrogen. Frozen sections (12 μm thick) were fixed with acetone and then blocked in skim milk for 10 min at room temperature and stained with the indicated Abs. The following Abs were used: anti-CD4 (BD Biosciences), biotinylated anti-CD11c (BD Bio-
sciences), polyclonal goat anti-NKp46 (R&D Systems), and rat anti-mouse metallophic macrophage (BMA BioMedicals, Augst, Switzerland). These Abs were visualized by direct coupling to Alexa Fluor 488 or through the use of appropriate secondary Abs coupled to Cy3 or streptavidin-Cy5. Samples were observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Image processing was performed using Keyence software. For quantitation of NK trafficking into white pulp, the numbers of NK cells per square millimeter of white pulp were counted in each group as described previously (28).

Mixed BM chimeras

Femurs and tibias were flushed with a syringe, and the suspension was passed through a 70-μm nylon mesh. RBCs were lysed, and both T cells and NK cells were removed by using biotinylated anti-CD4, -CD8, and -PK136 (anti-NK1.1) combined with Streptavidin Particles Plus-DM (BD Biosciences) and the BD IMag Cell separation system. For the generation of mice lacking DC-derived IL-12p35 or CD4 or CD40, B6(Ly5.1) mice were lethally irradiated (two doses of 500 rad, separated by 3 h) and were subsequently reconstituted with $10^6$ IL12p35−/− or CD4−/− or CD40−/− BM cells, respectively. All mice also received $5 \times 10^6$ CD11c−/−DTR-GFP BM cells (both CD45.2+). Control chimeras were reconstituted with $5 \times 10^6$ WT and $2 \times 10^6$ IL12p35−/− BM cells, respectively. All mice also received $5 \times 10^6$ CD11c−/−DTR-GFP BM cells and $5 \times 10^6$ WT and $5 \times 10^6$ 2IL12p35−/− BM cells. Mice were allowed to “rest” for 6–8 wk before use (29).

Real-time quantitative RT-PCR

Spleens were homogenized with Sepasol RNAI (Nacalai Tesque, Kyoto, Japan), and the total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg total RNA by reverse transcription (ReverTra Ace; Toyobo, Osaka, Japan) and 500 ng oligo(dT) primer (Invitrogen) for reverse transcription. Real-time quantitative RT-PCR was performed using the SYBR Green system (Applied Biosystems, Foster City, CA). Primers used for real-time quantitative RT-PCR are listed in Table I. The expression of cytokine gene expression were measured relative to GAPDH by using the 2−ΔΔCt method (30) and the total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg total RNA by reverse transcription (ReverTra Ace; Toyobo, Osaka, Japan). and the BD IMag Cell separation system. For the generation of mice lacking DC-derived IL-12p35 or CD4 or CD40, B6(Ly5.1) mice were lethally irradiated (two doses of 500 rad, separated by 3 h) and were subsequently reconstituted with $5 \times 10^6$ IL12p35−/− or CD4−/− or CD40−/− BM cells, respectively. All mice also received $5 \times 10^6$ CD11c−/−DTR-GFP BM cells (both CD45.2+). Control chimeras were reconstituted with $5 \times 10^6$ WT and $5 \times 10^6$ CD11c−/−DTR-GFP BM cells. Mice were allowed to “rest” for 6–8 wk before use (29).

Rapid splenic NK responses to tumor challenge in TLR-ligand- or DC-immunized mice

NK cells can be rapidly activated by certain TLR ligands and cytokine gene expression were measured relative to GAPDH by using the 2−ΔΔCt method (30). Intravenous immunization of WT mice with $1 \times 10^6$ BMDCs 4 wk prior to s.c. injection of MCA resulted in significant protection of mice from MCA-induced sarcoma formation (Fig. 2A, left panel). To determine the role of T cells in this activity, we repeated the experiment with Rag1−/− mice and found that the protection was lost (Fig. 2A, right panel). These results suggest that the control of sarcoma outgrowth in DC-immunized mice requires T cells.

Tumors from immunodeficient hosts are often highly immunogenic when transplanted into WT mice (34), because they are not subjected to the same immune pressure as tumors from immunocompetent hosts. Previous studies of MCA-induced sarcomas reported that ~40% of MCA-induced sarcomas derived from Rag1−/− mice are rejected in WT mice (34). To test whether this was the case in our model, we injected MCA-induced tumor cells derived from Rag1−/− mice (MCA1 and MCA2) s.c. into NOG (lacking lymphocytes and NK cells), Rag1−/−/−, WT, or WT mice that had been immunized with DCS 4 wk previously. As expected (34), all of the NOG and Rag1−/− mice experienced significant tumor growth. However, in WT mice, DC immunization significantly inhibited tumor outgrowth (5 of 11 mice rejected the tumor) as compared with untreated WT mice (2 of 11 mice rejected the tumor) (Fig. 2B).

**FIGURE 1.** DC therapy induces long-term activation of NK cells. A, C57BL/6 mice were injected with poly(I:C), CpG-ODNs, LPS, or syngeneic BMDCs s.c. Cells from the spleen and draining lymph node were isolated from nonimmunized or immunized mice 16 h or 3 wk later. The activation of NK cells (CD3− NK1.1+) was assessed by up-regulation of CD69 expression. B, Mice were immunized with poly I:C, CpG-ODN, LPS, or syngeneic BMDCs and, 3 wk later, were challenged with B16 tumor cells (i.v.). CD69 expression and IFN-γ production by tumor-reactive NK cells in the spleen were analyzed 16 h later in naive and immunized mice. Data are representative of two separate experiments (n ≥ 4/group).
To confirm a role for NK cells in tumor rejection, we selected the highly immunogenic MCA3 line, also derived from a Rag1-/- mouse, and transplanted it into WT mice that had been immunized with DCs or were untreated. Four weeks later, mice were challenged with 100 μg MCA s.c. in the hind flank. Tumor growth was measured every 7 d. The number of mice in each group is shown in parentheses. The asterisks denote a significant effect as determined by the Mann–Whitney U test at day 160 (*p < 0.001). B and C, Three sarcoma cell lines (MCA1, MCA2, and MCA3) were generated from MCA-treated C57BL/6 Rag1-/- mice. B, NOG, Rag1-/-, WT, and DC-immunized WT mice were injected s.c. with 1 × 10^6 cells from two of the sarcoma lines (MCA1, red; MCA2, black) into the hind flank of DC-immunized WT mice, and tumor growth was monitored. p < 0.05 for WT versus DC. p < 0.005 for NOG and Rag1-/- versus DC and NOG and Rag1-/- versus WT (Mann–Whitney U test). C, A total of 1 × 10^6 MCA3 cells were injected s.c. into the hind of WT mice that had been immunized 4 wk previously with DCs. Growth kinetics of MCA-3 sarcomas are shown. Mice were treated with rabbit serum or control IgG (panels 1, 3) or anti–asialo-GM1 Ab or anti-NK1.1 Ab to deplete NK cells (panels 2, 4) every 3 d beginning 2 d before tumor inoculation.

**FIGURE 2.** DC therapy prevents MCA-induced spontaneous tumors. A, C57BL/6 (left panel) or Rag1-/- C57BL/6 (right panel) mice were immunized with DCs or were untreated. Four weeks later, mice were challenged with 100 μg MCA s.c. in the hind flank. Tumor growth was measured every 7 d. The number of mice in each group is shown in parentheses. The asterisks denote a significant effect as determined by the Mann–Whitney U test at day 160 (*p < 0.001). B, NOG, Rag1-/-, WT, and DC-immunized WT mice were injected s.c. with 1 × 10^6 cells from two of the sarcoma lines (MCA1, red; MCA2, black) into the hind flank of DC-immunized WT mice, and tumor growth was monitored. p < 0.05 for WT versus DC. p < 0.005 for NOG and Rag1-/- versus DC and NOG and Rag1-/- versus WT (Mann–Whitney U test). C, A total of 1 × 10^6 MCA3 cells were injected s.c. into the hind of WT mice that had been immunized 4 wk previously with DCs. Growth kinetics of MCA-3 sarcomas are shown. Mice were treated with rabbit serum or control IgG (panels 1, 3) or anti–asialo-GM1 Ab or anti-NK1.1 Ab to deplete NK cells (panels 2, 4) every 3 d beginning 2 d before tumor inoculation.

As already mentioned, long-term NK cell reactivity requires CD4+ T cells and recipient DCs in vivo (18). To understand the role that CD4+ T cells play in the maintenance of long-term NK cell reactivity to tumors, we immunized MHC class II-/- mice with DCs. As expected, MHC class II-/- mice demonstrated a substantially lower level of killing as compared with WT mice, suggesting a role for CD4+ T cells (Fig. 3B). We also assayed IFN-γ secretion as a measure of NK cell activity and found IFN-γ secretion from the splenocytes of DC-immunized but not naive mice (Fig. 3C).

To further define the role of cytokines and costimulatory molecules in this process, we conducted a series of Ab-blocking experiments. Blocking MHC class II, but not MHC class I molecules, lead to a decrease in IFN-γ secretion, indicating that NK cell reactivity after DC immunization depends on CD4+ T cell–DC interactions (Fig. 3D). Furthermore, blocking IL-12, CD80, CD86, CD25, and IL-2 also resulted in a reduction in IFN-γ levels. Interestingly, blocking CD1d had no effect on IFN-γ production, thus demonstrating that invariant NKT cells do not con-
tribute to the long-term antitumor activity of NK cells after DC immunization.

CD4+ TEM cells in DC-immunized mice respond to syngeneic DCs with the production of IL-2, IFN-γ, and TNF-α

We next evaluated the functions of CD4+ T cells in DC-immunized mice. To rule out the possibility that T cell activation was a response to FCS in the culture media, we used BMDCs generated with rmGM-CSF with 1.5% mouse serum (ms-BMDCs) for immunization. NK cell cytotoxicity was confirmed in ms-BMDC–immunized mice (Supplemental Fig. 1A). When cell proliferation was analyzed after coculturing with syngeneic splenic DCs, CD4+ T cells from ms-BMDC–immunized mice, but not from naive mice, proliferated (Fig. 4A). In contrast, CD4+ T cells from both naive and ms-BMDC–immunized mice responded to allogeneic splenic DCs, although the DC-immunized mice showed some increase in responsiveness (Supplemental Fig. 1B). CD4+ T cell proliferation in response to syngeneic splenic DCs in ms-BMDC–immunized mice was completely blocked by anti-MHC Class II mAbs and was significantly reduced by a combination of mAbs to CD25 and IL-2 (Supplemental Fig. 1C). These findings demonstrate that DC vaccination sensitizes CD4+ T cells to respond to the Ag presented on the MHC class II molecules of syngeneic DCs. Interestingly, Palucka et al. (35) reported a similar phenomena in human melanoma patients treated with DC therapy. These investigators found that in vitro spontaneous proliferation of the CD4+ T cells in the patient’s PBMCs was dependent on CD11c+ DCs and could be inhibited with Abs to IL-2 and HLA-DR+. In addition, Martin-Fontecha et al. (36) also reported that CD4+ T cells migrating into the lymph nodes of BMDC-injected mice are prone to induce autoimmune disease. Therefore, it seems highly likely that DC vaccination is energizing CD4+ T–DC interactions.

We also detected elevated levels of IL-2 in 5% mouse serum containing cocultures of CD4+ T cells from ms-BMDC–immunized mice with either ms-BMDCs or FCS-DCs but no IL-2 in cultures of CD4+ T cells from naive animals (Supplemental Fig. 1D). There was no significant difference in levels of IL-2 secretion in response to either ms-BMDCs or FCS-DCs in ms-BMDC–immunized mice.

We verified IL-2 secretion by CD4+ T cells in DC-immunized mice but not naive mice by measuring intracellular cytokine
production (Fig. 4B). We then identified the T EM cell compartment (CD25^+CD44^hiCD62L^lo/CD4^+) as the CD4^+ T cell subset responsible for IL-2 production after restimulation with syngeneic DCs (Fig. 4C). Two-thirds of the CD4^+ T cells secreting IL-2 also expressed CXCR3 (Fig. 4C), and many also produced TNF-α and expressed CD40L, with some production of IFN-γ (Fig. 4D).

CD4^+ T EM cells in DC-immunized mice did not produce IL-4, IL-10, or IL-17 (data not shown), suggesting these cells have a polarized Th1 phenotype. CD4^+ T EM cells from DC-immunized mice produced IL-2 in response to syngeneic but not allogeneic DCs (data not shown). Interestingly, CD4^+ T EM cells from both naïve and DC-immunized mice produced less IL-2 even after stimulation with anti-CD3 mAb plus anti-CD28 mAb compared with CD4^+ non-T EM cells. Thus, CD4^+ T EM cells from DC-immunized mice had a cytokine profile that was unique in comparison with CD4^+ non-T EM subsets. Furthermore, the CD4^+ T EM cells in C57BL/6 mice immunized with DCs produced IL-2 after injection of tumor cells (Supplemental Fig. 2).

**IL-2 secreted by CD4^+ T EM cells in DC-immunized mice activates NK cells**

As shown in Fig. 4D, both IL-2 and IFN-γ are produced by CD4^+ T EM cells in response to syngeneic DCs. To determine which cytokine is responsible for long-term NK cell responses after DC immunization, CD4^+ T cells were isolated from C57BL/6, IFN-γ^−/−, or IL-2^−/− mice and transferred into Rag1^−/− mice. Recipient mice were then vaccinated with DCs. Two weeks later, the mice were challenged i.v. with B16 melanoma cells, and after 16 h, NK cells were analyzed for IFN-γ production. NK cells secreted IFN-γ in the mice were reconstituted with WT and IFN-γ^−/− CD4^+ T cells but not in mice reconstituted with IL-2^−/−CD4^+ T cells (Fig. 5A). These adoptive transfer studies demonstrate that IL-2 secretion by CD4^+ T EM cells after DC immunization is necessary for the generation of activated NK cells.

To evaluate whether the CD4^+ T EM subset from DC-vaccinated mice directly mediates NK cell responses in vivo, we transferred CD4^+ T EM cells or CD4^+ non-T EM cells from either non- or DC-immunized mice into Rag1^−/− mice (Fig. 5B). NK cells making IFN-γ were detected in mice that received bulk CD4^+ T cells or CD4^+ T EM cells but not in mice that received CD4^+ non-T EM cells (Fig. 5B). Taken together, these results show that CD4^+ T EM cells produce IL-2 and are essential for the long-term reactivation of NK cells in response to tumors.

**CXCL10-producing DCs mediate NK cell recruitment and antitumor effects**

Upon challenge with B16 melanoma cells, NK cells in DC-immunized but not naïve mice trafficked from the splenic red pulp into the white pulp, especially into the T cell-rich periarterial lymphatic sheaths (PALS) (Fig. 6A, second row, right panel). When white pulp NK cells were quantified as NK cells per square millimeter, NK cell homing to the PALS was shown to be sig-
FIGURE 5. NK cell activation mediated by CD4+ TEM cells in DC-immunized mice. A, A total of 5 × 10^6 CD4+T cells were isolated from C57BL/6, IFN-γ−/−, and IL-2−/− mice and transferred into Rag1−/− mice, which were vaccinated with 1 × 10^6 DCs on the following day. Two weeks later, the mice were challenged with 5 × 10^6 B16 melanoma cells i.v., and 16 h later, the spleens were analyzed for IFN-γ production by CD3+ NK1.1+ NK cells. B, As in A, but 1 × 10^6 CD4+ T cells or 5 × 10^5 CD4+ T cell subsets, either CD4+ TEM (CD4+CD44hi CD62L−) cells or CD4+ non-TEM (naive CD4+ T or central memory CD4+ T) cells from non- or DC-immunized mice were transferred to Rag1−/− mice. NK cells producing IFN-γ were analyzed after challenge with B16 melanoma. Data are representative of three separate experiments.

significantly increased upon tumor challenge (Fig. 6B). We therefore turned to the local secretion of chemokines to understand NK recruitment in our tumor challenge model.

CXCR3, which is expressed by NK cells in the steady state is downregulated after migration to white pulp (Fig. 6C), suggesting that NK cell migration to PALS is dependent on CXCR3 expression. To better define the role of chemokines in the activation of NK cells, we injected DC-immunized mice with pertussis toxin (PTX), which inhibits the GTP-binding proteins Gi and Go and thereby blocks signaling from chemokine receptors (37) but does not affect the steady-state migration of NK cells to the red pulp area (28). When DC-immunized mice were treated with PTX and challenged with tumor cells, NK cells no longer migrated to the white pulp of the spleen or produced IFN-γ (Fig. 6B, 6C), suggesting that chemokine-dependent trafficking to the PALS is required for NK cell activation.

These findings suggest that a signal produced in DC-immunized mice, but not naive mice, attracts NK cells to the PALS upon tumor-induced inflammation. To identify the signal, we used quantitative PCR to screen for expression of the NK cell trafficking chemokins. After challenge with B16 melanoma cells, DC-immunized mice demonstrated 5–20 times higher levels of CXCL9, CXCL10, and CXCL11 as compared with naive mice or DC-immunized mice not injected with tumor cells (Fig. 6D). CXCL9, CXCL10, and CXCL11 are CXCR3 ligands that act primarily on activated Th1 and NK cells, including their migration to inflamed tissues and endothelial cells (38). By real-time PCR, we were able to demonstrate that CXCL10, but not others chemokines, was produced at 6 h (Fig. 6E) by DCs rather than other cell types in the spleen and thus likely contributes to the observed increase in NK cell numbers. We tested these findings by administrating of anti-CXCL10 Ab into DC-immunized mice prior to tumor challenge. We found that the recruitment of NK cells as well as their IFN-γ production was abrogated by the addition of anti-CXCL10 Ab (Fig. 6B, 6C). In addition, CXCR3 on NK cells was not downregulated by anti-CXCL10 Ab.

Signaling between CD4+ TEM cells and DCs is a potential mechanism for the long-lived reactivation of NK cells seen in our studies. To test this, we cocultured CD4+ T cells and DCs from MHC Class II−/−, CD80/86−/−, CD40−/−, and IL-12−/−/− mice. We found that IL-2 secretion after the culture of CD4+ T cells with splenic DCs depends on MHC Class II and CD80/CD86 but not CD40 and IL-12 (data not shown). To examine this in vivo, we immunized CD40−/− mice with DCs, challenged them with a tumor, and looked at NK cell recruitment to the PALS by immunofluorescence microscopy. We found that NK cell recruitment to the PALS in CD40−/− mice was much less than in WT mice (Fig. 6A, third row, right panel, 6B), and the secretion of chemokines was decreased as well, demonstrating its dependency on the CD40 signal (Fig. 6D). This is despite the fact that CD4+ T cells from DC-immunized CD40−/− mice still produced IL-2 at the levels equal to WT mice (data not shown). Therefore, the expression of CXCL10 by DCs is decreased in DC-immunized CD40−/− mice indicated that CD40L signaling to endogenous DCs is crucial for the recruitment of NK cells to the PALS.

A crucial role for endogenous DC signaling following challenge with tumor cells

When splenic DCs from naive mice were cultured with CD4+ TEM cells from DC-immunized mice, the DCs upregulated CD40, CD80, CD86, and B7-DC and downregulated CD119 (Supplemental Fig. 3). However, the DCs did not mature when cultured with CD4+ non-TEM cells, suggesting that endogenous DCs could only be matured by CD4+ TEM cells. To examine this response in vivo, we challenged DC-immunized and naive mice with B16 tumor cells i.v. Consistent with the in vitro results, endogenous DCs from DC-immunized mice upregulated CD86 in response to tumor challenge (Fig. 7A).

We next examined the interaction between endogenous DCs and NK cells after their trafficking to the PALS following i.v. challenge with B16 melanoma cells. Rather than simply migrating randomly into the PALS, the NK cells were seen in close contact with the mature CD11c+ DCs (Fig. 7B). Thus, CXCL10-producing endogenous DCs attract NK cells and CD4+ TEM cells into the PALS, forming a cluster and leading to NK cell activation.

In the cascade of events leading to NK cell activation, other signals in addition to IL-2 and CXCL10 could be required to activate DCs. To identify whether other molecules are required for this response, CD4−/−, MHC class II−/−, CD40−/−, IL-12p35−/−, IFN-α/βR−/−, and MyD88−/− mice were immunized with DCs and challenged 4 wk later with B16 melanoma cells. We found that IFN-γ secretion by NK cells after B16 challenge was dependent on CD4, CD40, class II, and IL-12 but not IFN-α/β and MyD88 (Supplemental Fig. 4).

We also examined the molecules on endogenous DCs that were required for generating NK cell tumor reactivity after DC immunization. BM-mixed chimeric mice were generated by lethally irradiating B6 (Ly5.1) mice and reconstituting them with 5 × 10^6 BM cells from CD4−/−, CD40−/−, MHC class II−/−, or IL-12p35−/− mice. All recipient mice also received 5 × 10^6 BM cells from CD11c diphtheria toxin receptor (DTR) mice, in which CD11c-expressing DCs express the receptor for diphtheria toxin (DT) and thus can be eliminated in vivo by administering DT. After challenge with B16 tumor cells, we assayed for IFN-γ se-
FIGURE 6. NK cells are recruited to splenic white pulp in DC-immunized mice upon injection of tumor cells. One month after DC immunization, non- or DC-immunized mice were injected with B16 tumor cells or nothing. In some experiments, the mice were treated with PTX or anti-CXCL10 Ab before tumor challenge. A, Spleen sections were stained with anti-metallophilic macrophage (anti–MOMA-1) (blue), anti-NKp46 (red), and anti-CD4 (green) mAbs, and samples were observed under a fluorescence microscope. Images were captured using a ×20 objective lens. Scale bars, 100 μm. Data are representative of four separate experiments. B, The number of NK cells per square millimeter of white pulp was analyzed with Keyence software. *p < 0.001, WT DC-B16 versus others (Mann–Whitney U test). Data are from four separate experiments (n ≥ 4/group) (mean and SEM). C, CXCR3 expression on NK cells and IFN-γ secretion by NK cells in PTX (200 ng/mouse) or anti-CXCL10 Ab (500 μg/mouse)-treated, or untreated, DC-immunized mice or naive mice were analyzed by flow cytometry. Data are representative of four separate experiments. D, Quantitative RT-PCR analysis of the expression of NK cell-related chemokines in the spleen from untreated or DC-treated, WT, or CD40−/− mice 16 h after B16 challenge, presented relative to the expression in an unstimulated naive spleen, and set as 1. *p < 0.05 (CXCL9, DC-B16 versus nonimmunized, nonimmunized-B16, or DC; CXCL10, DC-B16 versus nonimmunized, nonimmunized-B16, or DC; CXCL11, DC-B16 versus nonimmunized, nonimmunized-B16, or DC) (Mann–Whitney U test). Data are mean ± SEM of two separate experiments (n = 4/group). E, Quantitative RT-PCR analysis of CXCL10 expression in splenic CD11c+ DCs or CD11c− cells from untreated or DC-treated mice 6 h after B16 challenge and presented relative to the expression in unstimulated naive CD11c+ DCs from non-immunized mice set as 1. *p < 0.05, CD11c+ from DC-B16 versus others (Mann–Whitney U test) Data are mean ± SEM of two separate experiments (n = 4/group).
cretion and found that it was defective in the class II−/−, CD40−/−, and IL-12−/− DC BM-chimeric mice but not in the CD4−/− chimeras (Fig. 7C). Therefore, both CD40 signaling to DCs by CD4+ TEM cells and IL-12 secreted by DCs are essential for long-term NK cell reactivation against tumors.

Discussion
These studies provide a mechanism for the development of prolonged NK cell antitumor reactivity in mice after DC immunization. This memory-like response is a newly discovered feature of NK cell-mediated tumor immunosurveillance. Our studies show that NK cell reactivation is controlled by newly generated CXCL10-expressing DCs that continuously recruit NK cells and CD4+ TEM cells, thereby maintaining NK cell responses.

After injection of tumor cells, the cascade of NK cell reactivation is supported by the interaction of endogenous DCs and self-reactive CD4+ TEM cells. A recent report using an influenza infection model showed that inflammatory cytokines and chemokines released during memory CD4+ T cell and DC interactions can enhance innate immune responses, leading to early control of virus (39). Hochweller et al. (40) recently showed that DC–T cell interactions in the steady state, even without exogenous Ag, can help in the subsequent immune response via recognition of self-Ag on the MHC of DCs. Also, Zwickey et al. (41) previously demonstrated that the self-peptide-MHC class II complex formation and presentation increased with DC activation. These studies all support our conclusions that a DC–CD4+ TEM cell interaction underlies the sustained NK cell activation. In DC-immunized mice, after an injection of tumor cells, endogenous DCs might overexpress self-Ag induced by “danger signals” such as heat shock proteins, high mobility group box-1, adenosine triphosphate, or uric acid released from tumor cells themselves (42). DCs could also express cross-reactive Ags derived from tumor cells in vivo. Further studies will be needed to define the Ags involved in this system.

IL-2 released from CD4+ TEM cells is one of the important factors in the modulation of NK cell responses in this immunological cascade. Mice immunized with DCs demonstrated an expansion of CD4+ TEM cells capable of producing IL-2, TNF-α, and IFN-γ. To our knowledge, this is the first demonstration that IL-2 produced by CD4+ TEM cells is essential for the development
of NK cell reactivity in vivo, although a role for IL-2 was previously reported in an in vitro study (43). IL-21 also activates NK cells to secrete IFN-γ (44); however, we found that neither IL-21 nor IL-2–producing follicular Th cells were necessary for establishing NK cell reactivity following tumor administration (data not shown). Nakashita et al. (45) recently showed that IFN-γ released from CD4+ TEM cells leads to the migration of CD8+ CTLs. However, our data showed that IFN-γ from CD4+ TEM cells is not essential for NK cell activation (Fig. 5A), reinforcing that NK cells and CD8+ T cells have different requirements for activation.

To our knowledge, the current study is the first demonstration of CD4+ TEM cell-mediated NK cell activation against tumors that is dependent on CD40. Following tumor challenge, it is CD40 signaling between DCs and CD4+ TEM cells that is essential for NK cell trafficking to the PALS and subsequent NK cell activation. Previously, it has been reported that activated NK cells expressing CD40L directly recognized and killed CD40-expressing cells, such as CD40-transfected tumor cells or DCs in a T cell-independent manner (46–48). However, a previous study from our laboratory showed that antitumor NK cell response required CD4+ T cells (18). In the current study, we confirmed that NK cells do not express CD40L (data not shown). Also, as shown in Fig. 6, after CD40–CD40L interactions with CD4+ TEM cells, DCs attract NK cells to the PALS. Apparently, without recruitment into the T cell-rich PALS area, which was impaired by PTX or anti-CXCL10 Ab treatment, NK cells could not be activated.

A key step in this process is the rapid secretion of CXCL10 by DCs after CD40 activation by CD4+ T cells. The importance of CD40 signaling was underscored in our in vivo model in which very few NK cells from CD40−/− mice migrate to the white pulp after tumor challenge (Fig. 6A, 6B). In addition, injection of tumor cells might induce release of CXCL9 and CXCL11 from endothelial and stroma cells (49–51). CXCR3+/−/− mice have extremely low numbers of NK cells, leading to speculation that this receptor may also be important for NK cell homeostasis (52). However, an interaction between CXCL10 and NK cell activation by tumor cells has not been studied, except in a CXCL10-deficient model (53). Collectively, our studies demonstrated that during tumor challenge of DC-immunized mice, CXCR3 receptor-expressing NK cells and CD4+ TEM cells are efficiently recruited to CXCR3 ligand-expressing DCs, resulting in the formation of cell clusters, in which DCs come into close contact with NK cells (Fig. 7B). In this tumor model, CD40L-expressing CD4+ TEM cells could also contribute to further maturation of DCs with the subsequent release of IL-12. As a result of CD40 signaling and CD4+ TEM cell activation, the synergy between IL-2 and IL-12 leads to full activation of NK cells in the PALS and secretion of IFN-γ. In contrast to our findings, Guarda et al. (54) observed that adaptive immunity dampens innate immune responses through selective regulation of CD40-mediated NLRP1 and NLRP3 inflammasomes by CD4+ TEM cells. This process was dependent on caspase-1 (54). However, in our studies, NK cells from caspase-1–deficient mice immunized with DCs secreted IFN-γ at similar levels to that of WT mice (data not shown), suggesting that preactivation of NK cells is not regulated by inflammasomes. We found instead evidence that adaptive immunity shapes NK cell antitumor immunity.

We previously reported that strong adaptive immunity was induced by activated NK cells in situ through in vivo DC maturation (4, 8, 27). We have now shown that DC immunization leads to NK cells capable of rapid reactivation against tumor cells. Therefore, DC immunization, by stimulating both adaptive and innate immunity, consolidates the two arms of the immune system for comprehensive tumor immunotherapy. These findings further support the potential benefits of developing DC-based strategies for the treatment ofrecalcitrant cancers.

Acknowledgments

We thank Y. Shozaki, M. Kawaguchi, H. Fujimoto, and Y. Hachiman (RIKEN, Research Center for Allergy and Immunology) for providing technical assistance, K. Nishida (RIKEN, Research Center for Allergy and Immunology) for technical advice, and Drs. K. Bickham, C. Munz (University of Zurich), P.D. Burrow (University of Alabama), and R.M. Steinman (Rockefeller University) for critical reviewing.

Disclosures

The authors have no financial conflicts of interest.

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