Memory Type 2 Helper T Cells Induce Long-Lasting Antitumor Immunity by Activating Natural Killer Cells

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Abstract

Functionally polarized helper T cells (Th cells) play crucial roles in the induction of tumor immunity. There is considerable knowledge about the contributions of IFN-producing Th1 cells that supports the role of cytotoxic cluster of differentiation (CD8) T cells and natural killer (NK) cells, but much less is known about how IL-4-producing Th2 cells contribute to tumor immunity. In this study, we investigated the cellular and molecular mechanisms employed by memory Th2 cells in sustaining tumor immunity by using a mouse model system wherein ovalbumin (OVA) is used as a specific tumor antigen. In this model, we found that OVA-specific memory Th2 cells exerted potent and long-lasting antitumor effects against NK-sensitive OVA-expressing tumor cells, wherein antitumor effects were mediated by NK cells. Specifically, NK cell cytotoxic activity and expression of perforin and granzyme B were dramatically enhanced by the activation of memory Th2 cells. Interleukin 4 (IL-4) produced by memory Th2 cells in vivo was critical for the antitumor effects of the NK cells, which IL-4 directly stimulated to induce their perforin- and granzyme-B–dependent cytotoxic activity. Our findings show that memory Th2 cells can induce potent antitumor immunity through IL-4–induced activation of NK cells, suggesting potential applications in cellular therapy for cancer patients. Cancer Res; 71(14); 1–9. ©2011 AACR.

Introduction

There is ample evidence that many tumors express antigens that can be recognized by the adaptive immune system (1) and potentially can be used to induce an antitumor immune response. Adoptive transfer of T cells has good potential as an immunotherapy for cancer. First, because of the ability of subsets of T cells to directly kill tumor cells and also indirectly activate other cells such as granulocytes to do so (2–7). Second, in the case of memory T cells, they are able to persist for long periods and provide antigen-specific protection from some diseases for the lifetime of the individual. Therefore, memory T cells may be able to be used as both therapeutic and preventive agents against the development and growth of cancer cells (8, 9). Furthermore, the infiltration of T cells into the tumor is known to be a strong predictive biomarker for the survival of ovarian and colorectal cancer patients (10, 11). Recently, the genetic manipulation of T cells to obtain antigen specificity and the maintenance of effector function was used in clinical trials for cancer and other diseases. However, the results suggest that further consideration of the translation of therapeutic approaches from animal models to human patients may be required (12–14).

The majority of cancer immunotherapy studies have focused on the generation of cluster of differentiation (CD8) CTLs that recognize tumor antigens in association with MHC class I molecules. In this context, the induction of tumor-specific CD4 T cells has been largely ignored, except when optimal activation and development of CD8 CTLs is thought to depend on help from CD4 T cells (15, 16). It is generally thought that only type I helper T (Th1) cells provide help to CD8 CTLs (17). However, there are a number of reports suggesting that both CD4+ Th1 and Th2 cells may act independently of CD8 CTLs. Effector Th2-mediated antitumor immune responses seem to be dependent on the production of IL-4 (3) and the recruitment of polymorphonuclear cells such as eosinophils (2–7), often in collaboration with macrophages (2–4). Interestingly, effector Th2 cell therapy is effective in a CTL-resistant tumor model (7).

The successful differentiation and maintenance of functional memory Th cells depend on several cellular and molecular processes (18). We established an in vivo experimental “memory Th1/Th2 mouse” system by adoptively transferring in vitro differentiated effector Th1/Th2 cells into syngeneic mice (19). These in vivo generated functionally polarized...
memory Th1/Th2 cells possess phenotypic and functional properties that are typical of memory cells. The levels of Th2 cytokine production in memory Th2 cells are significantly higher than those of effector Th2 cells (19). However, the importance of memory Th2 cells in the antitumor response has not been well defined.

We herein investigated the cellular and molecular mechanisms underlying memory Th2 cell-mediated antitumor immunity. We identify an important cellular network in which IL-4 produced by activated memory Th2 cells induces potent and long-lasting antitumor effects in vivo through the activation of natural killer (NK) cells. IL-4 seems to activate NK cells directly in addition to the reported pathway, that is, via the maturation of dendritic cells (DC; ref. 20). Thus, memory Th2 cells may have therapeutic potential for cell therapy in cancer patients.

Materials and Methods

Animals and cell lines

Heterozygous MLL-deficient (MLL+/−) mice were provided by Dr. Toshihisa Komori (Nagasaki University, Japan; ref. 21). BALB/c, C57BL/6, and BALB/c nu/nu mice were purchased from CLEA Inc. IL-4–deficient (IL-4−/−) mice were provided by Dr. Manfred Kopf (Institute of Integrative Biology, Zurich, Switzerland; ref. 22). STAT6-deficient (Stat6−/−) mice were provided by Drs. Shizuo Akira (Osaka University, Osaka, Japan; ref. 23). OVA-specific T-cell receptor (TCR; DO11.10) transgenic (Tg) mice were provided by Dennis Loh (Nippon Roche Research Center, Kanagawa, Japan; ref. 24). All mice used in this study were maintained under specific pathogen-free conditions. All animal care was conducted in accordance with the guidelines of China University. The A20-OVA cell line was provided by Dr. M.J. Bevan.

Reagents

The ovalbumin (OVA) peptide (residues 323–339; SQAV-HAAHAEINAEGR) was synthesized by BEX Corporation and was endotoxin free (<0.01 U/mL).

Generation of effector and memory Th1/Th2 cells

The generation of effector Th1/Th2 cells was described previously (25–27). In brief, splenic KJ1+CD4+ T cells from OVA-specific TCR DO11.10 Tg mice were stimulated with an OVA peptide (Loh15; 1 μmol/L) plus adenosomatous polyposis coli (APC)-irradiated splenocytes under Th1 or Th2 culture conditions for 6 days in vitro. Th1-condition; IL-2 (25 μ/mL), IL-12 (10 μ/mL), and anti-IFN-γ monoclonal antibody (mAb). Th2-condition: IL-2 (25 μ/mL), IL-4 (10 μ/mL), anti-IL-12 mAb, and anti-IFN-γ mAb. For the generation of memory Th1/Th2 mice (19, 27), the effector Th1/Th2 cells [2(−3) × 107] were transferred i.v. into syngeneic BALB/c recipient mice. In most experiments, mice were used as memory Th1 and Th2 mice 4 to 5 weeks after cell transfer.

Tumor model

Mice were challenged by s.c. injection of A20-OVA cells or A20 cells (2 or 6 × 106) in 100 μL of PBS on day 0. For depletion of NK or CD8 T cells, animals were injected intraperitoneally (i.p.) with 50 μL rabbit anti-Asialo GM1 (Wako chemical), or 200 μg rat anti-CD8 antibody (53.6.7), or 200 μg anti-NKG2D antibody (eBioscience) at days −1, 1, and 5. Rabbit serum IgG (500 μg; Sigma-Aldrich) or rat serum IgG (200 μg; Sigma-Aldrich) was used as a control. The size of tumors was assessed using microcalipers and was expressed as tumor volume calculated by the following formula: (tumor volume; mm3) = 0.4 × length (mm) × width (mm)2 as described previously (28).

Isolation of splenocytes, lymph node cells, and tumor-infiltrating lymphocytes for flow cytometric analysis

Splenocytes and lymph node (LN) cells were passed through a nylon mesh, centrifuged, and red blood cells were lysed. Tumors were minced and then treated for 30 minutes with collagenase (20 μg/mL; Worthington) and trypsin inhibitor (0.3 mg/mL; Sigma-Aldrich). Tumor mononuclear cells were separated by centrifugation on Percoll (GE Healthcare). Cell surface staining was done as previously reported (29). An NK cell–enriched population (whole NKp46+ cells) was isolated from splenocytes and tumor-infiltrated mononuclear cells by using an NKp46 cell isolation kit (Miltenyi Biotec). For the preparation of B220+ cell–depleted NKp46+ cells, B220+ cells were depleted using anti-B220-Biotin (BD Bioscience) before applying the NKp46 cell isolation kit. CD11c−NKp46+ cells were purified from whole NKp46+ cells stained with anti-CD11c mAb (N418; ebioscience) by using a cell sorter (FACSaria; Becton Dickinson). CD4 and CD8 cells were purified using direct magnetic beads (Miltenyi Biotec). For intracellular staining of CD4 cells, the cells were stimulated with phorbol-12-myristate-13-acetate (PMA; 20 ng/mL) plus ionomycin (500 nmol/L) for 4 hours, fixed, permeabilized, and then incubated with the appropriate staining reagents. For intracellular staining of NK cells, cells were permeabilized using a Foxp3 staining buffer set (ebioscience) and then incubated with the appropriate staining reagents. The reagents used in this study were anti-KJ1.26–phycocerythrin (PE), anti-CD4–allophycocyanin, anti-DX5–PE, anti-TCRβ–allophycocyanin, anti-IL-4–allophycocyanin, anti-CD8–fluorescein isothiocyanate (FITC), anti-CD69–FITC, and anti-IFNγ–FITC purchased from BD Biosciences, and anti-NKp46–PE (29A1.4), anti-granzyme B–FITC (16G6), and antiperforin–allophycocyanin (eBiosOMAK-D) purchased from ebioscience. Flow cytometric analysis was done on a FACSCalibur flow cytometer (Becton Dickinson), and the results were analyzed using the FlowJo software program (Tree star, Inc.).

51Cr release cytotoxicity assay

The chromium-51 (51Cr) release assay was conducted as previously described (30). In brief, the cytotoxic activity of splenocytes, NK cells, or tumor-infiltrating lymphocytes (TIL) stimulated with IL-2 (100 μ/mL), IL-4 (100 μ/mL) or Loh15 antigenic peptide (0.3 μmol/L) was assessed on A20-OVA, A20, and YAC-1 cells labeled with sodium chromium (51Cr). For cytokine blockade, anti-IL-2 antibody (JES6-1A12), anti-IL-4 antibody (11B11), anti-IL-5 antibody (TREK5), or anti-IFNγ antibody (R4-6A2) were added. For blocking the function of perforin or granzyme B, Concanamycin A...
(CMA; Sigma-Aldrich) or z-AAD-CMK (Sigma-Aldrich) was used, respectively.

**Proliferation assay**

A total of $2 \times 10^5$ cells were stimulated in 200 µL cultures with medium, IL-2 (100 µg/mL), and IL-4 (100 µg/mL) for 5 days. Proliferation was measured by $[^{3}H]$thymidine (37 kBq/well) incorporation for the final 16 hours of culture (31).

**Statistical analysis**

The significance between 2 groups was determined by 2-tailed Student’s t test. Differences in survival were analyzed using the log-rank test.

**Results**

**Inhibition of tumor growth by antigen-specific memory Th1/Th2 cells in vivo**

To investigate the antitumor effect of memory Th1/Th2 cells, we used a "memory Th1/Th2 mouse" system in which OVA-specific $\alpha$ITCR DO11.10 Tg CD4 T cells were stimulated with OVA peptide and antigen-presenting cells under Th1 or Th2 culture conditions in vitro and adoptively transferred into syngeneic mice (memory Th1/Th2 mice; ref. 19). Four weeks after cell transfer, memory Th1, Th2, and control mice were s.c. injected with $2 \times 10^5$ (low dose) or $6 \times 10^5$ (high dose) A20-OVA tumor cells, which express OVA as a tumor model antigen (6). As shown in Figure 1A and Supplementary Figure S1A, tumor growth was inhibited in both memory Th1 and Th2 mice and these mice showed significantly prolonged survival with either dose of A20-OVA inoculation. Interestingly, memory Th2 mice showed a more potent antitumor effect than memory Th1 mice. In contrast, the growth of parental A20 cells was not inhibited in OVA-specific memory Th1 or Th2 mice (Fig. 1B) and mice transferred with polyclonal effector Th1/Th2 cells prepared in vitro showed no inhibition of tumor growth (Supplementary Fig. S2A); these mice also showed no significant prolongation of survival (Supplementary Fig. S1B and S2B). These results indicate that antigen-specific memory Th1 and Th2 cells induce potent antitumor effects. The inhibition of tumor growth and increased survival were observed even when mice were challenged with tumor cells 9 months after effector Th2 cell transfer (Fig. 1C and Supplementary Fig. S1C). Moreover, memory Th2 mice were able to inhibit tumor growth even after repetitive challenge with A20-OVA cells over a 180-day period (Supplementary Fig. S3A and B). These results indicate that the antigen-specific antitumor activity induced by memory Th2 cells was maintained very efficiently in the normal host mice in our experimental system.

**Depletion of NK cells resulted in a reduction in the antitumor effect mediated by memory Th2 cells**

In terms of an adaptive immune response to cancer, much research has focused on the role of cytotoxic CD8 T cells and the production of Th1 cytokines by CD4 and CD8 T cells (32–34). In addition, NK cells are innate immune lymphocytes that are important for early host defense against infectious pathogens and tumors through a contact-dependent cytotoxicity mechanism (35, 36). To determine whether CD8 T cells and NK cells are required for the memory Th2 cell–mediated antitumor response, we depleted CD8 cells or NK cells by i.p. injection of anti-CD8 mAb, anti-Asialo GM1 Ab, or anti-NKG2D antibody, respectively (Fig. 2A and Supplementary Fig. S5). Injection of anti-Asialo GM1 Ab did not reduce the number of CD8 T cells within the splenocyte population (Supplementary Fig. S4). Anti-Asialo GM1 Ab and anti-NKG2D
Ab injection significantly reduced the antitumor effects of memory Th2 cells, whereas a moderate reduction was observed following administration of anti-CD8 mAb as compared with control Ig groups. These results indicate that NK cells are a predominant cell type involved in the antitumor effects induced by memory Th2 cells.

Next, the distribution of NK cells and memory Th2 cells in the tumor region and the associated lymphoid organs were examined. TILs were harvested 2, 5, 10, and 20 days after the injection of A20-OVA cells (Fig. 2B). The proportion of NKp46+ cells in TILs of memory Th2 mice was gradually increased up to day 10. The number of KJ1.26+ memory Th2 cells in the TILs of memory Th2 mice peaked at day 5, a few days earlier than that of the peak of NKp46+ cells. On day 20, we could not isolate TILs in memory Th2 mice because the tumor was eradicated completely. These results may indicate that NKp46+ cells exist for a long time in the tumor microenvironment as far as tumor cells are present. In addition, on day 10, the proportion of NKp46+ memory Th2 cells showed significantly higher (4.7% vs. 0.5%) in memory Th2 mice, whereas those in the spleen, ndLN, and dLN were similar.

Next, to examine whether the cytotoxic activity of NK cells in the TILs of tumor-bearing memory Th2 mice is enhanced, we purified NKp46+ cells from the spleen and TILs, and their cytotoxic activity was measured (Fig. 2D). The cytotoxic activity of NKp46+ cells in the spleen and TILs of memory Th2 mice was significantly higher than that of control mice. Moreover, the cytotoxic activity of TILs was significantly decreased by the injection of NKG2D mAb (Supplementary Fig. S6). These results indicate an increase in both numbers and activity of NK cells in the tumor region of memory Th2 mice.

**MLL+/− memory Th2 cells showed impaired antitumor effects compared with MLL+/+ memory Th2 cells**

We previously reported that expression of MLL is required for the maintenance of Th2 function and that the ability to produce Th2 cytokines is selectively reduced in MLL+/− memory Th2 cells (37). As expected, MLL+/− memory Th2 cells showed decreased ability to produce IL-4 (34.3% vs. 15.3%; Supplementary Fig. S7A). To investigate the effect of memory cells in which suboptimal Th2 responses develop, the memory mice were challenged with A20-OVA cells (Fig. 3A and B). Memory Th2 mice transferred with MLL+/+ effector Th2 cells showed significantly reduced inhibition of tumor growth and prolongation of survival as compared with memory Th2 mice transferred with MLL+/+ effector Th2 cells. The proportion of memory Th2 cells in the spleen, ndLN, and dLN was not significantly different between MLL+/+ and MLL+/− memory Th2 mice (Supplementary Fig. S7B). Interestingly, however, the proportion of MLL+/− memory Th2 cells in the tumor was higher than that of MLL+/+ memory Th2 cells (31.0% vs. 6.7%). The majority of infiltrated memory Th2 cells expressed CD69 in both MLL+/+ and MLL+/− groups (86.2% vs. 79.3%; Supplementary Fig. S7C).
Next, we examined the nature of tumor-infiltrated NK cells in MLL+/+ and MLL+/− memory Th2 mice (Fig. 3C and D). Activated NK cells contain cytotoxicity-related proteins, including perforin and granzymes; perforin facilitates the entry and/or trafficking of granzymes into target cells, whereas granzymes induce target tumor cell death (38). As can be seen in Figure 3C, obvious upregulation of both perforin and granzyme B in NKp46+ cells was detected in the MLL+/− group but not in the MLL+/+ group as compared with the control. The number of NKp46+ NK cells in the tumor was not significantly different between MLL+/− and MLL+/+ groups (Fig. 3D). These results indicate that NK cells migrated into the tumor microenvironment equivalently, although their activation with increased cytotoxicity-related proteins was not normally induced in the MLL+/+ memory Th2 mice, in which the production of Th2 cytokines from memory Th2 cells was impaired.

NK cell–mediated cytotoxicity is induced by activated memory Th2 cells in vitro

Next, to test whether NK cell cytotoxicity is induced by memory Th2 cells upon activation, whole splenocytes containing OVA-specific memory Th2 cells from memory Th2 mice were cultured with or without OVA peptide in vitro, and cytotoxicity against A20-OVA, A20, and YAC-1 cells was assessed by ⁵¹Cr release assay. Antigenic peptide treatment of splenocytes containing memory Th2 cells induced increased cytotoxicity against all cells as compared with control BALB/c or medium-treated memory Th2 mouse splenocytes (Fig. 4A). IL-2–treated splenocytes were used as a positive control and showed high cytotoxicity against all target cells. To investigate the role of CD4, CD8, and NK cells in the cytotoxicity induced by memory Th2 cells, these subsets were isolated. As shown in Figure 4B, the cytotoxicity against A20-OVA and YAC-1 cells of NK cells from memory Th2 mice was significantly increased as compared with NK cells from control BALB/c mice. No obvious increase in cytotoxicity was detected in CD4 or CD8 cells from memory Th2 mice. These results indicate that antigenic peptide-stimulated memory Th2 cells can induce NK cell–mediated cytotoxicity, although memory Th2 cells themselves are not cytotoxic.

Next, to examine the mechanisms underlying the induction of NK cell–mediated cytotoxicity induced by memory Th2 cells, neutralizing Abs to IL-2, IL-4, IL-5, or IFNγ were added to the culture (Fig. 4C). Surprisingly, neutralization of IL-4 drastically inhibited the cytotoxicity. Treatment of the culture with anti-IL-2 Ab also inhibited the ability to induce cytotoxicity but to a more moderate degree. Anti-IL-5 or anti-IFNγ Ab had no effect. The number of NK cells in the culture seemed to correlate with cytotoxic activity (Supplementary Fig. S8).

IL-4 induced cytotoxic activity in NK cells in vitro

It is reported that IL-4–treated DCs induces the activation of NK cells (20, 39). To investigate whether IL-4 acts directly on NK cells and induces their cytotoxic activity, NK cells were purified, stimulated with IL-4, and their cytotoxicity was assessed. NKp46+ cells were first prepared from splenocytes and then subjected to further purification by the depletion of B220+ cells or of CD11c+ cells. The phenotype of the purified NK cells is shown in Supplementary Figure S9. As shown in Figure 5A, significantly increased cytotoxic activity was detected in NKp46+ cells, NKp46+ cells depleted of B220+ cells, or NKp46+ cells depleted of CD11c+ cells, although the cytotoxicity induced by IL-4 was substantially lower than that of the positive control, IL-2–treated group. These results indicate that IL-4 acts directly on NK cells and induces their cytotoxic activity. We also noted that whole NKp46+ cells that contain CD11c+ DCs showed reproducibly more profound increases in cytotoxic activity than NKp46+ cells depleted of CD11c+ cells (×54.5 vs. ×11.6 in Fig. 5A).

Next, we examined whether IL-4–induced STAT6 activation is required for the induction of NK cell cytotoxicity, using Stat6+/− mice (Fig. 5B). The cytotoxicity induced in IL-4–

**Figure 3.** MLL+/− memory Th2 cells show reduced antitumor effects and NK cell activity compared with MLL+/+ memory Th2 cells. OVA-specific wt (MLL+/+) and MLL+/− memory Th2 mice were generated on the BALB/c background. A and B, mice were challenged with A20-OVA cells. A, tumor growth was monitored and is reported as mean tumor volume ± SE (n = 5 per group). *, P < 0.01 by Student’s t test. B, the percentage of living mice is indicated. *, P < 0.01 by log-rank test. C, at day 7, the expression of perforin and granzyme B in the tumor-infiltrated NKp46+ fraction was assessed by flow cytometric analysis. D, the proportion of NKp46+ cells is also shown (n = 5 per group). n.s., not significant.
treated Stat6−/− NK cells was very low as compared with control NK cells. Increased levels of both perforin and granzyme B were detected in the IL-4–treated NK cells as compared with control, although the levels were lower than those of IL-2–treated NK cells (Fig. 5C). IL-4 induced a significant NK cell proliferation in a [3H]thymidine incorporation assay as compared with medium control (Supplementary Fig. S10). We also observed increased FSChigh cells in the IL-4–cultured NK cells that were more prominently observed in the IL-2–treated group (Supplementary Fig. S11). Furthermore, IL-4–induced cytotoxicity in NK cells was inhibited by CMA, a perforin inhibitor, or z-AAD-CMK, a granzyme B inhibitor (Fig. 5D). These results indicate that IL-4 activates NK cells and induces a perforin and granzyme B–dependent cytotoxic activity.

Figure 4. IL-4–mediated NK cell cytotoxicity is induced by antigenic peptide-activated memory Th2 cells in vitro. Splenocytes derived from BALB/c (control) and memory Th2 mice were stimulated with medium, IL-2, or OVA-peptide for 2 days. A, the cytotoxicity was measured by 4-hour 51Cr release assays. B, the cytotoxicity of CD4, CD8, and NK cells isolated by magnetic beads was measured. C, indicated neutralizing Abs were added to the cultures of memory Th2 mouse splenocytes with peptide, and cytotoxicity against YAC-1 cells was measured. *, P < 0.01 by Student’s t test. Three independent experiments were conducted with similar results.

Figure 5. IL-4–induced cytotoxic activity in NK cells in vitro. A, splenic whole NKp46+, B220− NKp46+, and CD11c− NKp46+ cells were purified from BALB/c mice, stimulated with medium, IL-2, or IL-4 (100 μM/L) for 2 days, and cytotoxic activity was determined using YAC-1 cells. The number in the bar graph indicates fold increase compared with medium control. Four independent experiments were conducted with similar results. B, Stat6-deficient NK cells were stimulated with medium, IL-2, or IL-4, and cytotoxicity was measured. C, NK cells from BALB/c mice were stimulated with medium, IL-2, or IL-4 for 3 days and then expression of perforin and granzyme B was measured. Two independent experiments were conducted with similar results. D, BALB/c–derived NK cells were stimulated with IL-4, CMA (50 μmol/L), or dimethyl sulfoxide (DMSO) was added to the cell mixture of the cytotoxicity assay. Cytotoxicity against A20-OVA cells was measured. Two independent experiments were conducted with similar results. *, P < 0.01 by Student’s t test.
IL-4 regulates memory Th2 cell–mediated inhibition of tumor growth in vivo

To examine the role of IL-4 produced by memory Th2 cells in antitumor immunity in vivo, IL-4−/− × DO11.10 Tg and Stat6−− × DO11.10 Tg mice were generated (Fig. 6A). IL-4−/− memory Th2 cells and Stat6−− memory Th2 cells were substantially less effective in inhibiting the growth of OVA-expressing tumors and prolonging survival than wild-type (wt) memory Th2 mice (Supplementary Fig. S12A). To examine the role of recipient-derived IL-4 in memory Th2 cell–induced antitumor immunity, memory Th2 cells were generated in IL-4−/− mice, and these mice were challenged A20-OVA cells. As expected, wt Th2 cell–transferred IL-4−/− memory mice inhibited tumor growth and prolonged survival as effectively as control mice (data not shown). These results indicate that IL-4 plays a crucial role in memory Th2 cell–mediated tumor eradication in this model.

Finally, we assessed the antitumor effect of IL-4 treatment in memory Th2 mice transferred with MLL−/− effector Th2 cells. It has previously been shown that injection of IL-4 directly into a tumor results in tumor clearance (3) and, as expected, injection of IL-4 resulted in a decrease in tumor growth and prolongation of survival (Fig. 6B and Supplementary Fig. S12B). IL-4 injection in MLL−/− memory Th2 mice resulted in a significant inhibition of tumor growth and increased survival compared with PBS-injected MLL−/− memory Th2 mice. Importantly, the inhibition of tumor growth and increase in survival was similar to the marked benefit observed in MLL+/+ memory Th2 mice (Fig. 6B and Supplementary Fig. S12B). These results indicate that the decrease in antitumor effects of MLL−/− memory Th2 mice is positively compensated by IL-4 treatment.

Discussion

In this report, we identify an important cellular network in memory Th2 cell–mediated antitumor immunity, wherein IL-4 produced by activated memory Th2 cells induces a potent antitumor effect through the activation of NK cells (see Fig. 6C). IL-4–deficient or STAT6-deficient memory Th2 cells showed a defect in the induction of antitumor effects in vivo. Functionally impaired MLL−/− memory Th2 cells (37) also failed to induce full antitumor activity, and this effect was rescued by intratumoral injection of IL-4. Thus, memory Th2 cells may have the potential to be used as a cell therapy for cancer patients.

Effector Th2 cell–mediated antitumor activity has been shown particularly in collaboration with tumor-infiltrating granulocytes, such as eosinophils (6, 7). IL-4 in particular can induce the infiltration of eosinophils, macrophages, and, in some cases, neutrophils and lymphocytes into the tumor, and this correlates with tumor clearance (2–4). No significant difference in the number of eosinophils, macrophages, or neutrophils was detected between MLL−/− and MLL+/+ memory Th2 mouse groups; although the average number of tumor cells per section was significantly lower in MLL−/− memory

Figure 6. IL-4 regulates memory Th2 cell–mediated inhibition of tumor growth in vivo. A, OVA-specific wt, IL-4−/−, and Stat6−− memory Th2 mice were challenged with 6 × 10⁶ A20-OVA tumor cells. B, OVA-specific wt (MLL+/+) and MLL−/− memory Th2 mice were challenged with A20-OVA cells and injected with PBS or IL-4 in PBS (1 μg/mouse) intratumorally at day 1. Tumor growth was monitored and is reported as mean tumor volume ± SE (n = 6–8 per group). *, P < 0.01 by Student’s t test. Two independent experiments were conducted with similar results. C, the model shows how memory Th2–mediated immunity induces tumor clearance. Antigen-loaded DCs induce the activation of memory Th2 cells and then secretion of IL-4 and IL-5 from memory Th2 cells induces the infiltration of polymorphonuclear cells, such as eosinophils, to exhibit antitumor effects (left side of the schema). In addition, IL-4 produced by memory Th2 cells activates DCs (20) and also acts directly on NK cells and then NK cells induce antitumor activity in the tumor microenvironment (right side of the schema; red arrows). This newly identified pathway is shown in the current study.
Th2 mice than that of MLL\(^{+/−}\) memory Th2 mice (unpublished observation). Therefore, it is unlikely that in our memory Th2 mouse system, polymorphonuclear leukocytes are the primary effector cells acting on the target tumors. Terme and colleagues have previously shown that IL-4 acts on DCs to induce their maturation and that mature DCs induce proliferation and activation of NK cells (20). This report indicated for the first time that IL-4 positively regulates NK cell activation and proliferation via DC-NK cell cross-talk. In contrast, the current study shows another IL-4-mediated NK cell activation pathway, that is, IL-4 acts directly on NK cells and enhances their cytotoxicity. We have purified B220\(^{+}\) NK cells and CD11c\(^{+}\) NK cells by MACS (Miltenyi Biotec) and fluorescence-activated cell sorting, respectively, and stimulated them with IL-4 and shown their increased cytotoxic activity (Fig. 5A). It is reported that mouse NK cells express IL-4R and respond to IL-4 and differentiate into so-called NK2 cells that produce type 2 cytokines (40). Loza and colleagues also showed that human NK cells respond to IL-4 and become NK2-like cells (41). These results may indicate that IL-4 can directly modulate the function of NK cells. In addition, NKp46\(^{+}\) NK cells containing CD11c\(^{−}\) cells showed a more dramatic increase in the levels of cytotoxic activity (more than 50-fold) in NK cells in response to IL-4 (Fig. 5A). This may indicate that IL-4 confers NK stimulatory capacity to murine DCs as reported by Terme and colleagues (20).

Very recently, Brady and colleagues have shown that IL-4 decreases NKG2D-dependent function of NK cells, both in vitro and in vivo (42). They injected an IL-4-expressing vector (pOrf-IL-4) into Rag\(^{−/−}\) mice and measured the expression levels of several NK receptors, including NKG2D. Dramatically decreased expression of NKG2D and reduced NK cell cytotoxic activity were shown, and this was accompanied by very high levels of IL-4 in the serum of these mice (~80 ng/mL). We examined the expression of NKG2D on NK cells in the TILs, and only a slight reduction in NKG2D expression was detected (Supplementary Fig. S13). Thus, IL-4 produced in the tumor microenvironment of memory Th2 mice may not be as high as in the mice used by Brady and colleagues. Although it is not clear at this time, low and high levels of IL-4 may have a distinct effect on NK cells. Allavena and colleagues reported that IL-4 negatively regulates adhesion and transendothelial migration of human NK cells (43). Although we observed increased memory Th2 cell infiltration in the tumor region of MLL\(^{+/−}\) memory Th2 mice as compared with that in MLL\(^{+/+}\) memory Th2 mice (Supplementary Fig. S7B), no significant difference was detected in the infiltration of NK cells in the tumor region (Fig. 3D). In our mouse experimental system, no effect of IL-4 on the migration of NK cells was observed. This could be due to either the difference between mouse and human systems or the selective involvement of certain human NK cell subpopulations (44–46) in the IL-4-mediated regulation of NK cell migration.

In addition to NK cells, CD8 T cells are also involved in the memory Th2 cell–mediated antitumor effects identified in this study (Fig. 2A and B). These data are in agreement with previous studies that have identified a link between the production of IL-4 and CD8 T cell–mediated antitumor immunity (47). In addition, Schuler and colleagues reported that antitumor immunity induced by Th1 and CD8\(^{+}\) CTLs was impaired in IL-4–deficient mice because of the lack of DC maturation (48, 49), although the high-dose immunization did induce a moderate antitumor response in the IL-4–deficient mice. Thus, although a more comprehensive study is required, memory Th2 cells may induce Tc2 cells in an IL-4–dependent manner, or via the activation of NK cells, and may also facilitate the induction of CD8\(^{+}\) CTLs through the maturation of DCs, resulting in efficient antitumor effects against both NK-sensitive and -nonsensitive tumors.

In summary, memory Th2 cells are found to induce long-lasting antitumor effects via IL-4–mediated NK cell activation. Thus, memory Th2 cells may have the potential to be used as a cell-based therapy for cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Memory Th2-Mediated Antitumor Effect via NK Cell Activation


Memory Type 2 Helper T Cells Induce Long-Lasting Antitumor Immunity by Activating Natural Killer Cells

Masayuki Kitajima, Toshihiro Ito, Damon J. Tumes, et al.

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