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Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity¹

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Abstract

IL-17-secreting CD8 T cells (Tc17) have been described in several settings, but little is known regarding their functional characteristics. While Tc1 cells produced IFN- γ and efficiently killed targets, Tc17 cells lacked lytic function in vitro. Interestingly, the small numbers of IFN- γ -positive or IL-17/IFN- γ -double-positive cells generated under Tc17 conditions also lacked lytic activity and expressed a similar pattern of cell surface proteins to IL-17-producing cells. As is the case for Th17 (CD4) cells, STAT3 is important for Tc17 polarization, both in vitro and in vivo. Adoptive transfer of highly purified, Ag-specific IL-17-secreting Tc17 cells into Ag-bearing hosts resulted in near complete conversion to an IFN- γ -secreting phenotype and substantial pulmonary pathology, demonstrating functional plasticity. Tc17 also accumulated to a greater extent than did Tc1 cells, suggesting that adoptive transfer of CD8 T cells cultured in Tc17 conditions may have therapeutic potential for diseases in which IFN- γ -producing cells are desired.

A fairly recently described Th subset, CD4 T cells that IL-17 (Th17), has been shown to play an important role in several models of infectious and autoimmune diseases (1, 2).

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Culture of CD8 T cells in Th17 polarizing conditions results in IL-17 secretion, but the functional and phenotypic characteristics of IL-17-secreting CD8 T cells have not been systematically investigated. One recent study showed that CD8 T cells that lack both T-bet and eomesodermin are shunted into an IL-17-producing phenotype (3), and that these cells are nonfunctional in vivo, with double-knockout animals developing a viral wasting disease when infected with lymphocytic choriomeningitis virus. IL-17-secreting CD8 T cells can also be observed in mice deficient in T-bet alone, where they appear to play a role in allograft rejection (4). While these data suggest that IL-17-secreting CD8 T cells (Tc17) may represent a subset that is distinct from IFN- γ -secreting Tc1 CD8 T cells (5, 6), they are generated under somewhat artificial conditions and may say little about the existence or function of naturally occurring Tc17 cells.

Recent studies by the Dong group showed that CD4 Th subsets display considerable functional plasticity; that is, Treg cells can be converted to Th17 cells, and vice versa, under appropriate polarizing conditions (7). While the Th1 and Th2 subsets were previously considered to be less functionally plastic (8), recent studies show that Th2 cells can be converted into a unique IL-9-secreting subset (9). Supporting the concept of functional plasticity, complete CD4 T cell subset polarization is rarely observed in vivo. As Tc17 cells represent a recently described CD8 population, plasticity of this subset has yet to be investigated.

To address these issues, we polarized CD8 T cells toward IL-17 production (Tc17) in vitro and in vivo. As is the case for CD4 T cells (10, 11), expression of the transcription factor STAT3 appears to be important for Tc17 polarization. Phenotypic analysis shows that Tc17 cells do not express granzyme B and are not able to mediate lysis in vitro. However, adoptive transfer of highly purified, Ag-specific, IL-17-secreting Tc17 cells to Ag-expressing hosts demonstrated functional plasticity in this CD8 T cell subset, with loss of IL-17 expression and acquisition of IFN- γ expression after transfer, as well as increased accumulation and substantial pulmonary pathology as compared with Tc1 CD8 T cells. These studies suggest that adoptive transfer of Tc17 cells may have a role in the therapy of infectious disease and/or cancer.

Materials and Methods

Animals

B10.d2 and BALB/c mice were obtained from The Jackson Laboratory. C3HA^{high} mice express hemagglutinin (HA)³ as a self-Ag on a B10.d2 background was previously described (12). CD8⁺ TCR transgenic mice (Clone 4), which express a TCR recognizing a K^d-restricted HA epitope (⁵¹⁸IYSTVASSL⁵²⁶) (13), were either on a BALB/c background or were backcrossed >10 generations onto the B10.d2 background, with Thy1.1 as a congenic marker. Clone 4 mice on the B10.d2 background were backcrossed onto a STAT3^{fllox} background and then further crossed to CD4-Cre mice to obtain STAT3^{-/-} Clone 4 animals. All animal studies were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

CD8 T cell polarization

Spleens and peripheral lymph nodes were harvested from wild-type (WT) (B10.d2), Clone 4, or STAT3^{-/-} Clone 4 donors. Naive CD8 T cells (CD8⁺CD62L^{high}) were obtained via magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotec). CD3/CD28 Dynalbeads (Invitrogen) were used for polyclonal activation. For Ag-specific

³Abbreviations used in this paper: HA, hemagglutinin; ICS, intracellular staining; ROR, retinoic acid-related orphan receptor.

activation, irradiated splenocytes were pulsed with 2 $\mu\text{g/ml}$ HA class I K^d peptide (IYSTVASSL) and cocultured with naive HA-specific CD8 T cells at a ratio of 5:1. Culture medium used was IMDM (Invitrogen) supplemented with 1.0 mM sodium pyruvate (Sigma-Aldrich), 0.1 mM nonessential amino acids (Invitrogen), 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 0.25 μg amphotericin B (Cellgro), 50 μM 2-ME (Sigma-Aldrich), and 5% heat-inactivated FBS (Quality Biological). Skewing conditions were as follows: Tc0 (media alone), Tc1 (6 ng/ml IL-12, 10 $\mu\text{g/ml}$ anti-IL-4), Tc2 (20 ng/ml IL-4, 10 $\mu\text{g/ml}$ anti-IFN- γ), Tc17 (20 ng/ml IL-6, 5 ng/ml TGF- β , 20 ng/ml IL-1 β , 20 ng/ml IL-23, 10 $\mu\text{g/ml}$ anti-IFN- γ , and 10 $\mu\text{g/ml}$ anti-IL-4). Cells were cultured for 2 days and then rested for 3 days before restimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) before analysis or sorting. All cytokines were from Peprotec except for IL-23 (R&D Systems). Anti-IFN- γ was from eBioscience, and anti-IL-4 was provided by the National Cancer Institute Biological Resources Branch (Frederick, MD).

Flow cytometry and intracellular staining (ICS)

mAbs were purchased from BD Biosciences or eBioscience, with the exception of anti-IL-18R (R&D Systems). For ICS, cells were restimulated for 5 h as above in the presence of GolgiStop (BD Biosciences). For phos-pho-STAT3 ICS, 10⁶ cells were stimulated with 20 ng/ml IL-6 for 20 min, followed by fixation in 4% paraformaldehyde. After washing, cells were permeabilized with 90% methanol at 4°C then stained with phos-pho-STAT3 (BD Biosciences). Flow cytometry was performed using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Statistical analyses were performed using Prism 4 (Graph-Pad Software).

Tc subset sorting

After in vitro polarization and restimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 2 h, IL-17 or IFN- γ -secreting CD8 T cells were stained with bispecific CD45/cytokine mAbs according to the manufacturer's protocol (Miltenyi Biotec) and then sorted to >90% purity using a BD FACSARIA instrument. Propidium iodide staining was utilized to exclude dead/dying cells, and CD8 T cells were sorted into four discrete populations: IL-17⁺IFN- γ ⁻, IFN- γ ⁺IL-17⁻, IL-17/IFN- γ double-positive cells and double-negative CD8 T cells.

In vitro CTL assay

A20 lymphoma cells labeled with HA peptide (2 $\mu\text{g/ml}$) were used as targets. Clone 4 CD8 T cells on a BALB/c background polarized into Tc1 or Tc17 effectors were incubated for 4 h with 5×10^3 Cr⁵¹-loaded targets at the indicated ratios in triplicate wells. Percentage lysis was calculated as previously described (14).

Recombinant vaccinia virus and *Listeria monocytogenes*

A recombinant vaccinia virus strain encoding full-length HA (VacHA) from the influenza virus strain A/PR8/34 (H1N1) was a gift from Frank Guarnieri (The Johns Hopkins University) as previously described (15–17). VacHA was constructed by recombining HA gene into the thymidine kinase gene of WT vaccinia virus. VacHA was expanded on Hu-TK⁻ cells in the presence of 25 $\mu\text{g/ml}$ BrdU (Sigma-Aldrich), purified from cell lysates by sucrose banding, titered by plaque assay on BSC-1 cells, and stored at a concentration of 1×10^8 PFU/ml in the -80°C freezer. For a priming dose, VacHA was thawed on ice and diluted in PBS to 1×10^6 PFU per mouse in a volume of 200 μl for intraperitoneal injection. A *L. monocytogenes* mutant strain engineered to express the influenza HA class I epitope ⁵¹⁸IYSTVASSL⁵²⁶ (LMHA) was a gift from Cerus as previously described (18). Briefly, *L. monocytogenes* carrying the HA epitope (LM-actA⁻ INLB⁻ uvr⁻ PPL2⁻) was

derived from wild-type *L. monocytogenes* strain 10403S and was attenuated via in-frame deletions in the actA, INLB, uvr, and PPL2 genes, respectively. LMHA was grown in Difco brain-heart infusion medium (BD Diagnostic Systems), harvested at mid-log phase of growth, purified by standard methods, formulated in phosphate-buffered sodium chloride solution (PBS)/8% DMSO at a concentration of $<1 \times 10^{10}$ CFU/ml, and stored at -80°C . To prime the mice, LMHA was thawed on ice and diluted in PBS to 1×10^7 CFU per mouse in a volume of 200 μl , corresponding to 0.1 median lethality ($0.1 \times \text{LD}_{50}$), for i.v. injection.

Adoptive transfer

In initial adoptive transfer experiments, 2×10^6 CFSE-labeled, Thy1.1⁺, naive wild-type Clone 4 or STAT3^{-/-} Clone 4 CD8 T cells (B10.d2) were adoptively transferred into Thy1.2⁺ B10.d2 or C3HA^{high} recipients and harvested 4 days posttransfer as previously described (10, 18). In additional experiments, we transferred highly purified, four-way sorted CD8 T cells. For these studies, 1×10^5 Tc1 or Tc17 cells (purified based on cytokine profiles) were adoptively transferred to C3HA^{high} mice and harvested 7 days posttransfer. To model the CD8 T cell response to infectious agents, 1×10^6 PFU recombinant vaccinia virus carrying full length HA (VacHA) (15–17) or 1×10^7 CFU recombinant *L. monocytogenes* carrying HA Ag (LMHA) (18) were administered to B10.d2 mice 1 h after adoptive transfer (i.p. and i.v, respectively), and cells were harvested 4 days later, as described above.

Quantitative real-time PCR

Total RNA was extracted using the RNeasy Micro Kit (Qiagen), and cDNA was synthesized with the SuperScript III enzyme (Invitrogen). All primers were purchased from Applied Biosystems; reactions were performed in triplicate using an Applied Biosystems 7900 instrument.

Histopathology

Mice were anesthetized using avertin (0.5 mg/g body mass; Sigma-Aldrich), followed by intracardiac perfusion with PBS. The lungs were infused with Bouin's solution (Sigma-Aldrich) via trachea at a constant pressure for homogeneous expansion of lung parenchyma, and then fixed with 10% formalin. Tissues were paraffin-embedded and sectioned (5 μm), then stained with H&E for analysis. An inflammation score was assigned in a blinded manner (R.A.A.) as described previously (19). Briefly, a cumulative pathological score was calculated according to the following: peribronchiolar thickening (0, normal; 1, abnormal), $>50\%$ alveolar septum widening (0, normal; 1, abnormal), hemorrhage (0, normal; 1, abnormal). Negative control tissues were from C3HA^{high} mice that did not receive an adoptive CD8 T cell transfer.

Results

IL-17-secreting CD8 T cells are phenotypically distinct from Tc1 and Tc2 cells

Based on recent reports suggesting that IL-17-secreting CD8 T cells (Tc17) may have a nonfunctional phenotype in vivo (3), we optimized cell culture conditions to polarize Ag-specific CD8 cells from TCR transgenic mice (Clone 4) to secrete IL-17 so that we could perform further phenotypic and functional evaluation. As shown in Fig. 1A, optimized culture conditions were not dramatically different from those used to generate IL-17-secreting CD4 T cells (Th17) (1, 2, 10), with the possible exception that addition of IL-1 β did not appear to contribute markedly to Tc17 polarization (20). Notably, blockade of IL-4 and IFN- γ contributed significantly to Tc17 polarization (Fig. 1A, right panels). As compared with CD8 T cells polarized toward a Tc1 phenotype, Tc17 cells secreted more IL-17 and

less IFN- γ , although we were unable to completely abrogate IFN- γ secretion from Tc17 cells under any combination of conditions tested (Fig. 1, A and B). Interestingly, both Tc1 and Tc17 cells secrete significant quantities of TNF- α , and neither subset produces IL-10 (Fig. 1C). Quantitative real-time PCR analysis (Fig. 1D) suggested that these differences in IL-17 and IFN- γ secretion were at least partially controlled at the transcriptional level. These data also showed dramatic up-regulation of the transcription factor retinoic acid-related orphan receptor (ROR) γ t in Tc17 cells, suggesting a role for this molecule in IL-17 secretion by CD8 T cells, as is the case for CD4 T cells (21). Interestingly, although T-bet message was up-regulated in Tc1 as compared with Tc2 cells (22), its expression was only slightly decreased in Tc17 as compared with Tc1 cells, similar to a recent finding in an Ag-presenting cell-driven Tc17 subset (23), but different from a report on mixed lymphocyte culture (24). As expected, GATA-3 was highly expressed in Tc2 cells, but not Tc1 or Tc17 cells (25). Tc17 CD8 T cells also expressed a high level of IL-23R but not IL-12R β 2, consistent with the notion that these cells represent a population distinct from Tc1 CD8 T cells.

STAT3 is required for Tc17 polarization in vitro and in vivo

Since previous work by our group (10) and others (11) demonstrated a requirement for the transcription factor STAT3 in the generation of IL-17-secreting CD4 T cells (Th17), we postulated a similar requirement in Tc17 polarization. These experiments were facilitated by the fact that the CD4 promoter used to generate CD4-Cre mice is expressed at high levels in the thymus in CD4⁺CD8⁺ precursors, resulting in Cre-mediated excision in both CD4 and CD8 T cells (Fig. 2A). In vitro-polarized, Ag-specific (Fig. 2C) or bulk naive non-TCR transgenic CD8 T cells (Fig. 2B) from CD8 STAT3^{-/-} mice showed a marked attenuation of IL-17 secretion, although not as complete as was observed in CD4 T cells (10). This discrepancy most likely stems from incomplete deletion of STAT3 in the CD8 compartment (Fig. 2A). This in vitro finding by skewing CD8 T cells from the conditional STAT3 knockout mice is similar to a recent publication using small interfering RNA to silence STAT3 gene expression (26). Although IL-17-producing CD8 T cells has been found in different mouse models (3, 4, 26, 27) and in humans (28, 29), the in vivo requirement of STAT3 to produce IL-17 in CD8 T cells has not been shown. Therefore, we further explored this STAT3 requirement in vivo by adoptively transferring 2×10^6 HA-specific wild-type or STAT3^{-/-} CD8 T cells into mice expressing HA as a self-Ag (C3HA^{high}), or as an infectious Ag (VacHA and LMHA). These data showed nearly complete abrogation of IL-17 secretion by the STAT3^{-/-} CD8 T cells in vivo (Fig. 2D). Ag-specific STAT3^{-/-} CD8 cells were not markedly different from wild-type cells in terms of proliferation (as assayed by CFSE dilution); >90% of either wild-type or STAT3^{-/-} cells divided in response to self-Ag or infectious Ag by day 4. Survival of these cells was not markedly affected either, as persistence in nontransgenic (B10.d2) hosts was not grossly altered. Thus, as is the case for CD4 T cells (10, 11), STAT3 appears to be required for IL-17 secretion by CD8 T cells both in vitro and in vivo.

In vitro polarization determines Tc1 and Tc17 phenotype and function

We next sought to determine whether Tc17 cells displayed a cell surface phenotype distinct from Tc1 cells. Indeed, cell surface expression of IL-18R, CD45RB, CD38, CD103, and CCR6 were different between Tc17 and Tc1 cells (Fig. 3A). However, while these markers distinguish Tc1 from Tc17 cells, none was capable of discriminating between the IL-17- and IFN- γ -secreting populations that emerge under Tc17 polarizing conditions. In terms of CTL functional molecules, we found that expression of granzyme B was markedly attenuated in both IL-17- and IFN- γ -producing Tc17 populations, while other functional markers were not grossly different between Tc17 and Tc1 cells (Fig. 3B). Additionally, the IL-17- and IFN- γ -

secreting cells from Tc17 conditions were nearly identical in expression of these functional molecules.

We next optimized four-way sorting techniques to obtain >98% pure IFN- γ -secreting Tc1 cells, as well as each of the four distinct populations that emerge under Tc17 skewing conditions (Fig. 4A). Postsort analyses showed that ~1% of the sorted Tc17 IL-17 producers were IFN- γ -positive, and ~4% of the sorted Tc17 IFN- γ producers were IL-17-positive. In-depth quantitative real-time PCR analyses of these cells revealed a complex pattern of cytokine, transcription factor, and effector molecule transcription. We found a 300,000-fold relative increase in IL-17 message in the sorted IL-17-producing cells, as well an approximate 15,000-fold increase in IFN- γ message in IFN- γ -producing cells. As was the case for bulk-cultured Tc17 cells (Fig. 1D), we found a significant up-regulation of ROR γ t message in all of the populations that secreted IL-17. These results also support our earlier data regarding T-bet message, which was not statistically up-regulated in Tc17 IFN- γ producing cells as compared with Tc1 cells. Perforin and granzyme B message levels were significantly decreased in IL-17-secreting Tc17 cells, with the possibility that these cells might lack lytic function. To assay the functional capacity of these sorted cells, we performed classical in vitro CTL analyses, using HA-pulsed A20 lymphoma cells as targets (Fig. 4C). Consistent with the observed reduction in effector molecule levels, Tc17 cells were unable to mediate significant lysis in vitro, regardless of their cytokine production patterns. Taken together, these results are consistent with the notion that, regardless of cytokine secretion pattern, Tc17 CD8 T cells represent a cell population without classic CTL function.

Tc17 cells show functional plasticity in vivo

We next tested whether Tc1 and Tc17 phenotypes were stable; that is, could they be maintained after adoptive transfer into an Ag-expressing environment? We found that after adoptive transfer into C3HA^{high} mice, sorted, cytokine-secreting Tc1 cells were rapidly lost, as previously described in a pmel-1 tumor Ag system (30), but Tc17 cells appeared to persist to a greater extent (Fig. 5A). Surprisingly, both IL-17- and IFN- γ -secreting Tc17 cells persisted (Fig. 5B), and significant IFN- γ secretion was detected after transfer of either population (Fig. 5A). In fact, the most robust IFN- γ secretion came from in vitro purified IL-17-secreting Tc17 cells. Such robust conversion is similar to that recently described for CD4 Th17 cells in an in vivo diabetes model (31). Additionally, negligible IL-17-secreting cells were detected after transfer, either in the lung (Fig. 5C) or the draining lymph nodes (Fig. 5D). These data indicate that IL-17-secreting Tc17 cells generated in vitro can convert to IFN- γ secretion in a self-Ag environment and are consistent with the notion that T cell phenotypes may be functionally plastic (7, 9, 31, 32). Although we cannot completely deplete IFN- γ producers from Tc17 IL17 secretors, or completely eliminate IL-17 producers from Tc17 IFN- γ secretors before adoptive transfer (Fig. 4A), a side-by-side comparison using Tc17 IL-17 and Tc17 IFN- γ generated from the same culture condition enables us to exclude the possibility of a dramatic expansion of a small population of IFN- γ secretors in the purified IL-17-secreting Tc17 population, since greater numbers of lung-infiltrating clonotypic cells were detected after transfer of IL-17-secreting Tc17 than for IFN- γ -secreting Tc17 IFN- γ transfer (mean of absolute number \pm SEM: Tc17 IL-17 = 1271 \pm 164.8 vs Tc17 IFN- γ = 644.0 \pm 148.9; p = 0.0379) (Fig. 5B). Thus, IL-17-secreting Tc17 cells have a greater in vivo expansion/persistence capacity than IFN- γ -secreting Tc17 cells. Both Tc17 populations expressed CD107 after transfer (Fig. 5E), consistent with potential restoration of effector function.

Adoptive transfer of Tc17 cells results in pulmonary pathology

We next tested whether the observed *in vivo* conversion of Tc17 cells from IL-17 to IFN- γ secretion (Fig. 5) resulted in pulmonary pathology in our self-Ag model. These studies were complicated by the relatively small numbers of highly purified cytokine-secreting CD8 cells (1×10^5) available for adoptive transfer (Fig. 4A). Surprisingly, H&E sections from recipients of Tc17 IL-17-secreting cells revealed infiltration, hemorrhage, widened alveolar septae, and peribronchiolar thickening (Fig. 6A). Blinded scoring of these sections confirmed that inflammation was significantly increased in mice receiving IL-17 secreting Tc17 cells (Fig. 6B).

Discussion

CD4 T cell subset producing IL-17 (Th17) has been shown to play important roles in inflammatory and autoimmune diseases (1, 2). However, few studies have focused on IL-17-secreting CD8 T cells (Tc17), especially with regard to their phenotype and function (3, 4, 26–29, 33). In the current study we specifically generated IL-17-secreting CD8 cells (Fig. 1) and further purified this subset to characterize its phenotype (Figs. 3 and 4, A and B) and function *in vitro* (Fig. 4C) and *in vivo* (Fig. 5). As is the case for CD4 cells, we showed that CD8 T lymphocytes can be polarized to secrete IL-17 under conditions similar to those used to differentiate Th17 cells. Using a CD4-Cre \times STAT3^{fllox} system, we showed for the first time that the transcription factor STAT3 appears to be required for CD8 T cells to be polarized toward IL-17 production both *in vitro* and *in vivo* (Fig. 2). We also showed that the transcription factor ROR γ t, a crucial transcription factor for Th17 differentiation, is up-regulated in Tc17. We found that Tc17 differentiation can occur *in vivo* as well; naive Ag-specific CD8 cells produced IL-17 after adoptive transfer into mice that express their cognate Ag as a self-Ag in the lung (Fig. 2D).

A previous study using knockout mice suggested that naturally arising or differentiated Tc17 cells might prove to be nonfunctional (3). In mice lacking both T-bet and eomesodermin, CD8 T cells secreted IL-17 but not IFN- γ , and mice developed a chronic viral wasting syndrome when infected with lymphocytic choriomeningitis virus (3). Similarly, when extrinsic IL-12 and type I IFN signals were absent, a population of IFN- γ /IL-17-double-positive CD8 T cells emerged in response to *L. monocytogenes* infection (33). However, using an allograft rejection model (4), the Bishop group showed that, in T-bet-deficient mice, IL-17-producing CD8 T cells do appear to be functional and play a role in graft rejection. Further data suggesting that IL-17 producing CD8 T cells play a functional role *in vivo* came from studies of contact dermatitis; here, Ab-mediated neutralization of IL-17 mitigated dermatitis *in vivo*, and depletion studies suggested that CD8 T cells were a major source of this cytokine (27). *In vitro*, our data seemed to corroborate data from previous reports (3, 24, 26), as *in vitro*-polarized Tc17 cells lacked granzyme B expression (Fig. 3B) and did not mediate lysis in an *in vitro* CTL assay (Fig. 4C). However, our *in vivo* results painted a somewhat different picture. After transfer into animals where HA is widely expressed as a self-Ag, HA-specific IL-17-secreting Tc17 cells expanded significantly, with the emergence of an IFN- γ -secreting phenotype (Fig. 5). This switch in cytokine production was accompanied by a significant increase in accumulation as compared with Tc1 CD8 T cells and substantial pulmonary inflammation, suggesting a functional role for these cells *in vivo*.

The *in vivo* conversion of Tc17 IL-17 secretors to IFN- γ -secreting cells is surprising and does not appear to be explained by differential expansion of the small (<2%) population of IFN- γ -secreting precursors in the highly purified adoptive transfer product, since sorted IFN- γ -producing cells (from Tc17 conditions) did not out-proliferate IL-17-secreting Tc17 cells under identical, side-by-side *in vivo* conditions. Additionally, this conversion occurred

rapidly (<1 wk), arguing against a massive expansion of a small subpopulation contaminating the purified cells used for transfer. Instead, our data are consistent with the notion that Tc17 cells demonstrate functional plasticity, as has recently been reported for CD4 T cells by three separate groups (7, 9, 31, 32). In the most relevant example, the Weaver group recently showed that IFN- γ -producing CD4 cells could emerge from Th17 precursors in an IL-12-driven manner (32). Additionally, the Dong group showed functional interconversion between regulatory CD4 T cells and Th17 in vitro (7). Finally, the Stockinger group demonstrated that Th2 cells could be converted to a unique IL-9-secreting phenotype under appropriate conditions (9) and, using a similar methodology to that employed here, showed that highly purified TCR-transgenic CD4 Th17 cells could convert to Th1 cells in a self-Ag diabetes model (31). So, although functional plasticity of CD4 T cells has been reported, our data are the first of which we are aware showing a similar plasticity in CD8 T cells in vivo. Note that during the review process for this paper, the Restifo group obtained similar results in a tumor model system using bulk-cultured (but not highly sorted and purified) Ag-specific CD8 T cells (23). The molecular mechanisms underlying this plasticity have yet to be elucidated, but may involve differential expression of transcription factors or epigenetic modifications within effector cytokine gene promoter and enhancer regions, as has been demonstrated for CD4 T cells (34).

Overall, these studies provide novel data regarding the plasticity and function of IL-17-secreting CD8 T cells (Tc17). Polarization of CD8 T cells in the presence of IL-6 and TGF- β results in populations that secrete IL-17 and IFN- γ (Fig. 1), and these two populations cannot be readily differentiated by cell surface phenotype (Fig. 3A). As is the case for IL-17-secreting CD4 T cells (Th17) (10, 11), the transcription factor STAT3 appears to be required for Tc17 polarization. After transfer into animals where HA is widely expressed as a self-Ag, HA-specific IL-17-secreting Tc17 demonstrated a novel functional plasticity, with the emergence of an IFN- γ -secreting phenotype and the development of pulmonary pathology. Our present data suggest that, similar to CD4 T cells (35), adoptive transfer of IL-17-secreting CD8 T cells in an infectious disease or tumor setting may have therapeutic benefit.

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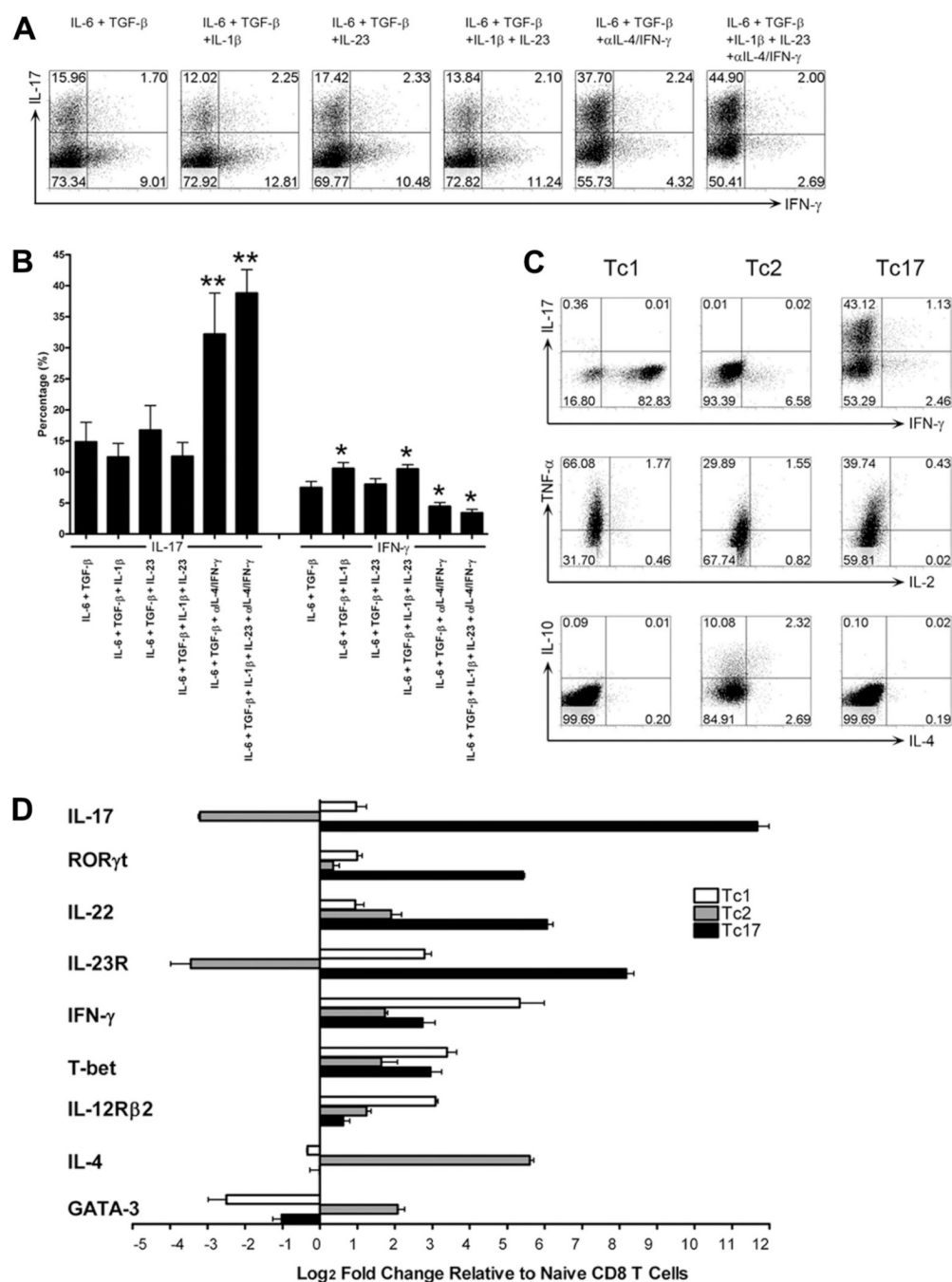


FIGURE 1. In vitro polarization of IL-17-secreting CD8 T cells (Tc17)

A, HA-specific CD8 T cells were activated in vitro for 2 days with cognate peptide plus irradiated APC in the presence of indicated cytokines. ICS was performed after 3 days of rest followed by 5 h of restimulation with PMA/ionomycin. B, Statistical analysis of IL-17 and IFN- γ production from polarized HA-specific CD8 T cells. Means \pm SEM from four independent experiments are shown. *, $p < 0.05$ and **, $p < 0.01$ vs IL-6 + TGF- β . C, Comparison of cytokine secretion profiles of in vitro polarized Tc1, Tc2, and Tc17 CD8 cells. D, Quantitative real-time PCR analysis of CD8 T cell subsets; data represent fold change relative to naive (CD8+CD62L^{high}) cells. Means \pm SEM are shown, performed in triplicate, repeated four times.

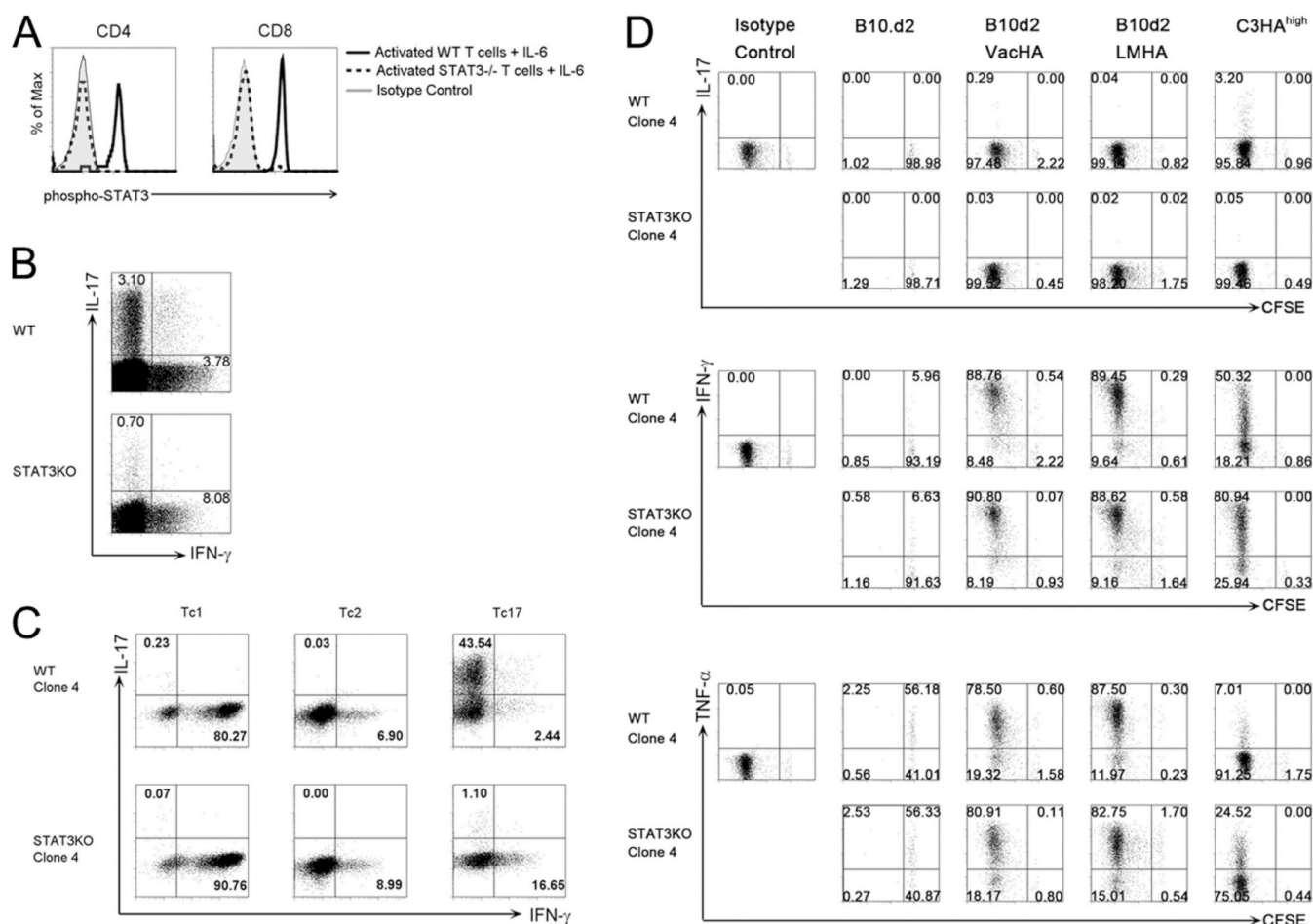


FIGURE 2. STAT3 is required for Tc17 differentiation in vitro and in vivo

A, Phospho-STAT3 expression is abrogated in both CD4 and CD8 T cells from CD4-Cre \times STAT3^{fllox} mice. B, Polyclonal naive CD8 T cells from wild-type and CD8 STAT3KO mice activated in vitro with CD3/CD28 microbeads under Tc17 skewing condition. ICS was performed post-PMA/ionomycin restimulation. C, HA-specific (Clone 4) CD8 T cells from wild-type and CD8 STAT3KO donors stimulated in vitro with specific peptide and irradiated APC under Tc1, Tc2, and Tc17 skewing conditions. D, WT but not STAT3KO Clone 4 CD8 T cells produce IL-17 after recognition of self-Ag in vivo. CFSE-labeled donor cells (gated on Thy1.1) analyzed 4 days after adoptive transfer to indicated recipients: B10.d2 = WT, VacHA = WT + vaccinia expressing HA, LMHA = WT + *Listeria* expressing HA, C3HA^{high} = transgenic animals widely expressing HA as self-Ag. Doses of infectious agents: VacHA, 1×10^6 PFU i.p., LMHA- 1×10^7 CFU i.v. Data are representative of two independent experiments using three to five mice per group.

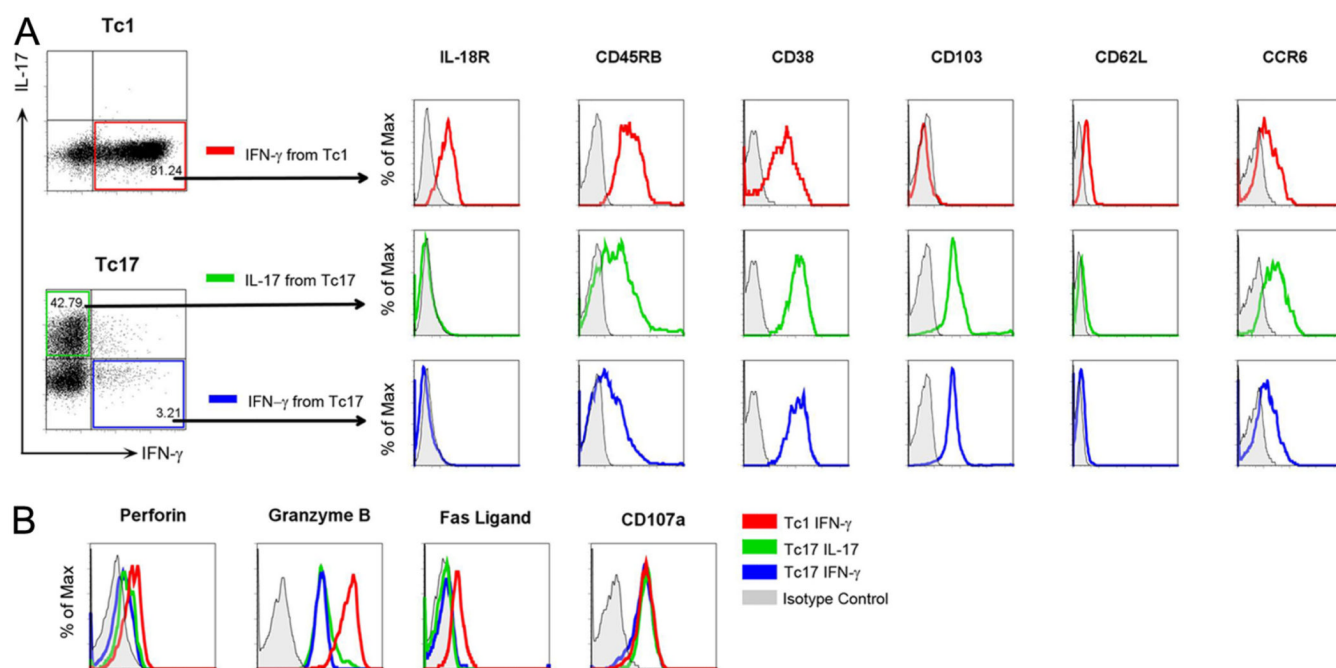


FIGURE 3. Phenotypic and effector molecule analysis of Tc1 and Tc17 subsets

A, Cell surface phenotype of clonotypic Tc1 cells as compared with IL-17- or IFN- γ -secreting CD8 cells differentiated in vitro under Tc17 conditions. Isotype controls are shown in gray. B, Expression of effector molecules in Tc1 vs IL-17 or IFN- γ -producing Tc17 cells. All experiments were repeated three times.

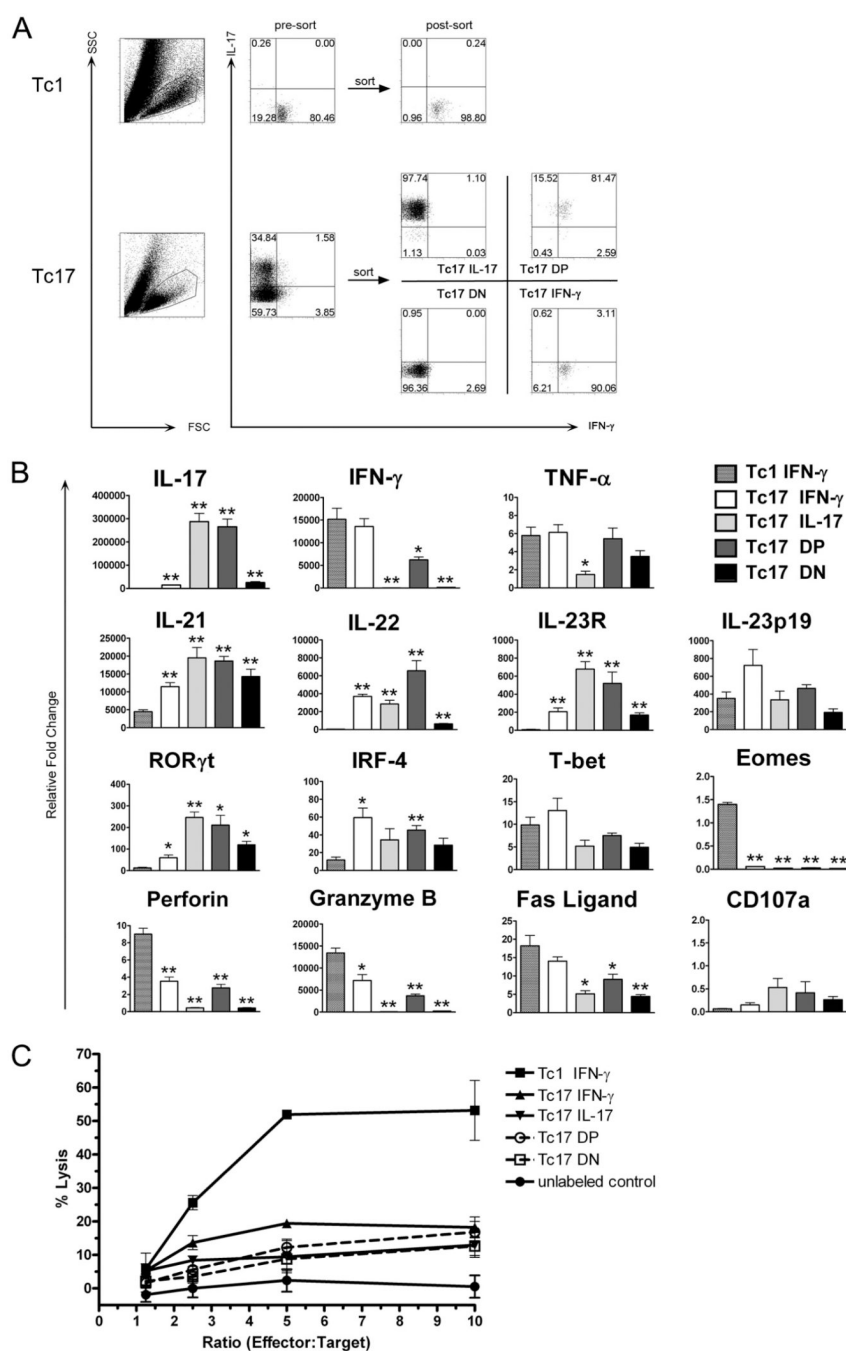


FIGURE 4. Differential gene expression and in vitro CTL function of Tc1 and Tc17 cells
A, Sorting strategy. After in vitro differentiation, clonotypic CD8 T cells were restimulated with PMA/ionomycin for 2 h, followed by staining for cytokine expression and four-way sorting to isolate populations of Tc17 IL-17, IFN- γ , IL-17/IFN- γ double-positive (DP) and double-negative (DN) CD8 T cells as well as Tc1 IFN- γ producers. **B**, Quantitative real-time PCR analyses of sorted, cytokine-expressing Tc1 cells as compared with Tc17 cells that secrete IL-17, IFN- γ , both, or neither. Expression level relative to naive (CD8⁺CD62L^{high}) cells calculated using $\Delta\Delta C_T$ with 18S as an internal control. Reactions were performed in triplicate and repeated four times (*, $p < 0.05$ and **, $p < 0.01$ vs Tc1 IFN- γ). **C**, In vitro lytic function of Tc subsets. In vitro polarized HA-specific CD8 T cells were sorted by

cytokine secretion assay, and 4-h ^{51}Cr -release assay was performed using A20 cells pulsed with HA class I peptide as targets, with unlabeled A20 as negative control. Mean \pm SEM of triplicate wells, repeated three times.

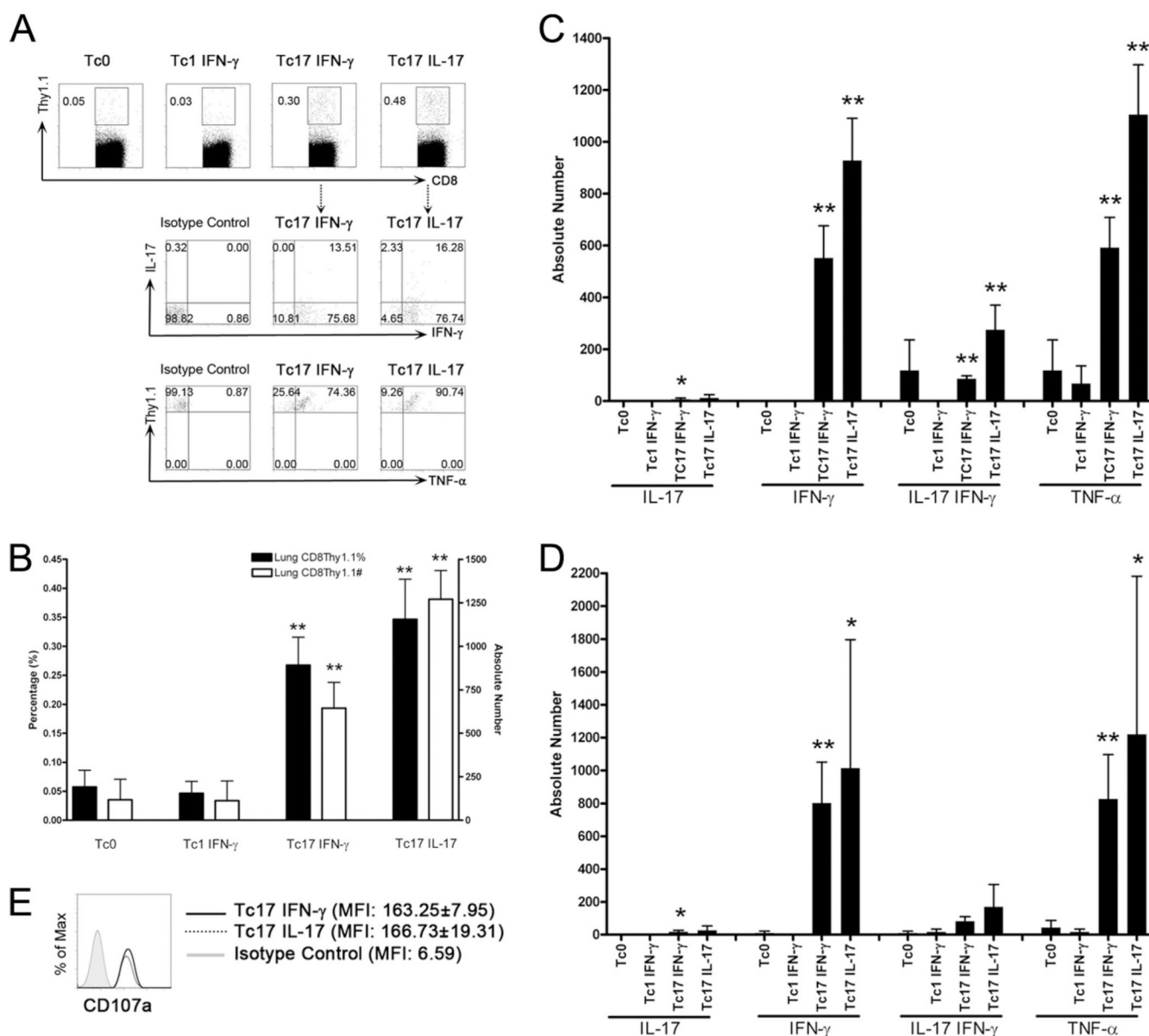


FIGURE 5. In vivo plasticity and persistence of Tc17 cells

A, FACS analysis of lung-infiltrating lymphocytes. Seven days after adoptive transfer of 1×10^5 sorted Tc1 or Tc17 cells, recipient mice were sacrificed and donor cells (Thy1.1) from a CD8 gate were analyzed by ICS after ex vivo restimulation; $n = 3-5$ mice per group, representative FACS plots shown. Experiments were repeated twice. B, Absolute number and percentage of Tc17 vs Tc1 cells in the lungs of C3HA^{high} mice (*, $p < 0.05$ and **, $p < 0.01$ vs Tc1 IFN- γ). Means \pm SEM are shown; $n = 3-5$ mice per group. C and D, Cytokine production by clonotypic Tc17 vs Tc1 cells in the lungs (C) or hilar draining lymph nodes (D) of C3HA^{high} mice (*, $p < 0.05$ and **, $p < 0.01$ vs Tc1 IFN- γ). Means \pm SEM are shown; $n = 3-5$ mice per group. E, CD107a expression on Tc17 lung-infiltrating lymphocytes. Mean fluorescence intensity (MFI) \pm SEM is shown.

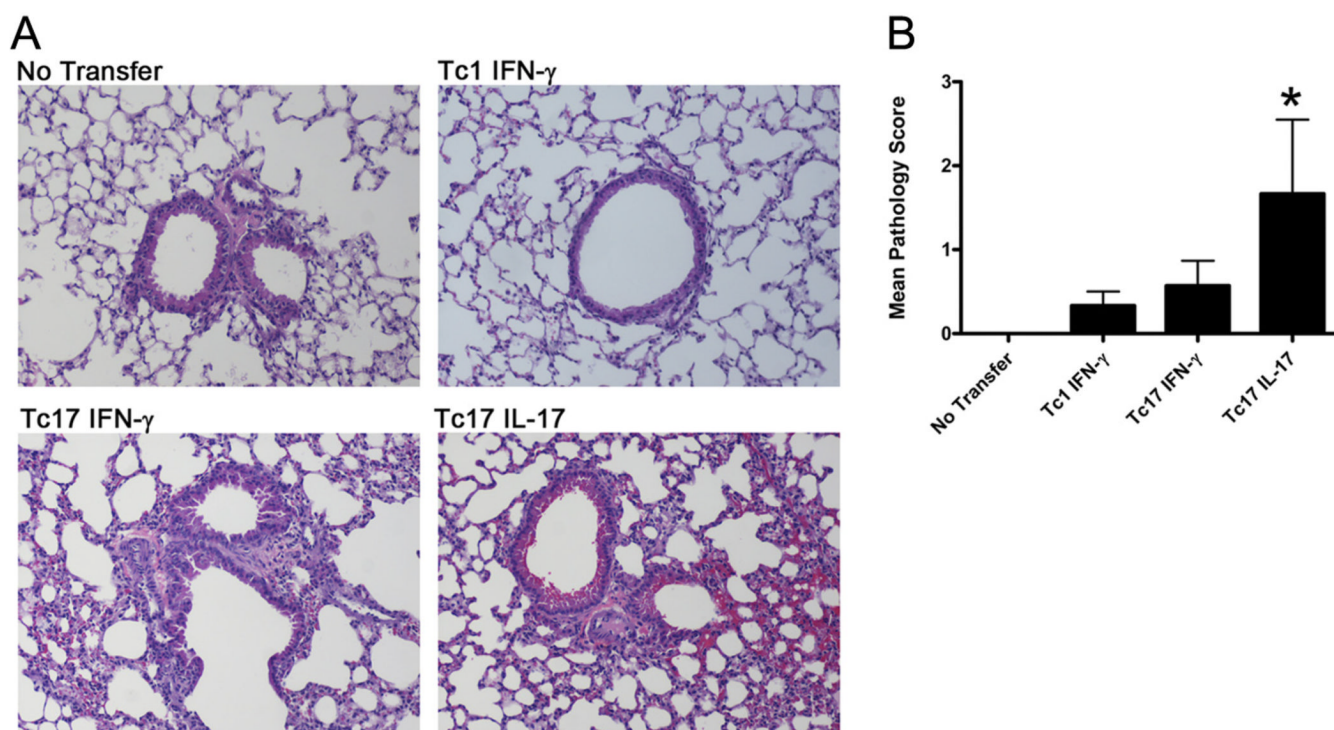


FIGURE 6. Adoptive transfer of IL-17-secreting Tc17 cells results in pulmonary pathology
A, In vitro-polarized HA-specific CD8 T cells were adoptively transferred into C3H/HA^{high} mice. Seven days after adoptive transfer of 1×10^5 sorted Tc1 or Tc17 cells, recipient mice were sacrificed and lung tissues were stained with H&E. Representative sections at $\times 20$ magnification shown. **B**, Mean pathology score (0–3) is shown; 6–12 mice per group. Mean \pm SEM shows *, $p < 0.05$ vs Tc1 IFN- γ .