

Th17 and Th17-stimulated CD8⁺ T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity

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Abstract CD4⁺ Th17 cells induce antitumor immunity leading to the eradication of established tumors. However, the mechanism of antitumor immunity and CTL activation by Th17 cells and the distinct role of Th17 and Th17-activated CTLs in antitumor immunity are still elusive. In this study, we generated ovalbumin (OVA)-specific Th17 cells by cultivating OVA-pulsed dendritic cells with CD4⁺ T cells derived from transgenic OTII mice in the presence of IL-6, IL-23, TGF- β , and anti-IFN- γ antibody. We demonstrated that Th17 cells acquired major histocompatibility complex/peptide (pMHC)-I and expressed ROR γ t, IL-17, and IL-2. Th17 cells did not have any direct in vitro tumor cell-killing activity. However, Th17 cells were able to stimulate CD8⁺ CTL responses via IL-2 and pMHC I, but not IL-17 signaling, which play a major role in Th17-induced preventive immunity against OVA-expressing B16 melanoma. Th17 cells stimulated the expression of CCL2 and CCL20 in lung tumor microenvironments promoting the recruitment of various inflammatory leukocytes (DCs, CD4⁺, and CD8⁺ T cells) stimulating more pronounced therapeutic immunity for

early-stage (5-day lung metastases or 3 mm, s.c.) tumor than for well-established (6 mm, s.c.) tumor. The therapeutic effect of Th17 cells is associated with IL-17 and is mediated by Th17-stimulated CD8⁺ CTLs and other inflammatory leukocytes recruited into B16 melanoma via Th17-stimulated CCL20 chemoattraction. Taken together, our data elucidate a distinct role of Th17 and Th17-stimulated CD8⁺ CTLs in the induction of preventive and therapeutic antitumor immunity, which may greatly impact the development of Th17-based cancer immunotherapy.

Keywords Th17 · pMHC I complexes · CD8⁺ CTL · CCL2/20 · Antitumor immunity

Introduction

Effector CD4⁺ T cells are classically divided into two lineages based on distinct cytokine secretion profiles: the IFN- γ -producing Th1 lineage and IL-4/IL-13-producing Th2 lineage. Recently, a lineage of effector Th17 cells that produce IL-17A and IL-17F and express the transcription factor ROR γ t through activation of STAT3 by IL-6 and IL-23 have been identified [1]. IL-17 cytokines are strongly proinflammatory and induce the expression of several chemokines such as CCL2, CCL7, and CCL20. Transgenic overexpression of IL-17A in the lungs provokes proinflammatory gene expression and tissue infiltration of leukocytes [2]. In contrast, inhibition of IL-17A expression impairs host defense against bacterial infection [3] and resistance to autoimmune diseases [4, 5].

Th17 cells and IL-17 expression have been found in various human tumors [6–10]. However, the involvement of IL-17 and Th17 cells in antitumor immunity is still controversial. For example, transgenic IL-17 expression

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either induced tumor regression through enhanced antitumor immunity in immune-competent mice [11, 12] or promoted tumor progression through an increase in inflammatory angiogenesis in immune-deficient mice [13]. It has been demonstrated that Th17 cells were indirectly linked to antitumor immunity [14]. Tumor-specific Th17 polarized cells were found to inhibit the growth of well-established melanoma via IFN- γ production [15]. However, the nature of Th17 cell's role in the context of antitumor immunity still remains largely unknown. Th17 cells have been shown to stimulate antitumor immunity in both prevention and therapeutic models by recruiting DCs, granulocytes, and CD4⁺ and CD8⁺ T cells [16]. However, (i) the molecular mechanism of CD8⁺ CTL activation by Th17 cells, (ii) the precise role of Th17 secreted IL-17 and different types of recruited leukocytes in Th17-induced antitumor immunity, and (iii) the potentially distinct role of Th17 and Th17-stimulated CD8⁺ CTLs in Th17-induced antitumor immunity are still unknown.

Intercellular membrane transfer through trogocytosis plays an important role in immune modulation [17]. We have recently demonstrated that CD4⁺ T cells derived from ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic OTII mice, which were activated in vitro with OVA-pulsed DCs (DC_{OVA}), differentiated into Th1 cells [17]. These Th1 cells acquired peptide major histocompatibility complexes I (pMHC I) and CD80 from DC_{OVA} by DCova activation and became capable of directly stimulating CD8⁺ CTL responses via endogenous IL-2 and acquired pMHC I and CD80 signaling [18, 19]. However, whether Th17 cells with distinctive phenotype from Th1 cell have a similar stimulatory effect as Th1 cells on the stimulation of CD8⁺ CTL responses is elusive.

In this study, we generated ROR γ t-expressing and IL-17-secreting OVA-specific Th17 cells by cultivation of OTII CD4⁺ T cells with OVA-pulsed DC_{OVA} in the presence of IL-6, IL-23, TGF- β , and anti-IFN- γ antibody. We then immunized C57BL/6 mice with these Th17 cells to assess the potential stimulatory effect on CD8⁺ T-cell responses and antitumor immunity in preventive and therapeutic models against OVA-expressing B16 melanoma (BL6-10_{OVA}).

Results

Th17 acquires pMHC I complexes by DC_{OVA} activation

Transgenic mouse OT II CD4⁺ T cells activated with irradiated DC_{OVA} in the presence of IL-23/IL-6/TGF- β and anti-IFN- γ antibody expressed cell surface FasL, intranuclear ROR γ t, and intracellular perforin and IL-17 by flow

cytometric and RT-PCR analysis (Fig. 1a, c). By using double staining for IL-17 and IFN- γ , polarized Th17 and Th1 cells expressed intracellular IL-17 and IFN- γ , respectively (Fig. 1b). These Th17 cells also secreted IL-2 (2.8 ng/ml), IL-6 (4.5 ng/ml), IL-17 (1.8 ng/ml), and TGF- β (0.2 ng/ml) by ELISA analysis, indicating that they are Th17 cells, which is consistent with a recent report showing that Th17 simultaneously expressed both IL-17 and IL-2 [20]. There was no CD11c⁺ DC_{OVA} contamination in these purified Th17 population (Fig. 1d). In addition, these Th17 cells did display pMHC I (Fig. 1a), indicating that they may acquire pMHC I from DC_{OVA} upon DC_{OVA} activation. This was confirmed by evidence that CD4⁺ T cells derived from pMHC I-negative (K^b-/-)DC_{OVA} activation did not express pMHC I (Fig. 1e).

Th17 stimulates in vitro CD8⁺ T-cell proliferation via IL-2 and pMHC I, but not IL-17 signaling

We previously demonstrated that DCova-activated Th1 with acquired pMHC I stimulated CTL responses via IL-2 and pMHC I signaling [18, 19]. To assess Th17's stimulatory effect, we performed ³H-thymidine incorporation assay. DCova-activated Th17 with acquired pMHC I also stimulated in vitro OTI CD8⁺ T-cell proliferation in a dose-dependent fashion. Interestingly, (K^b-/-)Th17 without acquired pMHC I or Th17 in the presence of anti-IL-2 Ab, but not anti-IL-17 Ab, failed to stimulate CD8⁺ T-cell proliferation (Fig. 2a), indicating that the in vitro Th17's stimulatory effect on CTLs is via IL-2 and pMHC I, but not via IL-17 signaling.

Th17-activated CD8⁺ T, but not Th17, cells have in vitro cytotoxicity

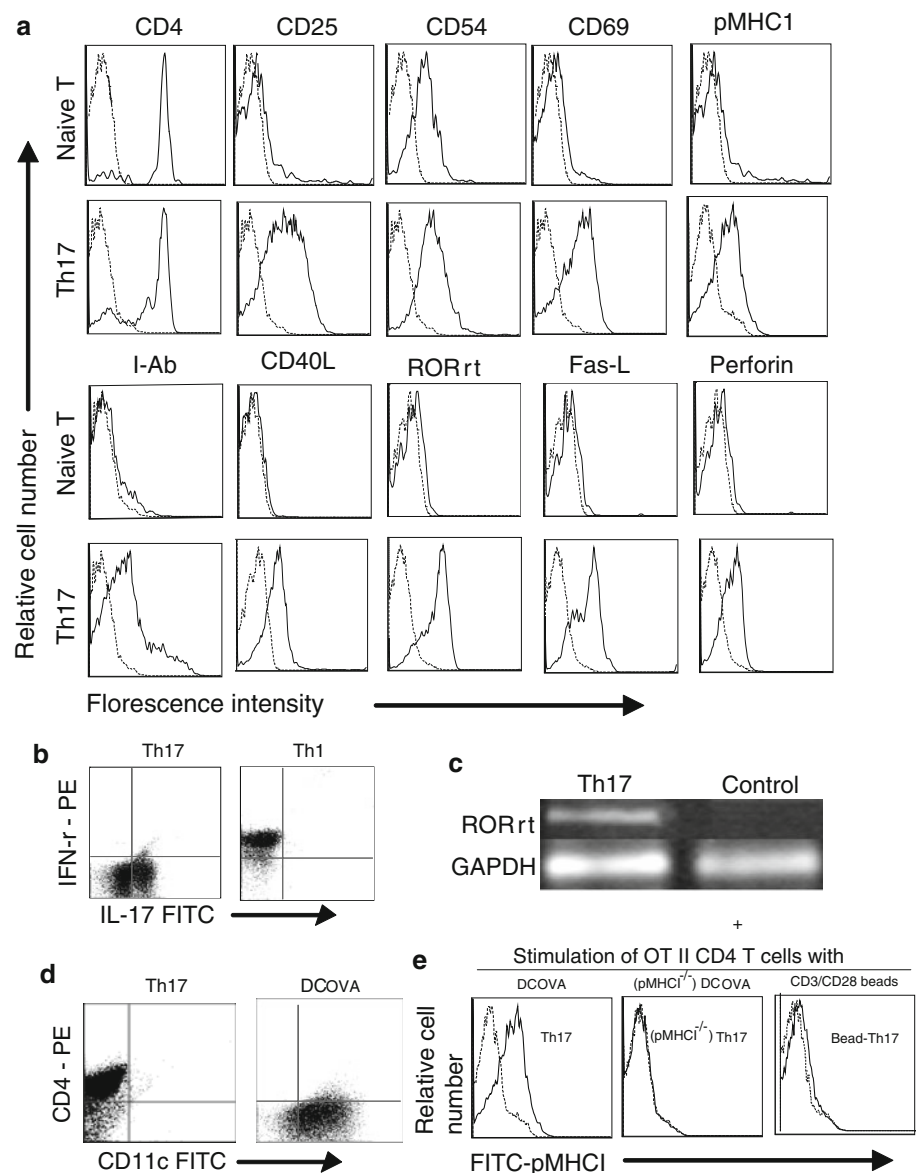
Since Th17 expressed cytotoxic FasL and perforin, they may have killing activity to pMHC II-expressing target cells. To assess their killing effect, we performed a chromium release assay. We found that Th17-activated CTLs showed killing activity to OVA-expressing EG7 tumor cells, and the killing activity was significantly ($P < 0.01$) or slightly ($P > 0.05$) reduced when T cells were preincubated with CMA or emetin, indicating that CD8⁺ T-cell-mediated killing activity is mainly via perforin pathway (Fig. 2b) [21]. In addition, we found that Th1 [19], but not Th17, killed OVAII peptide-pulsed LB27 cells (Fig. 2c), indicating that Th17 do not have any direct killing activity to tumor cells.

Th17 stimulates the host DC-independent CD8⁺ CTL responses

To assess DCova-activated Th17's ability to induce in vivo CD8⁺ T-cell proliferation, we i.v. transferred B6 mice with

Fig. 1 Phenotypic analysis of OVA-specific Th17 cells.

a Naïve CD4⁺ T cells and in vitro DC_{OVA}-activated Th17 were stained with a panel of antibodies (*solid lines*) and analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated antibodies were used as controls (*light dotted lines*). **b** In vitro DC_{OVA}-activated Th1 and Th17 cells were double stained with FITC-anti-IL-17 Ab and PE-anti-IFN- γ Ab and analyzed by flow cytometry. **c** RNA extracted from DC_{OVA}-activated Th17 and ConA-stimulated CD4⁺ T (control) cells were analyzed by RT-PCR to assess the expression of ROR- γ t. **d** DC_{OVA} and Th17 were stained with PE-anti-CD4 and FITC-anti-CD11c Abs and analyzed by flow cytometry. **e** Th17, (K^{b-/-}), Th17 cells, and CD3/CD28 bead-activated Th17 (bead-Th17) cells were stained with FITC-anti-pMHC I antibody (*solid lines*), and irrelevant isotype-matched antibody was used as control (*dotted lines*). One representative experiment of two experiments is shown



DCova and Th17 and then performed an OVA-specific tetramer staining assay to detect OVA-specific CD8⁺ T-cell proliferation [19]. As shown in Fig. 2d, DCova and Th17 stimulated OVA-specific CD8⁺ T cells accounted for 1.43 and 0.98% of the total CD8⁺ T-cell population, respectively. To assess whether the host DCs are involved in Th17-stimulated CTL responses by the uptake of antigen epitopes of Th17, we i.v. transferred the transgenic DTR-CD11c mice with DT treatment for complete depletion of endogenous DCs and macrophages (Clin Exp Immunol 141: 398, 2005) with Th17 and then performed an OVA-specific tetramer staining assay to detect OVA-specific CD8⁺ T-cell proliferation. We found that Th17-stimulated OVA-specific CTL responses in PBS- and DT-treated DTR-CD11c mice with and without endogenous APCs

were similar (Fig. 2d), indicating that Th17 stimulates the host DC-independent CD8⁺ CTL responses.

Th17 stimulates CTL-mediated preventive antitumor immunity via IL-2 and pMHC I, but not via IL-17 signaling

To elucidate the molecular mechanism of Th17-stimulated CTL responses, DCova-activated Th17, (IL-2^{-/-})Th17 with IL-2 deficiency, (K^{b-/-})Th17 without acquired pMHC I, and CD3/CD28 bead-activated Th17 (bead-Th17) without pMHC I expression (Fig. 1e) were used in the in vivo proliferation and cytotoxicity experiments. To assess the involvement of IL-17, Th17 cell transferred mice were treated with anti-IL-17 Ab to block IL-17 effect. As shown

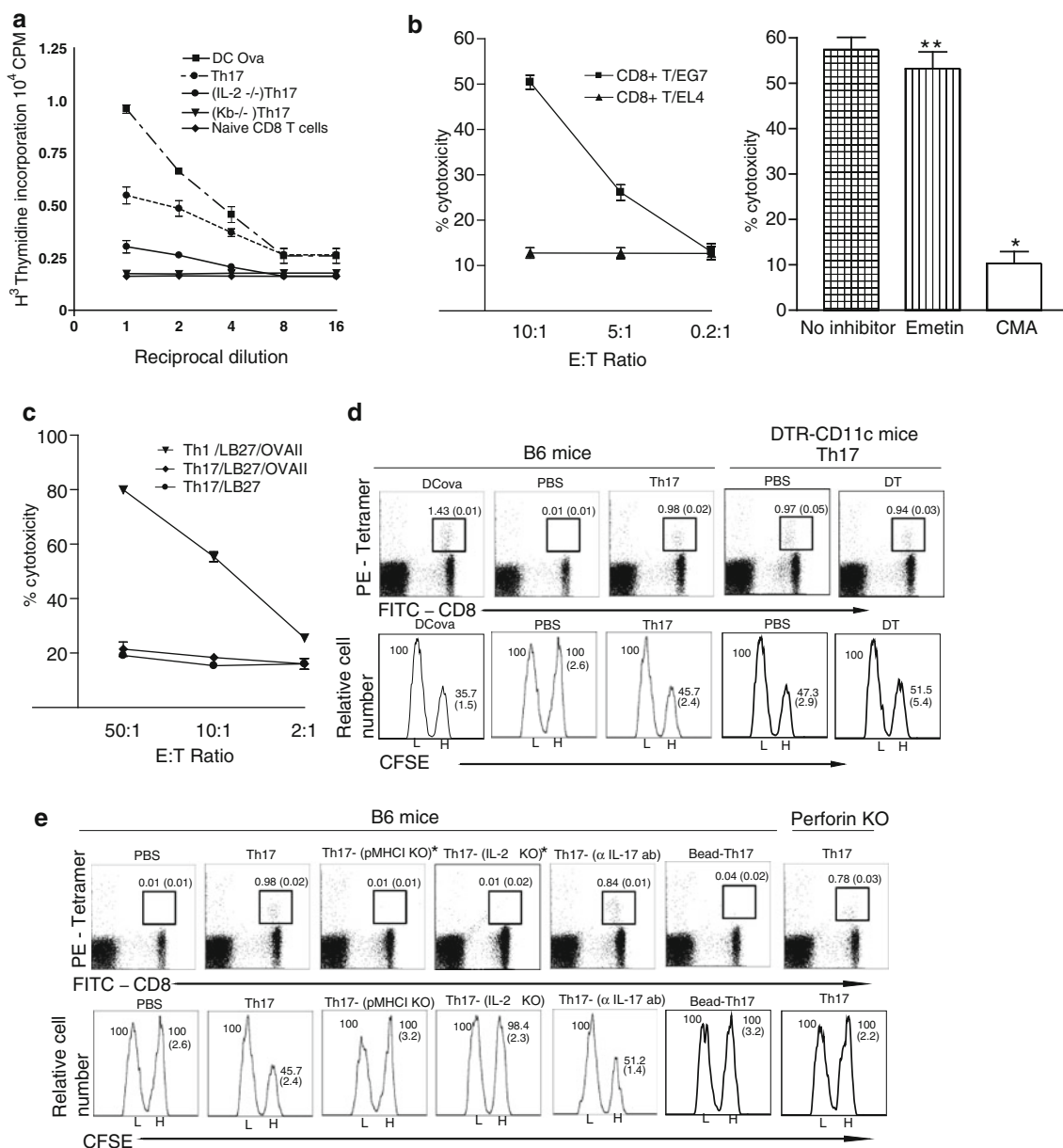


Fig. 2 Functional effect analysis of OVA-specific Th17 cells. **a** Irradiated DC_{OVA}, Th17, (IL-2^{-/-})Th17 and (K^b-/-)Th17, and their 2-fold dilutions were co-cultured with OTI CD8⁺ T cells. After 2 days, the proliferative responses of CD8⁺ T cells were determined by overnight ³H-thymidine uptake assay. **b** Th17-activated CD8⁺ T cells with or without preincubation of concanamycin A (CMA, 1 μ M) or emetin (5 μ M) for 2 h were used as effector (E) cells, while ⁵¹Cr-labeled EG7 and EL4 cells were used as target (T) cells. **c** DC_{OVA}-activated Th17 and Th1 were used as effector (E) cells, while ⁵¹Cr-labeled OVAII-pulsed LB27 cells and LB27 cells were used as target (T) cells. **P* < 0.01 and ***P* > 0.05 versus cohorts of ‘no inhibitor’ group (*Student’s t test*). **d** In tetramer staining assay, the tail blood samples of wild-type B6 or DT-treated DTR-CD11c mice (6 per group) transferred with Th17 were stained with PE-H-2K^b/OVA I (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8) and then analyzed by flow cytometry. **e** In tetramer staining assay, the tail

blood samples from wild-type B6 mice (6 per group) transferred with DCova-activated Th17, Th17 with various KO and CD3/CD28 bead-activated Th17 (bead-Th17) or from anti-IL-17 Ab-treated B6 mice transferred with Th17 or perforin^{-/-} mice were stained with PE-H-2K^b/OVA I (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8) and then analyzed by flow cytometry. The value represents the percentage of tetramer-positive CD8⁺ T cells in the total CD8⁺ T-cell population with standard deviation in parenthesis. In *in vivo* cytotoxicity assay (in both panel d and e), the residual CFSE^{high} (H) and CFSE^{low} (L) target cells remaining in the recipients’ spleens (6 per group) were analyzed by flow cytometry. The value in each panel represents the percentage of CFSE^{high} vs CFSE^{low} target cells remaining in the spleen. (*n* = 6, average \pm SD). **P* < 0.05 versus cohorts of mice immunized with Th17 (*Student’s t test*). One representative experiment of two is shown

in Fig. 2e, the stimulatory effect was significantly reduced in mice transferred with ($K^b^{-/-}$)Th17, bead-Th17, and ($IL-2^{-/-}$)Th17 ($P < 0.05$), but not in Th17-transferred mice with the treatment of anti-IL-17 Ab, indicating that endogenous IL-2 and acquired pMHC I, but not IL-17 signaling influence in vivo Th17's stimulatory effect. In in vivo cytotoxicity assay, we found that Th17-transferred mice showed substantial loss of OVA-specific CFSE^{high} cells, indicating that Th17 can stimulate CD8⁺ T-cell differentiation into effector CTLs with killing activity for OVA-specific target cells in vivo. To assess the pathway responsible for the killing activity of CD4⁺ Th17-stimulated CD8⁺ T cells in vivo, we also transferred CD4⁺ Th17 cells into perforin^{-/-} mice and repeated the above-stated tetramer staining and in vivo cytotoxicity assays. We found that OVA-specific CD8⁺ T-cell in vivo proliferation in C57BL/6 and perforin^{-/-} mice were similar (Fig. 2e). However, CD8⁺ T-cell-induced killing activity to OVA-specific CFSE^{high} target cells was lost in perforin^{-/-} mice (Fig. 2e), indicating that the in vivo CD4⁺ Th17-stimulated CD8⁺ T-cell-induced killing activity to OVA-specific target cells is via perforin-dependent pathway. Interestingly, ($IL-2^{-/-}$)Th17-, ($K^b^{-/-}$)Th17-, and bead-Th17-transferred mice maintained their OVA-specific CFSE^{high} target cell numbers, indicating that in vivo stimulatory effect of Th17 is mediated by its IL-2 secretion and pMHC I targeting. To assess preventive antitumor immunity, we performed animal studies by i.v. injection of BL6-10_{OVA} cells into the above-transferred mice 6 days subsequent to transfer. We found that all mice (8/8) were free from metastasis, whereas all (8/8) Th17-transferred mice with treatment of anti-CD8 Ab completely lost their antitumor immunity (Exp I of Table 1), indicating that Th17-induced antitumor immunity is mainly mediated by CD8⁺ T cells. We also found that all (8/8) ($IL-2^{-/-}$)Th17- and ($K^b^{-/-}$)Th17-transferred, but not mice with treatment of anti-IL-17 Ab, lost their antitumor immunity (Exp I of Table 1), indicating that Th17's stimulatory effect on preventive antitumor immunity is also mediated by IL-2 (not IL-17) signaling and pMHC I targeting. To assess the long-term immunity, we also challenged Th17-transferred mice 60 days after the primary immunization. As shown in Exp II of Table 1, all transferred mice had a long-term protective antitumor immunity.

IL-17 is associated with DCova-activated Th17-induced eradication of early-stage (5 day) lung tumor metastases

To assess Th17's therapeutic effect, we i.v. injected C57BL/6 mice with BL6-10_{OVA} tumor cells. Five days after tumor cell injection, mice were i.v. transferred with DCova-activated Th17 cells with pMHC I expression and

Table 1 Vaccination with Th17 protects against lung tumor metastases in mice

Immunization ^a	Tumor-bearing mice (%)
Experiment I	
Th17	0/8 (0)
Th17($IL-2^{-/-}$)	8/8 (100)
Th17($K^b^{-/-}$)	8/8 (100)
Th17 + anti-IL17 Ab	0/8 (0)
Th17 + anti-CD8 Ab	8/8 (100)
Th17 + control Ab	0/8 (0)
PBS	8/8 (100)
Experiment II	
Th17-immunized mice	0/8 (0)
PBS-treated mice	8/8 (100)

^a In Experiment I, C57BL/6 mice (8 per group) were i.v. injected with Th17 or Th17 with gene deficiency or Th17 plus antibody. Six days after Th17 injection, mice were challenged with BL6-10_{OVA} cells. In Experiment II, C57BL/6 mice (8 per group) were i.v. injected with Th17. Sixty days after Th17 injection, mice were challenged with BL6-10_{OVA} cells. The mice were killed 3 weeks after tumor cell challenge, and lung metastatic tumor colonies were counted. One representative experiment of two is shown

CD3/CD28 bead-activated Th17 (bead-Th17) cells without pMHC I expression. Lung tumor colonies were numerated 10 days after transfer. Compared with untreated control mice, those mice transferred with DCova-activated Th17, but not with bead-Th17, had significantly fewer tumor foci ($P < 0.05$) (Fig. 3a), indicating that DCova-activated Th17 cells have efficient therapeutic effect on early-stage (5 day) tumor lung metastasis via acquired pMHC I signaling. To assess the role of Th17-secreted IL-17 in DCova-activated Th17-induced antitumor immunity, we treated immunized mice with anti-IL-17 Ab to block IL-17 signaling. We found that Th17-transferred mice with treatment of anti-IL-17 Ab had numerous tumor foci as the control mouse group (Fig. 3a), indicating that IL-17 is critically involved in DCova-activated Th17-induced therapeutic antitumor immunity.

Th17 induces recruitment of leukocytes into tumors via Th17-stimulated CCL2/CCL20 expression

To assess the potential Th17 stimulated CCL2/CCL20 expression, we first analyzed the expression of CCL2/CCL20 by lung cells using real-time PCR. We found that BL6-10 tumor cells and normal lungs did not express any CCL2/CCL20 chemokines (Fig. 3b). However, the expression of CCL2/CCL20 was greatly increased in lung cell fractionations containing both tumor and lung cells, indicating that Th17 cells stimulate expression of CCL2/

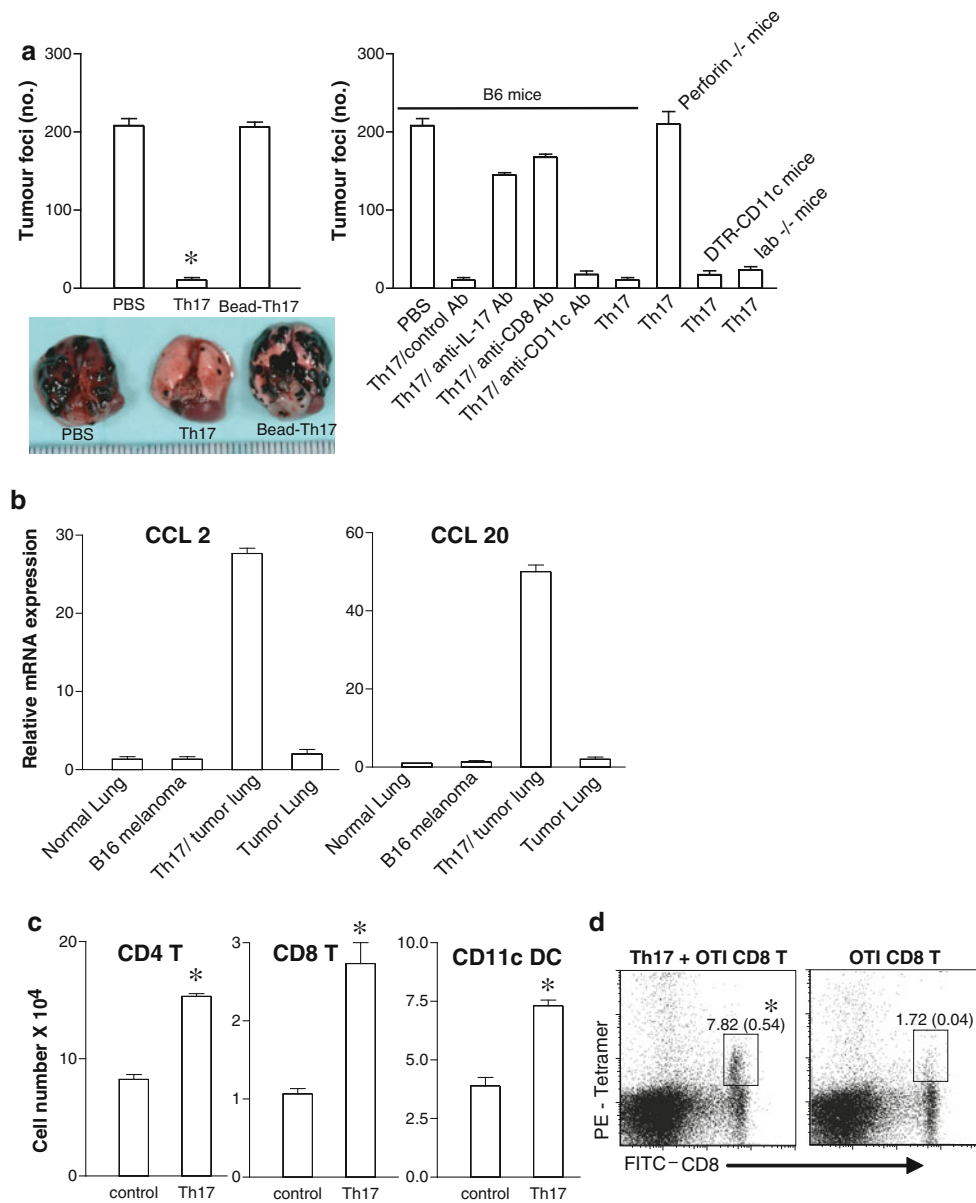


Fig. 3 Therapeutic effect of OVA-specific Th17 cells on eradication of established lung tumor metastasis. **a** C57BL/6 mice or $Ia^{b-/-}$ KO mice or toxin-treated DTR-CD11c mice or perforin^{-/-} mice bearing 5-day lung B16 melanoma were i.v. immunized with DCova-activated Th17 with or without various Abs against CD8, CD11c, IL-17 for depletion of host CD8⁺ T cells, DCs, and cytokine, respectively. C57BL/6 mice bearing 5-day lung B16 melanoma were also i.v. immunized with CD3/CD28 bead-activated Th17 (bead-Th17). Tumor colonies were counted on day 16 after tumor challenge ($n = 8$, average \pm SD). * $P < 0.05$, versus cohorts of tumor foci in mice injected with control Ab (Student's t test). **b** mRNA gene

expression analysis was assessed by RT-PCR. Data shown were normalized to the reference gene GAPDH. Graphs represent the average values of four mice after duplicate analysis per sample ($n = 4$, average \pm SD). **c** Total numbers of leukocytes from cell fractions of tumor-bearing lungs were calculated from percentages of live cells gated on CD45.2 ($n = 4$, average \pm SD). **d** 5-day lung tumor-bearing mice were transferred with 3×10^6 OTI CD8⁺ T cells and after 3 days percentages of OTI CD8⁺ T cells out of total CD8⁺ T-cell population in lungs were measured by gating on FITC-CD8 and PE-tetramer-positive T cells ($n = 4$, average \pm SD). One representative experiment of two is shown

CCL20. Further analysis of lung leukocyte fractionations by flow cytometry revealed that CD11c⁺ DCs, CD4⁺, and CD8⁺ T cells were significantly increased in Th17-treated mouse lungs (Fig. 3c) ($P < 0.05$). To assess the potential

recruitment of OVA-specific CD8⁺ T cells, we transferred DC_{OVA}-activated OTI CD8⁺ T cells into B6 mice bearing 5-day lung tumor metastasis and then numerated tumor-infiltrating CD8⁺ T cells by flow cytometry. We found that

Th17 significantly promoted tumor infiltration of OVA-specific CD8⁺ T cells indicating that Th17 cells promotes tumor infiltration of OVA-specific CD8⁺ T cells via CCL20 chemoattraction.

Th17-activated CD8⁺ T cells mediate therapeutic effect via perforin-dependent pathway on early-stage (5 day) lung tumor metastases

To assess the role of recruited DCs, CD4⁺, and CD8⁺ T cells, the above immunized C57BL/6 mice were treated with different antibodies to deplete CD8⁺ T cells or DCs. Alternatively, DTR-CD11c transgenic mice with the treatment of toxin to deplete the host DCs were transferred with Th17. To assess the role of recruited CD4⁺ T cells in Th17-induced therapeutic effect, we also i.v. transferred Ia^{b-/-} KO mice with Th17, which lack the host CD4⁺ T cells. We found that only the mice treated with anti CD8 Ab (host CD8⁺ T cells depleted), but not mice with depletion of host DCs (anti CD11c Ab or DTR-CD11c mice treated with toxin) or lacking host CD4⁺ T cells (Ia^{b-/-} KO mice), showed numerous tumor foci (Fig. 3a), indicating that Th17-stimulated CD8⁺ CTLs play an important role, but the host DCs and CD4⁺ T cells recruited into tumor tissues are not involved in Th17-induced therapeutic effect. To assess the molecular mechanism of CD8⁺ T-mediated killing, we repeated Th17 treatment in tumor-bearing perforin^{-/-} mice, where Th17-stimulated host OVA-specific CD8⁺ T cells are perforin deficient. We found that Th17-transferred perforin^{-/-} mice showed numerous tumor foci (Fig. 3a), indicating that Th17-stimulated CTLs mediate therapeutic effect via perforin-dependent pathway.

Th17-activated CD8⁺ T cells are potent in the eradication of early-stage (3 mm), but not well-established (6 mm), s.c. tumors

To determine whether Th17 protects against tumors in different tissues in addition to lung tissues, we s.c. inoculated B6 mice with BL6-10_{OVA} cells. We then treated mice bearing different sizes (early stage: 3 mm and well-established tumor: 6 mm) of B16 melanoma with Th17. We found that all tumor (3 mm)-bearing mice (8/8) died of tumor within 21 days without treatment, whereas all 8/8 tumor-bearing mice with treated with Th17 survived (Fig. 4a), indicating that DCova-activated Th17 cells have immunotherapeutic effect on early-stage tumors. To assess the role of IFN- γ expression, and host CD4⁺ and CD8⁺ T cells in the immunotherapeutic effect, we used IFN- γ ^{-/-}, Ia^{b-/-}, and H-2 K^{b-/-} KO mice in the above experiments. We found that DCova-activated Th17 cell-induced therapeutic effect was not affected in IFN- γ ^{-/-} and Ia^{b-/-} KO mice (Fig. 4b), indicating that Th17-induced therapeutic effect is not mediated via host IFN- γ and CD4⁺ T cells. However, its therapeutic effect was completely lost in H-2 K^{b-/-} KO mice lacking host CD8⁺ T cells (Fig. 4b), confirming that Th17-stimulated host CD8⁺ CTLs play a major role in Th17-induced therapeutic antitumor immunity. To assess the potential immunotherapeutic effect on well-established tumors, we repeated experiments in mice bearing BL6-10_{OVA} tumors (6 mm). We found that none (0/8) of the treated mice survived though their survival was significantly prolonged ($P < 0.05$) (Fig. 4c), indicating that DCova-activated Th17 cells, though having therapeutic effect, are not potent enough in well-established tumors.

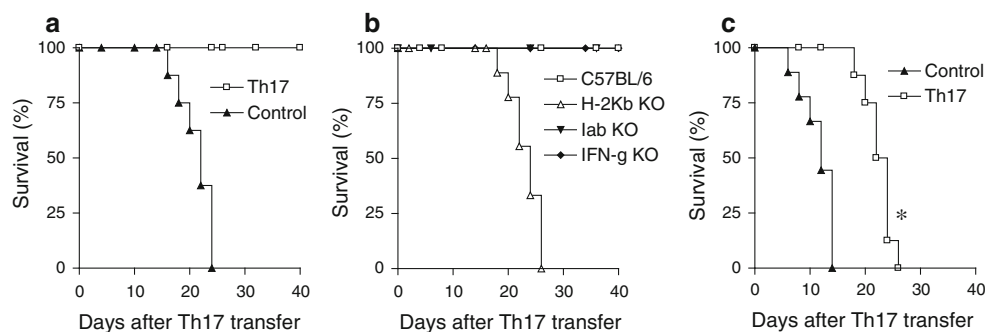


Fig. 4 Therapeutic effect of OVA-specific Th17 cells on the eradication of established s.c. tumors. **a** Wild-type C57BL/6 mice ($n = 8$) bearing early-stage (3 mm) B16 melanoma were i.v. immunized with DCova-activated Th17 cells or PBS as a control. **b** Wild-type C57BL/6, IFN- γ ^{-/-}, H-2 K^{b-/-}, and Ia^{b-/-} gene KO mice ($n = 8$) bearing early-stage (3 mm) B16 melanoma were i.v. immunized with DCova-activated Th17 cells. Tumor regression or growth was monitored. The evolution of the tumors in individual

mouse is depicted for their survival period. **c** Wild-type C57BL/6 mice ($n = 8$) bearing well-established (6 mm) B16 melanoma were i.v. immunized with Th17. Tumor size was measured daily using an engineering caliper. The evolution of the tumors in individual mouse is depicted for their survival period. * $P < 0.05$ versus cohorts of control group (Log rank test). One representative experiment of three is shown

Discussion

Th17 cells are an important inflammatory component and have been shown to promote inflammation in autoimmune diseases [22, 23]. Recent evidence suggests that Th17 cells are also involved in tumor immunology and may be a target for cancer immunotherapy [24]. We have previously demonstrated that Th1 cells acquired pMHC I complexes and co-stimulatory molecules from DC_{OVA} upon DC_{OVA} activation and became capable of stimulating OVA-specific CD8⁺ CTL responses via IL-2 and pMHC I signaling and antitumor immunity [15, 19]. In our current study, we demonstrated for the first time that (i) *in vitro* DC_{OVA}-activated Th17 cells expressing ROR γ t, IL-17, and IL-2 also acquire pMHC I complexes upon activation by DC_{OVA} and (ii) Th17 with cytokine profile distinct from Th1 is also capable of stimulating CD8⁺ CTL responses and long-term memory via IL-2 and pMHC I, but not IL-17 signaling. Endogenous IL-2 of Th17 cells is important in CTL induction, though IL-2 has been shown to constrain Th17 generation from CD4⁺ T precursors via STAT5 [25]. In addition, we also demonstrated that Th17-induced preventive antitumor immunity is mainly mediated by Th17-stimulated CTLs.

In the therapeutic model for early-stage (5 day) lung tumor metastases, we found that (i) it is Th17-activated CD8⁺ T cells that play a major role in the eradication of lung metastatic tumors and (ii) Th17 stimulated the expression of CCL2/20 in lung tumor microenvironments, which promoted the recruitment of various inflammatory leukocytes (DCs, CD4⁺, and CD8⁺ T cells) to induce therapeutic immunity. Our study elucidates the molecular mechanism of Th17's stimulatory effect on CD8⁺ CTL responses and also demonstrated that (i) it is the Th17-stimulated CTLs, but not Th17 cells, that themselves have direct *in vitro* killing activity to tumor cells, (ii) Th17-secreted IL-17, but not the host IFN- γ , is associated with Th17-induced therapeutic effect, and (iii) although Th17 cells promote tumor infiltration of various inflammatory leukocytes, the tumor-specific CD8⁺ T cells with killing activity via perforin pathway [26], but not DCs and CD4⁺ T cells recruited via CCL20 chemoattraction, play a major role in Th17-induced therapeutic effect. To date, adoptive T-cell immunotherapy for cancer by using *in vitro* expanded tumor-infiltrating CD8⁺ T cells has achieved some degree of success [27, 28]. However, one of the major obstacles in this therapy is only very limited number of transferred CD8⁺ T cells that eventually infiltrate into tumors [29, 30], which greatly affects its therapeutic efficacy. Therefore, Th17 cells may be useful in enhancing adoptive CD8⁺ T-cell immunotherapy for cancer.

Th17-polarized cells derived from TRP-1-specific TCR transgenic mice inhibited the growth of large s.c.

established B16 melanoma (~ 0.6 cm², equal to ~ 7 – 8 mm in diameter) after adoptive transfer of Th17 cells [15]. However, in our current study, we found that Th17 cells efficiently cured s.c B16 melanoma only in early stage (3 mm), but not well-established stage (6 mm), which is consistent with a previous report on the eradication of 6-day s.c. tumors [31]. The discrepancy in therapeutic efficiency may be due to the different treatment protocols in these reports. In our protocol, we simply *i.v.* transferred OVA-specific Th17 into B16 melanoma-bearing mice for direct assessment of Th17 therapeutic effect. In their protocol [15], they have combined adoptive transfer of Th17 with an extra-total body sublethal irradiation plus TRP-1 virus and IL-2 administration. Their complex protocol will definitely interfere with the assessment of Th17-mediated therapeutic effect. For example, sublethal irradiation will induce lymphopenia leading to proliferation and prolonged survival of transferred T cells [32, 33] and conversion of Th17 into Th1 [34, 35], whereas TRP-1 virus vaccine alone can activate both Th1 and CTL responses important for tumor rejection [36, 37].

Based upon previous reports and our own findings, we propose the following model for Th17-induced antitumor immunity: (i) Th17 directly stimulates tumor-specific CD8⁺ T-cell responses via pMHC I and IL-2 signaling, (ii) homing molecule (CXCR4, CCR6, and CD161)-expressing Th17 migrates into tumors [20, 38] by tumor environmental RANTES and MCP-1 chemoattraction [39], (iii) tumor-infiltrating Th17 stimulates tumor tissues to express CCL20 for recruiting CCR6-expressing tumor-specific CD8⁺ T cells into tumors via CCL20 chemoattraction, and (iv) tumor-specific CD8⁺ T cells exert direct killing activity to tumor cells via perforin/granzyme B pathway (Fig. 5).

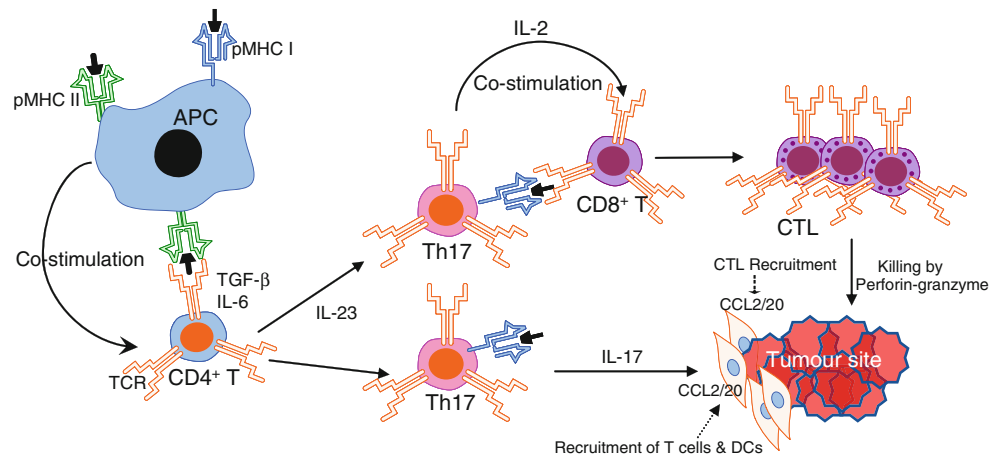
Taken together, our data demonstrate a distinct role of Th17 and Th17-stimulated CD8⁺ T-cell responses in preventive and therapeutic antitumor immunity, which may greatly impact the development of Th17-based cancer immunotherapy.

Materials and methods

Reagents, antibodies, cell lines, and animals

The biotin-labeled anti-CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40L (TRAP1), CD69 (H1.2F3), FasL (CD178), and major histocompatibility complex (MHC) class I (H-2 K^b) (AF6-88) antibodies (Abs) were purchased from BD Biosciences (San Diego, CA). The R-phycoerythrin (PE)-conjugated anti-mouse IFN- γ (XMG1.2), perforin (δ G9), and FITC-conjugated IL-17 (TC11-18H10) Abs were obtained from Pharmingen Inc. (Mississauga, Ontario,

Fig. 5 Mechanism of Th17-mediated antitumor immune responses. Tumor-infiltrating Th17 cells stimulate tumor microenvironment to express CCL2/20 leading to the recruitment of inflammatory cells such as CD8⁺ CTLs derived from direct Th17 stimulation via pMHC I and IL-2 signaling into the tumor site, which exert direct killing activity to tumor cells via perforin/granzyme B pathway



Canada). The PE-conjugated anti-mouse ROR γ t (ROR γ 2) Abs were purchased from BioLegend (San Diego, CA). The FITC-conjugated avidin was obtained from Jackson Immuno Research Laboratory Inc. (West Grove, PN). Peptides OVA I (OVA_{257–264}) specific for H-2K^b, OVA II (OVA_{323–339}) specific for I^a^b, and 3LL lung carcinoma antigen (Ag) Mut1 peptide specific for H-2K^b were synthesized by Multiple Peptide Systems (San Diego, CA). The FITC-labeled anti-CD8 Ab and PE-H-2K^b/OVA I tetramer were obtained from Beckman Coulter (Mississauga, Ontario, Canada). Recombinant cytokines were obtained from R&D Systems Inc. (Minneapolis, MN). Tumor cell lines including ovalbumin (OVA)-expressing thymoma (EG7) and BL6-10_{OVA} and I^a^b-expressing LB27 were available in our laboratory [40]. Wild-type C57BL/6, IL-2^{+/-}, perforin^{-/-}, I^a^b^{-/-} and H-2K^b^{-/-} knockout (KO), diphtheria toxin receptor (DTR)-CD11c transgenic mice [41], and OVA-specific T-cell receptor (TCR) transgenic OTI and OTII mice, which express TCR specific for OVA I and OVA II, respectively, were all purchased from the Jackson Laboratory (Bar Harbor, MA). Homozygous OTII/H-2K^b^{-/-} and OTII/IL-2^{-/-} mice were generated by backcrossing the designated gene KO mice onto OTII background. All mice were treated according to Animal Care Committee guidelines of University of Saskatchewan.

Preparation of dendritic cells

Bone marrow-derived dendritic cells (DCs) of C57BL/6 mice were generated in the presence of GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) as described previously [18, 19]. These DCs expressed MHC II, CD40, and CD80, indicating that they were mature DCs. They were then pulsed with OVA (0.3 mg/ml) overnight at 37°C and termed as DC_{OVA}. OVA-pulsed DCs generated from H-2K^b gene KO mice were referred to as (K^b^{-/-})DC_{OVA}.

Preparation of OVA-specific T cells

To generate OVA-specific Th17 cells, naïve CD4⁺ T cells (2×10^5 cells/ml) from OT II mice were stimulated for 3 days with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF- β (10 ng/ml), and anti-IFN- γ antibody (20 μ g/ml). These DC_{OVA}-activated Th17 cells were purified by positive selection using CD4 microbeads (Miltenyi Biotech, Auburn, CA). (K^b^{-/-})DC_{OVA}-activated CD4⁺ T cells derived from OTII mice were termed (K^b^{-/-})Th17, whereas DC_{OVA}-activated CD4⁺ T cells derived from OTII/IL-2^{-/-} mice were termed (IL-2^{-/-})Th17. Alternatively, naïve CD4⁺ T cells (2×10^5 cells/ml) from OT II mice were stimulated with CD3/CD28 T-cell expander Dynabeads (Invitrogen) at a ratio of 1:1 (bead:cell) for 4–5 days in the presence of IL-6 (10 ng/ml), IL-23 (10 ng/ml), and TGF- β (10 ng/ml) [42]. The cytokine profiles of the above various gene KO Th17 or CD3/CD28-bead-activated Th17 (bead-Th17) were similar to the cytokine profile of DC_{OVA}-activated Th17 cells except for the specific molecule deficiency (data not shown). Preparation of DC_{OVA}-activated OVA-specific Th1 cells expressing IL-2, IFN- γ , FasL, and perforin, but not IL-4 and OVA-specific CD8⁺ T cells, were previously described [19, 43].

Phenotypic characterization of OVA-specific Th17 cells

The above Th17 cells and Th17 cells with various gene KO were stained with antibodies and analyzed by flow cytometry. To measure intracellular expression of cytokines, Th17 were processed using an intracellular staining commercial kit (BD Biosciences, San Diego, CA) and stained with anti-perforin and ROR γ t Ab or double stained with FITC-conjugated anti-IL-17 Ab and PE-conjugated anti-IFN- γ Ab. Culture supernatants of Th17 re-stimulated

with irradiated (4,000 rads) OVAII peptide-pulsed LB27 cells [18, 19] were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA).

CD8⁺ T-cell proliferation assays

To assess the functional effect of OVA-specific Th17, we performed *in vitro* CD8⁺ T-cell proliferation assay. Two-fold serially diluted irradiated (4,000 rads) DC_{OVA}, Th17, (IL-2^{-/-}), Th17, and (K^{b-/-})Th17 cells (0.4×10^5 cells/well) were co-cultured with a constant number of naive OT I CD8⁺ T cells (1×10^5 cells/well). After culturing for 48 h, overnight thymidine incorporation was quantified by liquid scintillation counting. In *in vivo* proliferation assay, C57BL/6 or perforin^{-/-} mice (6 per group), or transgenic DTR-CD11c mice (6 per group) with a single dose (4 ng/g mouse body weight) of *i.v.* diphtheria toxin (DT) treatment 1 day before Th17 transfer were *i.v.* transferred with DC_{OVA} (1×10^6 cells), DC_{OVA}-activated Th17 (3×10^6 cells) or Th17 (3×10^6 cells) with various gene KO or CD3/CD28 bead-activated Th17 (bead-Th17) (3×10^6 cells). Six days subsequent to transfer, tail blood samples were stained with FITC-anti-CD8 Ab and PE-H-2 K^b/OVAI tetramer and analyzed by flow cytometry.

Cytotoxicity assays

In *in vitro* cytotoxicity assay, DC_{OVA}-activated OTII Th17 and Th1 were used as effector (E) cells, while ⁵¹Cr-labeled OVAII-pulsed LB27 cells were used as target (T) cells. In another experiment, Th17-activated OTI CD8⁺ cytotoxic (Tc) cells were used as effector (E) cells, while ⁵¹Cr-labeled EG7 were used as target (T) cells in a chromium release assay. CMA and emetin inhibitors were used in Th17-activated OTI CD8⁺ cytotoxic (Tc) cell cytotoxicity assay to inhibit perforin and FAS ligand-mediated cytotoxicity, respectively [19]. In *in vivo* cytotoxicity assay, 6 days following Th17 transfer, the Th-transferred C57BL/6 mice (6 per group) were *i.v.* injected with a 1:1 (OVA-specific CFSE^{high}:nonspecific control CFSE^{low}) mixture of splenocyte targets. Sixteen hours subsequent to target cell delivery, the proportion of CFSE^{high} and CFSE^{low} target cells remaining in the spleens was analyzed by flow cytometry [21].

Real-time RT-PCR

Total RNA was extracted from BL6-10_{OVA} cells, lung cell fraction from normal or tumor-bearing lungs with Qiagen RNeasy purification kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturer's protocols. The primer sets for real-time PCR analysis of ROR γ t, CCL2, and CCL20 were designed as previously described [16]. Qiagen

quantitative reverse transcription kit (Qiagen) was used to synthesize cDNA, which was then analyzed by real-time quantitative PCR in triplicates by using SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA, USA) in the Stepone Real-time PCR system (Applied Biosystems). Each gene expression was normalized to GAPDH expression level using comparative CT method.

Lung fractionation and cell analysis

Mouse lungs were digested with collagenase D (1 mg/ml, Worthington Biochemical, Freehold, NJ) at 37°C for 30 min and 5 min with 0.01 M EDTA for prevention of DC-T-cell aggregate formation [44]. The cells were separated using Histopaque (Sigma, St. Louis, MO). The middle section of the gradient, which was enriched with leukocytes, was counted and analyzed by flow cytometry, whereas the bottom fraction that was enriched with tumor cells and lung cells was used for RNA extraction.

Animal studies

In preventive immunity model, wild-type C57BL/6 mice (8 per group) were *i.v.* transferred with OVA-specific Th17 (3×10^6), Th17 (3×10^6) with various gene KO and Th17 (3×10^6) with various antibodies. Six days subsequent to transfer, mice were *i.v.* challenged with BL6-10_{OVA} cells (0.5×10^6). Mice were killed after 3 weeks, and the numbers of metastatic lung tumor colonies were counted [18]. In the lung tumor metastasis therapeutic model, C57BL/6 mice (8 per group) were *i.v.* injected with BL6-10_{OVA} tumor cells (0.5×10^6). After 5 days of tumor cell injection (5-day lung tumor metastasis), mice were *i.v.* injected with Th17 or CD3/CD28 bead-activated Th17 (bead-Th17) (3×10^6 cells/mouse) cells. To deplete CD8⁺ T cells or DCs or to block IL-17, C57BL/6 mice were *i.p.* injected with anti-CD8 or anti-CD11c or anti-IL-17 Ab (each, 0.5 mg/mouse) 1 day before Th17 transfer and followed by another two injections (once every 3 days). To assess the involvement of host CD4⁺ T cells or DCs or host CD8⁺ T-cell's perforin in therapeutic effect, Ia^{b-/-} KO mice (lacking CD4⁺ T cells) or DTR-CD11c transgenic mice (8 per group) with diphtheria toxin (DT) treatment (a single dose of 4 ng/g body weight of mouse; *i.p.*) to deplete host CD11c⁺ DCs or perforin^{-/-} mice were used [45]. Mice were killed on day 16 after *i.v.* injection of tumor cells. The metastatic lung tumor colonies were counted. In the *s.c.* tumor therapeutic model, C57BL/6 or K^{b-/-} or Ia^{b-/-} or IFN- γ ^{-/-} KO mice (8 per group) were *s.c.* injected with BL6-10_{OVA} tumor cells (0.5×10^6). When *s.c.* tumors reached 3 or 6 mm in diameter, these mice were *i.v.* injected with Th17 cells (3×10^6 cells). Tumor growth

was monitored by measuring tumor diameter using caliper; for ethical reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were killed.

Statistical analysis

Mouse survival was analyzed using *log rank* test [46, 47], and all other experiments were tested for statistical differences using unpaired, two-tailed, *Student's t* test. Differences were considered significant if $P < 0.05$.

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