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Tc17 Cells Are Capable of Mediating Immunity to Vaccinia Virus by Acquisition of a Cytotoxic Phenotype

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CD8 T cells can acquire cytokine-secreting phenotypes paralleling cytokine production from Th cells. IL-17–secreting CD8 T cells, termed Tc17 cells, were shown to promote inflammation and mediate immunity to influenza. However, most reports observed a lack of cytotoxic activity by Tc17 cells. In this study, we explored the anti-viral activity of Tc17 cells using a vaccinia virus (VV) infection model. Tc17 cells expanded during VV infection, and TCR transgenic Tc17 cells were capable of clearing recombinant VV infection. In vivo, adoptively transferred Tc17 cells lost the IL-17–secreting phenotype, even in the absence of stimulation, but they did not acquire IFN-γ-secreting potential unless stimulated with a virus-encoded Ag. However, examination of cells following infection demonstrated that these cells acquired cytotoxic potential in vivo, even in the absence of IFN-γ. Cytotoxic potential correlated with FasL expression, and the cytotoxic activity of postinfection Tc17 cells was partially blocked by the addition of anti-FasL. Thus, Tc17 cells mediate VV clearance through expression of specific molecules associated with cytotoxicity but independent of an acquired Tc1 phenotype. The Journal of Immunology, 2010, 185: 000–000.

CD8 T cells can acquire cytokine-secreting phenotypes parallel to those acquired by CD4 T cells and require transcription factors similar to those required for Th cell phenotypes. For example, the development of IFN-γ-secreting CTLs or Tc1 cells is promoted by IL-12, as well as the transcription factors T-bet and eomesodermin (1–4). Recent reports described the IL-17–secreting Tc17 phenotype. Tc17 cells require Stat3 and RORγt for their development (5, 6). Moreover, factors that promote the development of Tc1 cells inhibit the development of Tc17 cells (7, 8). Apart from the differences in cytokine secretion, Tc17 cells differ from Tc1 cells in that they are not cytotoxic (5, 9–11). In most reports, in vitro-generated polyclonal or TCR transgenic Tc17 cells lack cytotoxic activity in 51[Cr]-release assays. Tc17 cells have low expression of granzyme B (GrB), perforin, and FasL compared with Tc1 cells (5, 9, 11, 12).

Tc17 cells were shown to develop in vivo, during the development of experimental autoimmune encephalomyelitis and during influenza infection (5, 12). In vivo transfer of in vitro-derived Ag-specific Tc17 cells was shown to be efficacious in clearing lethal doses of influenza, in antitumor immunity, and in promoting inflammation, although these cells were not protective against a lymphocytic choriomeningitis virus infection (7, 8, 10, 12). How Tc17 cells mediate these functions is unclear. Although one report demonstrated cytotoxic activity of Tc17 cells that correlated with diabetogenic potential requiring IL-17A and IL-17F (7), most reports have suggested that the ability of Tc17 cells to promote immunity in vivo depends upon the ability of the cells to switch to a Tc1 phenotype (6, 10, 12). The instability of the IL-17–secreting phenotype, as well as the acquisition of an IFN-γ–secreting phenotype, even from highly purified IL-17–secreting CD8 T cells (6), is well documented. In the influenza model, the protective effect of Tc17 cells was partially dependent upon IFN-γ (12), although it is likely that other molecules are required for Tc17-mediated immunity.

In this report, we demonstrate that Tc17 cells develop during a vaccinia virus (VV) infection and can promote anti-VV immunity. As with other studies, we observed instability of the IL-17–secreting phenotype of adoptively transferred Tc17 cells. The loss of the IL-17–secreting phenotype occurred in the absence of stimulation in vivo, whereas the acquisition of IFN-γ–secreting potential required Ag and virus infection. However, the anti-viral activity was present in Ifng−/− Tc17 cells, suggesting that acquiring the Tc1 phenotype is not critical for anti-viral activity. Isolation of cells following adoptive transfer shows an increase in cytotoxic potential, suggesting that the in vivo environment during infection reprograms Tc17 cells to a unique effector phenotype.

Materials and Methods

Mice

The generation of Stat4−/− (13) and Thx21−/− (14) mice was described previously. The derivation of OT-I Ifng−/− (12), OT-I Stat4−/− (2), and Stat4−/− Thx21−/− (15) mice was previously described. All mice were used on a C57BL/6 background. C57BL/6 and BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN), OT-I Rag1−/− mice were purchased from Taconic Farms (Germantown, NY), and Ifng−/− and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BoyJ mice were obtained from the Indiana University Simon Cancer Center In Vivo Therapeutics Core. Mice were kept in pathogen-free conditions, and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; GrB, granzyme B; SF, SIINFEKL; VV, vaccinia virus; VV-WR, vaccinia virus-Western Reserve strain.
Viruses

VV (Western Reserve strain) and VV-SIINFEKL (originally provided by J. Yewdell and J. Bennick, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases/National Institutes of Health, Bethesda, MD) were propagated in the human osteosarcoma TK-143B cell line, followed by sucrose-gradient purification and titer determination by the VV Core Facility at the Indiana University School of Medicine, as described (16).

Tc cell differentiation

Total CD8⁺ cells were isolated from spleens and lymph nodes using a MACS isolation system (Miltenyi Biotec, Auburn, CA). For Tc cell differentiation, CD8⁺ cells were activated with soluble anti-CD3 (4 μg/ml; 145-2C11) and anti-CD28 (1 μg/ml; BD Pharmingen, San Jose, CA) in the presence of CD8⁺-depleted irradiated splenocytes (1:5). OT-I CD8⁺ cells were activated with soluble SIINFEKL peptide (1 μM; Biosynth International, Itasca, IL) and anti-CD28 (1 μg/ml). Tc17-primed cells were cultured with human TGF-β1 (2 ng/ml; R&D Systems, Minneapolis, MN), IL-6 (100 ng/ml; PeproTech, Rocky Hill, NJ), and anti–IFN-γ (10 μg/ml; R4/6A2 or XMG); Tc1-primed cells were differentiated with IL-12 (5 ng/ml; R&D Systems). After 3 d of incubation, cells were expanded in the presence of recombinant human IL-2 (20 U/ml; PeproTech) and analyzed after an additional 2 d of culture. In some experiments, cells underwent two rounds of stimulation. For the second 5-d culture, cells were replated

FIGURE 1. Tc17 cells are unstable and have increased GrB production in a second round of culture. WT (A) or Ifng⁻/⁻ (B) CD8⁺ T cells were primed under Tc17 conditions. After 5 d, Tc17 cells were restimulated in the presence of anti–IFN-γ alone or with the addition of the indicated cytokines or IL-12 alone for an additional 5 d. IL-17⁺, IFN-γ⁺, and GrB⁺ cells were detected using intracellular cytokine staining after the first or second round of stimulation.
and stimulated with soluble anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of CD8<sup>−</sup>-depleted irradiated splenocytes (1:5) with the same cytokine and neutralizing Ab concentrations. OT-I cells were activated with soluble SIINFEKL peptide (0.5 μM) and anti-CD28 (0.5 μg/ml).

**Tc cell analysis**

Before intracellular staining, CD4<sup>+</sup> or CD8<sup>+</sup> T cells were stimulated for 4 h with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences, San Jose, CA), and OT-I cells were stimulated for 4 h with SIINFEKL peptide (1 μM) in the presence of GolgiPlug. Cells were fixed for 10 min with 2% formaldehyde, permeabilized in FACS buffer containing 0.1% saponin for cytokine staining or 100% cold methanol for GrB and T-bet (eBiosciences). For surface staining, cells were stained for CD4, CD8, CD45.1, or CD45.2 (eBiosciences, San Diego, CA), following treatment with Fc Block (BD Biosciences), for samples containing APCs. All samples were analyzed using WinMDI software, with supernatants of isolated CD8<sup>+</sup> T cells stimulated with plate-bound anti-CD3 (4 μg/ml) or SIINFEKL peptide (1 μM) for 48 h. Quantitative PCR was performed using TaqMan assays, as previously described (15).

**Cytolytic activity analysis**

Cytotoxic activity of OT-I Tc17 and Tc1 effector cells was measured in a standard <sup>51</sup>Cr-release assay (17). In brief, 6 × 10<sup>3</sup> EL4 and EG.7 (EL4 cells transfected with chicken OVA) target cells were incubated with 200 μCi Na<sup>65</sup>CrO<sub>4</sub> (PerkinElmer, Waltham, MA) in 1 ml for 1 h at 37°C and washed three times with supplemented RPMI 1640 media. Effector cells were added to a round-bottom 96-well microtiter plate in triplicate and serially diluted 3-fold to generate E:T cell ratios ranging from 60:1–0.1:1. Target cells were added to the microtiter plate at a concentration of 2 × 10<sup>4</sup> cells/well. Reactions were conducted in a total volume of 200 μl/well. Plates were incubated for 6 h at 37°C, spun down, and 100 μl cell-free supernatant was harvested from each well. Radioactivity was counted in a PerkinElmer γ counter. The percentage of specific lysis was calculated as (cpm<sub>sample</sub> – cpm<sub>spontaneous</sub>)/(cpm<sub>maximum</sub> – cpm<sub>spontaneous</sub>) × 100. Spontaneous release represents the radioactivity released by target cells in the presence of media alone, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100 (Sigma-Aldrich). In some experiments, control Ig or anti-FasL (10 μg/ml; BioLegend, San Diego, CA) was added to cytotoxicity assays.

**FIGURE 2.** Tc17 instability is dependent on Stat4 and T-bet. WT, Tbx21<sup>−/−</sup>, Stat4<sup>−/−</sup>, or Tbx21<sup>−/−</sup> × Stat4<sup>−/−</sup> CD8<sup>+</sup> T cells were cultured under Tc17 conditions. After 5 d, Tc17 cells were restimulated in the presence of IL-12 (Tc1 conditions) for an additional 5 d. IL-17, IFN-γ, and GrB were detected using intracellular cytokine staining after the first round of stimulation (A, B; top panels) and the second round of stimulation (A, B, bottom panels).
VV infections

For standard infections, two to six mice per group were infected i.p. with a sublethal dose (1–5 × 10⁶ PFU/mouse) of VV-WR. Mice were sacrificed after 5–21 d of infection, and ovaries and spleens were collected. For adoptive-transfer experiments, two to seven mice per group were infected intranasally (2 × 10⁶ PFU/mouse) or i.p. (2 × 10⁶ PFU/mouse) with VV-SIINFEKL or VV-WR. Infection was performed 1 d before or after cell transfer. Mice were sacrificed 4–8 d postinfection, and spleens and ovaries were collected. VV was titered using plaque assays, as previously described (16).

Adoptive T cell transfer and isolation

Bone marrow-derived dendritic cells (BMDCs) were cultured as described in Lutz et al. (18) and stimulated with LPS (1 μg/ml; Sigma-Aldrich) in the presence or absence of SIINFEKL peptide (1 μM) for 24 h. Prepared BMDCs (1 × 10⁵) or 5-d differentiated OT-1 Tc17 or Tc1 cells (1 × 10⁵) were transferred via tail vein injection in 0.5 ml PBS. In some experiments, CD45.2+ cells were isolated from infected mice using anti-CD45.2 Abs and magnetic cell selection.

Results

Stat4 and T-bet are required for the switch of Tc17 cells to a Tc1 phenotype

Previous reports showed that the IL-17–secreting T cell phenotype is unstable, particularly in vivo. To explore this phenomenon further, we cultured in vitro-derived Tc17 cells for an additional round of differentiation under various culture conditions. Tc17 cultures maintained for a second round under Tc17-skewing conditions (TGF-β + IL-6) developed a higher percentage of IL-17–secreting cells but still maintained a small population of IFN-γ-secreting cells (Fig. 1A). Tc17 cells cultured for a second round in the absence of cytokines (condition labeled as “anti–IFN-γ”) demonstrated decreased IL-17 production and increased IFN-γ production, and culture of the cells in Tc1 conditions (IL-12) enhanced this phenotype (Fig. 1A). When IL-12 was added to cells under Tc17 conditions for the second round, the cells that developed were more heterogeneous, with a significant population positive for both IFN-γ and IL-17 (Fig. 1A). The decrease in IL-17 production was largely dependent upon endogenous IFN-γ production because culture of Ifng−/− CD8 Tc17 cells resulted in only a modest decrease in IL-17–secretting cells (Fig. 1B). However, IL-12 was able to repress IL-17 production in the absence of endogenous IFN-γ (Fig. 1B).

We also examined GrB expression in these cultures. After 1 wk of culture, only a small percentage of Tc17 cells demonstrated expression of GrB (Fig. 1A). The percentage of GrB+ cells increased after a second round of culture in Tc17 conditions (Fig. 1A). GrB+ cells increased if Tc17 cells were cultured in the absence of cytokines, an effect that was enhanced by culture with IL-12 (Fig. 1A). Moreover, IL-12 induced GrB expression even when cells were maintained in Tc17 culture conditions (Fig. 1A). The induction of GrB was partially dependent on IFN-γ, although IL-12 was able to induce GrB+ cells in the absence of endogenous IFN-γ (Fig. 1B). Thus, continued exposure to Tc17-skewing cytokines is required to maintain the Tc17 phenotype, whereas IFN-γ and IL-12 contribute to the conversion of Tc17 cells to a Tc1 phenotype.

Because IL-12 was able to alter the Tc17 phenotype in the absence of endogenous IFN-γ, we further explored the downstream mediators of this response by examining the ability of Tc17 cultures to switch phenotype in response to IL-12 when cells lacked expression of STAT4, T-bet, or both transcription factors. As previously described, differentiation of Stat4−/− and Tbx21−/− cells under Tc17 conditions showed a modest increase in IL-17–secreting cells (7, 8), although this was not further affected by double deficiency (Fig. 2A). Although culture of wild type Tc17 cells for a second round under Tc1 conditions resulted in increased IFN-γ production and decreased IL-17, cells deficient in T-bet were unable to completely repress IL-17 or induce IFN-γ (Fig. 2A). Stat4−/− cultures showed more repression of IL-17 compared with Tbx21−/− cultures, but less induction of IFN-γ. Cells that were doubly deficient in Stat4 and T-bet could neither repress IL-17 nor induce IFN-γ, suggesting that each factor has unique, but overlapping, functions in switching the phenotype of Tc17 cultures (Fig. 2A). Although IL-12 was also able to induce GrB in wild type cultures, an effect that was only modestly attenuated in Stat4−/− cultures, cells that were deficient in T-bet were defective in the induction of GrB (Fig. 2B). Cells that were deficient in both Stat4 and T-bet were capable of inducing GrB expression, and because IL-17 was not repressed in these cultures, there were more IL-17+GrB+ cells (Fig. 2B).

FIGURE 3. Expression of cytotoxicity-associated genes in Tc17 cultures. OT-1 CD8+ T cells were cultured under Tc1 or Tc17 conditions. After 5 d, Tc17 cells were restimulated under Tc1 or Tc17 conditions for an additional 5-d RNA was isolated from differentiated cells after 4 h of restimulation with SIINFEKL peptide. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β2-microglobulin mRNA and are relative to levels in Tc1 cells, with the exception of Il17a. A logarithmic scale is used to display the relative expression of all genes, with the exception of Il17a and Tnf.
Expression of cytotoxicity genes in Tc17 cells

Because Tc17 cultures switched to Tc1 conditions were able to acquire IFN-γ and GrB expression, we tested further whether other genes in these cultures that are associated with cytotoxicity demonstrated changes in expression. Similar to results of intracellular cytokine staining, Il17a was expressed in Tc17 cells but not in Tc1 cultures, and expression was diminished in Tc17 cells cultured for a second week in Tc1 conditions (Fig. 3). Ifng and Gzmb also showed similar patterns to intracellular staining: higher expression in Tc1 cells and acquired expression in Tc17 cells switched to Tc1 conditions, although still lower than in Tc1 cells. Expression of Tnf was not different between Tc1 and Tc17 cells. Somewhat surprisingly, Tc17 cells switched to culture under Tc1 conditions did not demonstrate increased expression of Prf1 or Tnfsf10 (encoding TRAIL) but rather maintained low expression, compared with Tc1 cells, regardless of culture condition (Fig. 3). In contrast to the pattern of expression of other genes, Fasl, which was expressed at considerably lower levels in cells cultured for one round in Tc17

FIGURE 4. Tc17 cells are induced in response to infection with vaccinia virus. A, CD8+ T cells were isolated from the spleens of BALB/c, C57BL/6, or C3H/HeJ mice and stimulated for 48 h before cell-free supernatants were used to measure IL-17 and IFN-γ protein levels using ELISA. Results are presented as the mean ± SEM of CD8+ T cells from three mice. B, C57BL/6 mice were infected i.p. with 5 × 10^6 PFU VV per mouse. Spleens and ovaries were respectively isolated for cytokine analysis following restimulation (B) or viral titers (C) at 5, 7, 10, 14, and 21 d postinfection. The data are mean ± SEM from four mice per time point. D, C57BL/6 mice were infected i.p. on days 0 and 21 with 1 × 10^6 PFU VV. Five days after the second infection, CD8+ and CD4+ T cells were isolated from the spleens and restimulated before analyzing IL-17 and IFN-γ production using intracellular cytokine staining. The data are mean ± SEM of five mice. F, Splenocytes were stimulated with SIINFEKL peptide for 48 h, and IL-17 and IFN-γ protein levels in cell-free supernatants were measured using ELISA. Statistical analyses in D and E were performed using the Student t test. *p < 0.05, compared with unstimulated condition.
conditions than in Tc1 cells, demonstrated expression comparable to Tc1 cells in Tc17 cultures that were switched to Tc1 or Tc17 culture conditions for the second round (Fig. 3).

**Tc17 cells in vivo**

Although considerable attention has been paid to the function of in vitro-derived Tc17 cells, little work has been performed on in vivo-derived IL-17–secreting CD8 T cells. To test the ability of CD8 T cells from different strains of mice to produce IL-17, we isolated CD8 T cells from naive BALB/c, C57BL/6, and C3H mice, stimulated them with anti-CD3, and assessed the production of IL-17 and IFN-γ using ELISA. Although the amounts of IL-17 were significantly lower than IFN-γ, IL-17 production was detectable in cultures from all three strains and was highest in C57BL/6 (Fig. 4A). To determine whether this population expanded in response to viral infection, we inoculated mice with VV and assessed IL-17 and IFN-γ production in CD4 and CD8 T cells (Fig. 4B). Although CD4 and CD8 T cells showed transient increases in IFN-γ–producing cells, with decreases following viral clearance (Fig. 4B, 4C), only CD8 T cells showed an increase in the number of IL-17–secreting cells (Fig. 4B). Although the number of IL-17+ cells was much lower than the number of IFN-γ–producing cells, the fold induction compared with uninfected mice was similar to that observed for IFN-γ–producing cells. We performed a similar experiment in mice that were infected twice with VV to examine the memory response; although there was no significant increase in IL-17–secreting CD4 T cells, there was a significant increase in IL-17–secreting CD8 T cells compared with uninfected mice (Fig. 4D).

To determine whether the increased numbers of IL-17–secreting CD8 T cells elicited in vivo were specific for viral Ag, we adoptively transferred naive OT-I T cells to recipient mice that were subsequently infected with VV expressing the OVA SIINFEKL peptide. Five days after intranasal or i.p. infection, splenocytes were stimulated with peptide and analyzed using intracellular cytokine staining and ELISA. A significant increase in epitope-specific IL-17–secreting cells was observed in mice following both routes of infection (Fig. 4E, 4F).

**Anti-viral activity of Tc17 cells in vivo**

We next tested the ability of Tc17 cells to mediate viral clearance. OT-I cells were differentiated in vitro to Tc1 or Tc17 phenotypes, and cultured cells were adoptively transferred to mice 1 d after i.p. infection with VV-SIINFEKL. At day 7 postinfection, viral titers in the ovaries were determined. Transferred Tc1 or Tc17 cells were equally capable of enhancing the clearance of virus (Fig. 5A). Although transferred Tc17 cells showed greater expansion than Tc1 cells, they lost IL-17–secreting potential and demonstrated increased production of IFN-γ; however, the levels were still lower than for Tc1 cells (Fig. 5B, 5C). Analysis of posttransfer cells following intranasal VV inoculation showed a switch to more IFN-γ production and less IL-17 production, although the polarization was less dramatic compared with i.p. infection (Fig. 5C, 5D).

**Acquisition of a Tc1 phenotype in vivo requires virus-encoded Ag**

Because we observed that Tc17 cells transferred to VV-infected mice altered their phenotype toward a potential for increased IFN-γ production and decreased IL-17 production, we wanted to determine whether virus-encoded Ag was required for the altered phenotype. Transfer of Tc17 cells into naive mice resulted in a time-dependent decrease in IL-17 production with no concomitant increase in IFN-γ production, whereas transferred Tc1 cells maintained the IFN-γ–secreting phenotype (Fig. 6A, 6B). When OT-1 Tc17 cells were transferred with BMDCs or SIINFEKL-pulsed BMDCs, there was a similar

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**FIGURE 5.** Adoptively transferred Tc17 cells promote VV-SIINFEKL clearance and convert to an IFN-γ–secreting phenotype. A–C, BoyJ (CD45.1+) mice were infected i.p. with 2 × 10⁵ PFU VV-SIINFEKL and infected i.v. with differentiated OT-I Tc17 or Tc1 cells (1 × 10⁶, CD45.2+) or PBS after 24 h. Six days later, ovaries were harvested for viral titer determination (A), and splenocytes were surface stained with Abs to CD45.1 and CD45.2 to identify transferred cells (B). Statistical analysis in A and B was performed using the Student’s t test. C, IL-17+ and IFN-γ+ cells were identified in differentiated OT-I Tc17 or Tc1 cells immediately after adoptive transfer (left panels) and in splenocytes 6 d after adoptive transfer (right panels). Transferred cells in the right panels are gated on CD45.1+CD45.2+ cells. Data are the mean ± SEM of four or five mice (A) or are representative experiments (B, C). D, Differentiated OT-I Tc17 or Tc1 cells (1 × 10⁶) or PBS was injected i.v. into BoyJ mice. One day later, mice were infected with 2 × 10⁶ PFU VV-SIINFEKL intranasally. IL-17+ and IFN-γ+ cells were determined in Tc17 or Tc1 cells immediately before adaptive transfer (left panels) and 5 d after adaptive transfer in isolated splenocytes (right panels). Transferred cells in the right panels are gated on CD45.1+CD45.2+ cells. The data are representative of six or seven mice.
FIGURE 6. VV-encoded Ag is required for Tc17 cells to convert to an IFN-γ-secreting phenotype in vivo. A, Naive BoyJ (CD45.1+) mice were injected i.v. with differentiated OT-I Tc17 or Tc1 cells (1 × 10⁶, CD45.2+) or PBS. After an additional 3, 6, or 10 d, spleens were isolated, and total splenocytes were stimulated with SIINFEKL peptide. IL-17+ and IFN-γ+ cells were identified in differentiated Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and 3, 6, and 10 d after adoptive transfer from splenocytes (right panels). Transferred cells in the right panels are gated on CD45.1+ CD45.2+ cells. The data are representative of two experiments. B, BoyJ mice were injected i.v. with untreated BMDCs, BMDCs pulsed with SF peptide (1 × 10⁶, CD45.1+), or PBS and were injected i.v. with differentiated OT-I Tc17 cells (1 × 10⁶, CD45.2+) 48 h later. After an additional 5 d, spleens were isolated, and total splenocytes were stimulated with SIINFEKL peptide. IL-17+ cells were identified in differentiated Tc17 cells immediately before adoptive transfer and 5 d after adoptive transfer from total splenocytes. Transferred cells are gated on CD45.1+ CD45.2+ cells. The data are the mean ± SEM of three or four mice and are representative of two or more experiments. Statistical analysis was performed using the Student t test. C and D, BoyJ mice were infected i.p. with 2 × 10⁷ PFU VV-WR and injected i.v. with differentiated OT-I Tc17 or Tc1 cells (1 × 10⁶) or PBS 1 d later. After an additional 6 d, spleens were isolated, and total splenocytes were stimulated with SIINFEKL peptide. C, IL-17+ and IFN-γ+ cells were analyzed in differentiated OT-I Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and 6 d after adoptive transfer in spleen (right panels). The transferred cells were gated on CD45.1+ CD45.2+ cells. Data are representative of five mice (C) or the mean ± SEM of five mice (D) and are representative of two or more experiments. SF, SIINFEKL.

loss of IL-17–secreting potential (significantly more loss of IL-17 in the mice receiving SIINFEKL-pulsed BMDCs versus naive mice) but little induction of IFN-γ (Fig. 6C, data not shown), despite increased cell expansion in mice that received SIINFEKL-pulsed BMDCs (1.1 ± 0.06% CD45.2+ cells in spleen versus only 0.31 ± 0.15% for BMDCs alone). We then tested whether VV infection alone, without relevant Ag, would alter the phenotype of the cells. Tc1 cells showed increased IFN-γ–secreting potential following infection (Fig. 6C, 6D). However, transferred Tc17 cells still displayed a marked reduction in the percentage of IL-17+ cells postinfection, with no increase in IFN-γ production (Fig. 6C, 6D). These results suggest that the combination of virus-encoded Ag and infection is required to promote conversion of Tc17 cells to the Tc1 phenotype in vivo.

**Acquisition of cytotoxic function by Tc17 cells in vivo**

To determine whether IFN-γ is required for Tc17-mediated viral clearance, we repeated the adoptive transfer experiment using wild type and Ifng−/− OT-I cells. Somewhat surprisingly, WT and Ifng−/− Tc17 cells were equally capable of clearing VV infection (Fig. 7A). Although Ifng−/− Tc17 cells demonstrated increased potential for production of IL-17 and modest increases in T-bet expression, neither wild type nor Ifng−/− Tc17 cells showed acquisition of GrB expression (Fig. 7B). Similar results were obtained using Stat4−/− Tc17 OT-I cells, suggesting that Stat4 was also not required for the anti-viral activity of Tc17 cells (data not shown). Thus, conversion to an IFN-γ-secreting phenotype is not a crucial component of Tc17-mediated viral clearance.

The ability of transferred Tc17 cells to mediate viral clearance, regardless of IFN-γ production, suggested that Tc17 cells might acquire a cytotoxic phenotype in vivo. As other investigators showed and we reproduced, after 1 wk of culture, Tc17 cells have acquired the IL-17–secreting phenotype is not a crucial component of Tc17-mediated viral clearance.

We then wanted to determine whether the acquisition of cytotoxic activity that potentially contributed to viral clearance also occurred in vivo. Transferred Tc1 and Tc17 OT-I cells were purified after VV-SIINFEKL infection, and function was tested in a cytotoxicity assay. Purified Tc17 cells demonstrated in vitro cytotoxic activity following adoptive transfer to virus-infected mice, although they were still less efficient than similarly purified Tc1 cells (Fig. 7D).
Because Fasl expression was increased in long-term Tc17 cultures (Fig. 3), and these cells acquired cytotoxic activity (Fig. 7C), we tested whether there was increased Fasl expression in cytotoxic Tc17 cells isolated posttransfer. We observed that Tc17 cells isolated posttransfer demonstrated Fasl expression comparable to in vitro differentiated Tc17 cells (Fig. 7E). To determine whether FasL is responsible for the cytotoxic activity of Tc17 cells following infection, we performed a cytotoxicity assay as in Fig. 7D, with the addition of control Abs or anti-FasL. Incubation with anti-FasL, but not the control Abs, resulted in a modest, although significant, decrease in Tc1-mediated cytotoxicity (Fig. 7F). Moreover, anti-FasL resulted in a 50% decrease in the cytotoxic activity of posttransfer Tc17 cells. Thus, Tc17 cells can acquire cytotoxic potential in vivo that is dependent, at least in part, upon FasL.

Discussion

Cytokines are critical effectors of T cell function, and CD8 T cells predominantly produce IFN-γ upon stimulation. However, CD8 T cells can acquire various cytokine-secreting potentials, including...
a phenotype resembling the Th17 phenotype, termed Tc17. Acquisition of this phenotype is directed by similar cytokines and transcription factors as for the Th17 subset. However, the function of these cells is not well defined. In several studies, Tc17 cells were shown to mediate anti-viral immunity and inflammation. It is not clear whether Tc17-mediated immunity occurs through the promotion of inflammation or acquisition of a cytotoxic phenotype.

One of the features of in vitro-derived Tc17 cells following in vivo injection is that these cells lose the IL-17–secreting phenotype. As shown in Th17 cells (19, 20), the development of Tc17 cells requires STAT3 and results in cells expressing RORγt (5, 6, 9, 11; data not shown). However, signals that generate the Tc1 phenotype seem to be dominant because retroviral expression of STAT3C or RORγt in developing Tc1 cultures resulted in only modest shifts of phenotype (data not shown). Using in vitro experiments, we determined that IFN-γ, STAT4, and T-bet contribute to the ability of Tc17 cells to switch phenotypes (Figs. 1, 2). IFN-γ and T-bet promote the repression of IL-17–secreting potential, whereas STAT4 is mostly dispensable for this function. This is consistent with the ability of T-bet to cooperate with eomesodermin in repressing CD8 T cell IL-17 production (8). In contrast, STAT4 is more critical than T-bet in the induction of eomesodermin in repressing CD8 T cell IL-17 production (8).

Importantly, the current study reveals that Tc17 cells, although initially noncytotoxic, acquire a cytotoxic phenotype and can mediate clearance of VV in vivo. The requirement for FasL and the specific contribution of each mechanism may depend on the pathogen. Importantly, it is clear that Tc17 cells do function after transfer in vivo, and in some experiments they function better than matched Tc1 cells (6, 10). In this report, we demonstrated that after extended culture in vitro, or following exposure to Ag-encoded virus in vivo, Tc17 cells can acquire a cytotoxic phenotype that is at least partially dependent on FasL.

Although IL-17–secreting CD4 T cells are established as an important component of inflammatory immunity, the role of Tc17 cells is less clear. Several studies showed that Tc17 cells expand during infection or inflammation and that they can function in vivo. Importantly, the current study reveals that Tc17 cells, although initially noncytotoxic, acquire a cytotoxic phenotype and can mediate clearance of VV in vivo. The requirement for FasL and the mechanisms of cytotoxic phenotype acquisition will be important to determine in the future.

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Disclosures
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References
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