RESEARCH ARTICLE

Antifungal Tc17 cells are durable and stable, persisting as long-lasting vaccine memory without plasticity towards IFN\(\gamma\) cells

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Abstract

Our understanding of persistence and plasticity of IL-17A\(^+\) memory T cells is clouded by conflicting results in models analyzing T helper 17 cells. We studied memory IL-17A\(^+\) CD8\(^+\) T-cell (Tc17) homeostasis, persistence and plasticity during fungal vaccine immunity. We report that vaccine-induced memory Tc17 cells persist with high fidelity to the type 17 phenotype. Tc17 cells persisted durably for a year as functional IL-17A\(^+\) memory cells without converting to IFN\(\gamma\)\(^+\) (Tc1) cells, although they produced multiple type 1 cytokines in the absence of residual vaccine antigen. Memory Tc17 cells were canonical CD8\(^+\) T cells with phenotypic features distinct from Tc1 cells, and were Ror(\(\gamma\))\(^hi\), TCF\(-1\)\(^hi\), T-bet\(^lo\) and EOMES\(^lo\). In investigating the bases of Tc17 persistence, we observed that memory Tc17 cells had much higher levels of basal homeostatic proliferation than did Tc1 cells. Conversely, memory Tc17 cells displayed lower levels of anti-apoptotic molecules Bcl-2 and Bcl-xL than Tc1 cells, yet were resistant to apoptosis. Tc1 cells required Bcl-2 for their survival, but Bcl-2 was dispensable for the maintenance of Tc17 cells. Tc17 and Tc1 cells displayed different requirements for HIF-1\(\alpha\) during effector differentiation and sustenance and memory persistence. Thus, antifungal vaccination induces durable and stable memory Tc17 cells with distinct requirements for long-term persistence that distinguish them from memory Tc1 cells.

Author summary

CD4\(^+\) T-cell deficient patients such as those with AIDS and idiopathic CD4\(^+\) T-cell lymphopenia are vulnerable to systemic fungal infections. We previously showed that CD8\(^+\) T cells can be exploited in CD4\(^+\) T cell deficient hosts for vaccine immunity against lethal fungal pneumonia in mice and that IL-17A production by these cells (Tc17) is essential. Existing dogma holds that IL-17A producing CD4\(^+\) T cells (Th17) are highly plastic,
unstable, and convert into IFNγ producing cells, losing the capacity to produce IL-17A, which is the signature feature of Tc17 cells. Here, we show that vaccine-elicited antifungal Tc17 cells are maintained as stable and long-lasting memory cells that resist conversion into IFNγ cells (Tc1) and protect CD4⁺ T cell deficient hosts against lethal pulmonary fungal infection. Antifungal Tc17 cells displayed features that define classical memory cells. However, memory Tc17 exhibited different requirements than Tc1 cells in the factors that promote T cell survival, including anti-apoptotic molecules Bcl-2 and Bcl-xL, and HIF-1α, which aids survival of cells in lower oxygen conditions found during inflammation. Thus, our study reveals that fungal vaccination elicits a durable, stable population of Tc17 cells with distinct features of survival needed for preventing infection in immunocompromised hosts.

**Introduction**

Memory Th1 and Tc1 (IFNγ⁺ CD8⁺ T cells) cells have been well characterized. Following the expansion phase of an immune response, a pool of memory precursor cells (~10%) survives the contraction phase, and slowly differentiates into long-lasting memory cells[1]. Memory Th1 and Tc1 cells are maintained stably for years, often lifelong, even in the absence of cognate antigen [2–4]. Maintenance of memory Th1 and Tc1 is chiefly regulated by IL-7 for survival and IL-15 for intermittent homeostatic turnover[5–7]. Type 1 memory precursors are CD127hi, KLRG-1lo, CD27hi, CD122hi, Bcl-2hi, T-betlo, Eomeshi and TCF-1hi[8,9], and the expression of CD62L and CCR7 distinguish “central memory” vs. “effector memory” T-cell subsets[10].

In contrast to Th1 and Tc1 cells, attributes of Th17 cells are less well understood and the persistence of memory Th17 cells is debated. In vitro polarized Th17 cells that are adoptively transferred persist and portray stem-cell like features[11–14]. Conversely, in vivo induction and persistence of Th17 cells depends on route and type of infection. Th17 cells induced upon mucosal *Listeria monocytogenes* infection are short-lived due to their CD27lo phenotype [15,16], whereas subcutaneous injection of *Mycobacterium tuberculosis* antigens induces long-lasting Th1 and Th17 memory cells[16]. Acute cutaneous infection with *Candida albicans* induces Th17 cells that quickly “switch off” IL-17 production[17], while oropharyngeal infection leads to induction of Th17 cells that persist for at least weeks[18]. Th17 cells are known to persist for a longer time under chronic inflammatory conditions, implying a role for lingering antigen and inflammation[19].

Unlike Th1 and Th2 cells, Th17 cells are poised to be highly plastic, presumably due to their epigenetic instability[20–22]. In vitro polarized Th17 cells either convert to Th1 cells or express both Th1 and Th17 cytokines after adoptive transfer into mice, possibly due to deprivation of signals from polarizing cytokines[23]. Host immune status also may impact Th17 plasticity since lymphopenia promotes expansion of Th1 cells[24]. In some models, immunization of mice with adjuvanted antigen drives Th17 responses, but the cells fail to maintain their phenotype and convert into Th1 cells. For example, in EAE models, Th17 cells show plasticity towards IFNγ and GM-CSF production and augment severity of the disease[25,26]. Similarly, *Candida*-specific human memory Th17 cells co-express IFNγ, and staphylococcus-induced Th17 cells express IL-10 upon re-stimulation[27].

Similar to Th17 cells, Tc17 cells form a distinct subset with widely ranging responses implicated in immunity, immunopathology and systemic autoimmunity [28]. Effector Tc17 cells mediate potent anti-viral, anti-fungal, and anti-tumor activity[29–31]. Tc17 effectors also potentiate autoimmune actions of Th17 cells during EAE[32] and exacerbate other autoimmune
disorders due to their ability to express multiple cytokines e.g. IL-22, GM-CSF, M-CSF, IFNγ and IL-3[28]. Like Th17 cells, in vitro polarized Tc17 show plasticity towards IFNγ production[33], and donor Tc17 cells become plastic “inflammatory” iTc17 in recipients with GVHD[34].

Vaccination in the absence of CD4+ T cells induces Tc17 cells that confer resistance against lethal experimental fungal pneumonia[30], a feature that can be translated to immune-compromised individuals with CD4+ T cell lymphopenia that are susceptible to opportunistic fungal infection[35]. While memory Tc17 cells are required in this type of immunity, the persistence and plasticity of these cells have not been investigated.

In this study, we investigated the persistence, fidelity, plasticity and functionality of memory Tc17 cells by using an experimental fungal vaccine that is effective against lethal pneumonia in CD4+ T cell deficient hosts. We found that antifungal memory Tc17 cells are durable and persist long term. Memory Tc17 cells also showed little conversion into IFNγ producing cells, although the cells expressed multiple cytokines. We also found that memory Tc17 cells retain expression of RORγt and portray a phenotypic profile distinct from memory Tc1 cells. Memory Tc17 cells displayed higher proliferative renewal than did Tc1 cells, and lower levels of the anti-apoptotic molecule Bcl-2, which was not required for maintenance of these Tc17 cells. Memory Tc17 cells required HIF-1α for their homeostasis, whereas memory Tc1 cells did not require this factor. Our work provides new insight into distinct features that characterize and promote persistent, stable antifungal Tc17 cells induced upon vaccination.

Results

Anti-fungal memory Tc17 cells are long-lived

We first investigated the fate and longevity of memory Tc17 cells after vaccination. We used “fate-mapping” mice (IL-17AcreRosa26eYFP)[17] where IL-17A-induced cells become indelibly marked with eYFP irrespective of the fate of IL-17A expression. Similar to a published report[17], ~30–50% of the eYFP+ cells expressed IL-17A (S1A Fig). Likewise, ~30–40% of total IL-17A+ cells also expressed eYFP[17]. To assess the persistence of memory Tc17 cells, we vaccinated IL-17A fate-mapping mice and enumerated the percent and absolute numbers of CD8+ eYFP+ cells in tissues (Fig 1A & 1E; S1B Fig). Effector CD8+ eYFP+ cells persisted as memory cells maintaining their phenotype up to 11 months post vaccination (Fig 1A). We previously noted that live vaccine yeast persist for ~7 weeks[36]. To exclude that persistent vaccine antigen accounts for the longevity of memory Tc17 cells, we purified effector CD8+ T cells and adoptively transferred them into naïve wild-type (WT) and TCRα-/- recipient mice. At serial time points, we enumerated CD8+ eYFP+ T cells in draining lymph nodes (dLNs) and spleens (Fig 1C & 1F; S1C Fig). The intake of CD8+ eYFP+ cells was ~10% as expected (at day 1 post-transfer) and the remaining cells persisted stably even after 3 months rest in both immune-sufficient (WT) and -deficient (TCRα-/-) recipients (Fig 1F). Thus, antifungal memory Tc17 cells induced by vaccination persist stably even in the absence of vaccine antigen.

Fidelity of anti-fungal memory Tc17 cells

One of the cardinal features of memory T-cell subsets is their ability to retain expression of signature cytokines. Effector Th17 cells are known to either cease IL-17A expression or convert into an IFNγ+ subset[17,23]. However, Th17/Tc17 cells are essential for immunity against many fungal and extracellular bacterial infections[37], and we have shown that loss of IL-17A signaling ablates vaccine-immunity[30,38]. Here, we investigated whether persistent, memory CD8+ eYFP+ T cells retain the ability to produce IL-17A. We found that ~30% (dLNs) to ~45% (spleen) of CD8+ eYFP+ cells expressed IL-17A (Fig 1B) following vaccination and, with a
**Long-term persistence of anti-fungal Tc17 memory**

Fig 1. Long-term persistence, fidelity and plasticity of memory Tc17 cells. Naive IL17a<sup>Cre</sup>R26R<sup>eYFP</sup> mice were vaccinated with ~10<sup>5</sup> CFU of #55 strain of B. dermatitidis. The draining lymph nodes (dLNs) and spleens were harvested on indicated days. Following staining, cells were analyzed by flow cytometry. Percent (A) and absolute numbers (E) of eYFP<sup>+</sup> CD8<sup>+</sup> T cells. Percent (B) cytokine producing cells among eYFP<sup>+</sup> CD8<sup>+</sup> T cells. N = 5 mice/group. (C & F) Percent and absolute numbers of eYFP<sup>+</sup> cells in naive WT or TCR<sup>α</sup>-/- recipient mice in the spleens were analyzed by flow cytometry on indicated days following adoptive transfer of purified effector CD8<sup>+</sup> T cells from IL17a<sup>Cre</sup>R26R<sup>eYFP</sup> donor mice (day 23 post-vaccination, 12.7x10<sup>4</sup> eYFP<sup>+</sup> cells/mouse). Percent (D) cytokine producing cells among eYFP<sup>+</sup> CD8<sup>+</sup> T cells. N = 3–5 mice/group. Data is representative of at least two independent experiments. Mice were injected with GK1.5 throughout the experiment to deplete CD4<sup>+</sup> T cells.

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long period of rest after vaccination, the IL-17A expressing eYFP<sup>+</sup> cells increased to 50%-60%, suggesting continuous maturation of memory Tc17 cells with loss of non-producers. We also observed similar frequencies of IL-17A<sup>+</sup> cells among adoptively transferred CD8<sup>+</sup> eYFP<sup>+</sup> cells in WT and TCR<sup>α</sup>-/- recipient mice (Fig 1D), suggesting that persistent vaccine antigen is dispensable for maintenance and fidelity under lymphopenic conditions. Thus, IL-17A expression in vaccine-induced memory Tc17 cells not only endured, but also increased during the memory phase.

**Antifungal memory Tc17 cells do not show plasticity towards IFNγ<sup>+</sup> cells**

In many studies involving infection, tumor, and autoimmunity, Th17 cells are either short-lived or convert into IFNγ producing cells[23]. Given the requirement that we previously
observed for effector Tc17 cells following vaccination[30], we investigated whether antifungal memory Tc17 cells portray such plasticity. We detected significant numbers of eYFP⁺ IFNγ⁺ cells immediately after vaccination, especially in the spleen (Fig 1B), most of which did not produce IL-17A. However, the percentage of eYFP⁺ cells expressing IFNγ actually diminished over time. By day 346 post-vaccination <1% of eYFP⁺ cells in the dLN and ~3% of eYFP⁺ cells in the spleen were IFNγ⁺ (Fig 1B). Similar results were found in adoptively transferred CD8⁺ eYFP⁺ T cells, with <10% of them producing IFNγ in both WT and TCRα⁻/⁻ mice (Fig 1D).

Next, we evaluated the effect of IL-12 and/or IFNγ on plasticity of memory Tc17 cells in an in vitro experiment. As expected, IL-12 stimulation significantly enhanced the frequency of IFNγ⁺ cells among eYFP⁻ CD44hi (Tc1) cells (S2A Fig). However, memory Tc17 cells were resistant to IL-12- or IFNγ-induced signals for plasticity towards IFNγ production (S2B Fig). Thus, our data suggest that vaccine-induced anti-fungal memory Tc17 cells do not display plasticity towards IFNγ, even in the absence of persistent vaccine antigen or in a lymphopenic environment.

**Functional memory Tc17 cells for anti-fungal immunity**

We and others have shown that effector Tc17 cells contribute to fungal resistance in the absence of CD4⁺ T cells[18,30,39]. Previously, we showed that effector Tc17 cells are obligatory for anti-fungal immunity, whereas type I cytokines (IFNγ, TNFα and GM-CSF) can be compensated[30,40]. Here, we investigated whether vaccine-induced memory Tc17 cells retain their recall responses and function in antifungal resistance over an extended period after vaccination. To test this issue, mice were rested for ~5 months after vaccination before we challenged them with a lethal strain of yeast. Upon pulmonary challenge, memory Tc17 cells efficiently recalled into the lung and expressed IL-17A (Fig 2A). However, memory eYFP⁺ Tc17 cells did not express IFNγ, suggesting a stable Tc17 lineage commitment without plasticity. To analyze the role of IL-17A in resistance, we neutralized soluble IL-17A with anti-IL-17A mAb throughout the infection after challenge. Unvaccinated mice had a higher fungal burden than vaccinated controls. Among vaccinated mice, the IL-17A neutralized group had a ~100-fold higher fungal burden than the mice treated with control antibody (Fig 2; P≤0.001), suggesting that memory Tc17 cells contributed significantly to vaccine-immunity in the absence of CD4⁺ T cells. Thus, vaccine-induced antifungal memory Tc17 cells persist and maintain the capacity to mediate protective immunity.

**Antifungal memory Tc17 cells express type I cytokines other than IFNγ**

We next asked whether CD8⁺ eYFP⁺ cells can produce cytokines other than IFNγ. Previously, we showed that type I cytokines such as GM-CSF and TNFα augment antifungal vaccine immunity[40]. Here, using cells portrayed in Fig 1, we assessed multi-cytokine production by memory CD8⁺ eYFP⁺ cells by analyzing the percentage of mutually exclusive single, double, triple and quadruple cytokine producing CD8⁺ eYFP⁺ cells (e.g. IL-17A, IFNγ, GM-CSF, and TNFα) (Fig 3). Effector eYFP⁺ cells were largely single IL-17A producers, while most memory IL-17A⁺ eYFP⁺ cells expressed more than one cytokine with the largest pool in the spleen being triple producers by 137 days post vaccination (Fig 3A). Notably, single or multicytokine IFNγ producing eYFP⁺ cells were lost during transition to the memory phase. Adoptively transferred effector eYFP⁺ cells were more often double producers than triple producers (Fig 3B), suggesting that prolonged antigen exposure may bias towards multi-cytokine producing memory Tc17 cells (Fig 3A). About 30% of eYFP⁺ cells produced none of these cytokines. We asked whether these cells had higher PD-1 expression, an indicator of a dysfunctional or exhausted phenotype. Only 12% of non-producers were PD-1⁺ (S3A Fig) similar to the
Fig 2. Functional role of memory Tc17 cells in resistance to fungal pneumonia. Recall: (A) Effector CD8+ T cells from vaccinated IL-17a+R26R<sup>eYFP</sup> were purified from the dLNs and spleen on day 16 post vaccination and adoptively transferred into naive WT recipients. After 5 months of rest, mice were challenged intratracheally with an isogenic virulent strain and after 4 days, lungs were harvested to analyze recall responses by flow cytometry. Values denote percent ± SD. Data is representative of 2 independent experiments. N = 4 mice/group.

Resistance: (B) Naive WT mice were vaccinated with attenuated B. dermatitidis #55 strain and rested for ~5 months. Vaccinated mice and unvaccinated controls were challenged intratracheally with a virulent strain of B. dermatitidis (10<sup>4</sup> CFU of #26199). Anti-IL-17A or control IgG antibody (300 μg) was administered intravenously every other day starting from day -1 of challenge. Lung homogenate was enumerated for CFU. Whisker plots
proportion of producers expressing PD-1. We also looked at other T cell phenotypes: ≤5% of CD8+ eYFP+ cells produced IL-22, a cytokine often associated with Th17 responses, and ≤1% expressed FoxP3+, which is associated with regulatory T cells (S3B Fig). We also evaluated

![Diagram](https://doi.org/10.1371/journal.ppat.1006356.g002)

**Fig 3. Multicytokine producing CD8+ eYFP+ T cells.** Cells from mice portrayed in Fig 1 were analyzed for expression of IL-17A, IFNγ, TNFα and GM-CSF cytokines. (A). Naïve IL17aCreR26RlqR mice were vaccinated with #55 strain. The draining lymph nodes (dLN)s and spleens were harvested on indicated days enumerate multi-cytokine producing CD8+ cells by flow cytometry. (B). Naïve IL17aCreR26RlqR mice were vaccinated, effector CD8+ T cells were purified (as described in Fig 1) and transferred into naïve WT or TCRα−/− mice. On indicated days, cells were analyzed for multicytokine producing CD8+ T cells by flow cytometry. Values in the pie diagrams represent percent cytokine-expressing cells among all cytokine-producing cells.

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whether memory IL-17 producing CD8+ T cells, similar to Th17 cells, co-express IL-21 [41] along with IL-1R, IL-23R and Stat3. We found that Tc17 cells did not express IL-21, but they did express higher levels of IL-1R, IL-23R and Stat3 compared to Tc1 cells (S3C Fig) suggesting a distinct differentiation program in contrast to Th17 cells. Thus, vaccine induced memory Tc17 cells have the ability to produce multiple type I cytokines that confer fungal immunity, yet without undergoing conversion towards IFNγ production.

Antifungal memory Tc17 cells are canonical CD8+ T cells with distinct phenotypic attributes

We evaluated the lineage specificity of CD8+ eYFP+ cells by looking at expression of CD8β, TCRβ and CD3ε on CD8α+ eYFP+ cells. CD8α+ eYFP+ cells equivalently co-expressed a CD8β chain (Fig 4A), a cardinal feature of conventional CD8+ T cells. Similarly, CD8α+ eYFP+ cells were TCRβ+ and CD3ε+, suggesting that memory Tc17 cells are indeed classical CD8+ T cells.

We characterized memory Tc17 cells for their phenotypic attributes and contrasted them with Tc1 cells (CD8+eYFP-) (Fig 4B & S4 Fig). Tc17 (eYFP+) cells expressed high levels of CD127 (IL-7Rα, essential for memory homeostasis); however, they were CD62Llo and Ly6Clo, suggesting they are “effector” memory not “central” memory cells [10,42]. As expected for effector memory cells, none of the antifungal memory cells expressed the terminal differentiation marker KLRG-1 [36,43]. Further, memory Tc17 cells retained high expression levels of chemokine receptor CCR6 and costimulatory molecule, CD43, similar to effector Tc17 cells [30]. In concordance with other studies [13,44], many of the Tc17 cells were CD27lo [13,15,30]. Interestingly, most of the activated (CD44hi) T cells (both eYFP+ and eYFP-) expressed integrin CD103 (αE), the alpha chain of integrin αEβ7 that selectively marks tissue “resident” memory (T_RM) cells [45].

We also compared the transcription factor profile of memory Tc17 cells with Tc1 cells (Fig 4C). Memory Tc17 cells retained high expression of the prototypical transcription factor Rorγt, and expressed low levels of transcription factors T-bet and Eomes typically associated with Tc1 cells. Notably, the expression levels of TCF-1, a LEF/TCF family member associated with memory and stem-cell like activity [13,46], was similar or higher in memory Tc17 cells vs. memory Tc1 (CD44hi eYFP+) cells. Likewise, the frequencies of many phenotypic attributes of Tc17 cells differed significantly from eYFP CD44hi cells (Tc1) and naïve CD8+ T cells (S4 Fig). Collectively, our data suggest that memory Tc17 cells are canonical CD8+ T cells that display distinct phenotypic attributes and constitute an “effector memory” population.

Basal homeostatic proliferation of memory Tc17 cells is higher than memory Tc1 cells

Th17 cells generally display more proliferative renewal than Th1 cells, but homeostatic renewal of Tc17 cells is less understood [47]. We have shown that antifungal effector Tc17 cells undergo significantly higher proliferation than effector Tc1 cells during the expansion phase after vaccination [39]. Here, we assessed the proliferative ability of early and late memory Tc17 and Tc1 cells (Fig 5A & S5 Fig). Consistent with other studies [48], memory Tc1 cells became quiescent, and the proliferation of memory Tc1 cells (≈20%) was ~3.7 times lower than effector Tc1 cells (≈75%) as measured by BrdU uptake over a 12-day pulse. Although memory Tc17 cells also showed decreased basal homeostatic proliferation compared to effector Tc17 cells (~1.8-fold reduction), proliferation remained significantly higher in memory Tc17 cells than memory Tc1 cells (49% vs. 20%). Thus, vaccine-induced memory Tc17 cells appear to have a higher proliferative renewal than memory Tc1 cells.
Fig 4. Phenotype and transcription factor profile of CD8\(^+\)eYFP\(^+\) T cells. Splenocytes from vaccinated IL17a\(^{Cre}\)R26R\(^{eYFP}\) mice (day 46 post-vaccination) were surface stained ex vivo for (A) CD8\(\beta\), TCR\(\beta\), CD3\(\epsilon\), and NK1.1 expression on CD8\(^+\)eYFP\(^+\) cells and
Homeostatic turnover of CD8⁺ T cells involves a similar number of cells undergoing apoptosis in order to maintain constant numbers[48]. Based on our BrdU data, we expected that memory Tc17 cells would display higher levels of apoptosis than Tc1 cells. Surprisingly, eYFP⁺ cells did not display staining for active caspase3/8 (Fig 5B), albeit the cells were tested at a single time-point (76 days after vaccination). Likewise re-stimulation with anti-CD3 and -CD28 to enhance apoptosis revealed that memory Tc17 cells were indeed resistant to TCR signal-induced apoptosis (S6 Fig). We also measured the levels of anti-apoptotic factors (e.g. Bcl-2, Bcl-xL, Mcl-1) and observed that memory Tc17 cells expressed significantly lower levels of Bcl-2 and Bcl-xL and similar levels of Mcl-1 compared with memory Tc1 cells (Fig 5C). Memory Tc17 cells tended to express greater levels of these anti-apoptotic factors than naïve CD8⁺ T cells. Thus, antifungal memory Tc17 cells are maintained with higher levels of proliferation renewal than Tc1 cells, and although memory Tc17 cells express lower levels of the anti-apoptotic factors Bcl-2 and Bcl-xL, they have greater resistance to apoptosis.

Memory Tc17 cell homeostasis is independent of anti-apoptotic factor Bcl-2

Memory Tc17 cells express higher levels of Bcl-2 than memory Th1 cells[12,13], linked to resistance to cell death[12]. However, our data revealed that antifungal memory Tc17 cells expressed lower levels of Bcl-2 than memory Tc1 cells; the Bcl-2 expression levels in memory Tc17 cells were nevertheless on par with naïve T cells (Figs 4C & 5C), which require Bcl-2 for survival[49]. To investigate the role of Bcl-2 for memory Tc17 cell homeostasis in vivo, we inhibited Bcl-2 chemically with ABT-199 in vaccinated mice and analyzed memory CD8⁺ T cells. The total numbers of CD8⁺ T cells, both activated and naïve, were significantly reduced in lymph nodes but not spleens (S7A Fig), indicating that Bcl-2 inhibition reduced lymph node size. Bcl-2 inhibition also significantly decreased the frequency and total numbers of IFNγ⁺ (Tc1) cells in the lymph nodes (Fig 6A), but not in the spleens. Central memory T cells are enriched in the lymph nodes suggesting that ABT-199 preferentially affects survival of central memory IFNγ⁺ (Tc1) cells as previously described[50]. Despite the significant reduction in the total CD8 T-cell population and memory Tc1 cells in the lymph nodes upon Bcl-2 inhibition (S7A Fig), the total number of IL-17A⁺ CD8 T-cells was unchanged in the draining lymph nodes (Fig 6A). The frequencies and total numbers of CD8⁺ eYFP⁺ (Tc17) cells were unaffected in the spleens, as with Tc1 cells. Although Tc1 cells in the lymph nodes responded to Bcl-2 inhibition with increased proliferation, as assessed by Ki67 staining, Tc17 cells did not (S7B Fig). Collectively, these results suggest that Bcl-2 is required for the maintenance and survival of antifungal central memory Tc1 cells, but not Tc17 cells.

HIF-1α is required for differentiation but not for sustenance of effector Tc17 cell response

Hypoxia-inducible factor 1α (HIF-1α) plays a role during the induction and survival of Th17 cells[12,51]. HIF-1α also regulates the expression of Bcl-2 indirectly through Notch signaling in Th17 cells[12]. To our knowledge, the role of HIF-1α for generation of antifungal effector Tc17 (or Tc1) cells has not been investigated. We first investigated the expression levels of HIF-1α in CD8⁺ eYFP⁺ vs. eYFP⁻ cells. Basal expression of HIF-1α in both effector and

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(B) cytokine, chemokine, adhesion, co-stimulatory and terminal differentiation markers/receptors. C. Naive IL17aCreR26R eYFP mice were depleted of CD4⁺ T cells and vaccinated with strain #55. On day 346 after vaccination, splenocytes were re-stimulated and stained for surface markers, Bcl-2, intracellular cytokines and transcription factors. CD8⁺ T cell populations were analyzed by flow cytometry. Values represent mean florescence intensity (MFI) ±SD.

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memory CD8+ eYFP+ cells was low, but increased upon re-stimulation (S8A & S8B Fig). We next investigated the functional role of HIF-1α on Tc17 and Tc1 cells by using the chemical inhibitor Echinomycin to block HIF-1α during the expansion phase, begun 4 days after...
vaccination. The proportion of effector Tc1 cells was significantly reduced by inhibition of HIF-1α, whereas the proportion of effector Tc17 cells was unaffected, indicating that HIF-1α is required for effector Tc1 cells but not for the sustenance or function of effector Tc17 cells (Fig 7A). In a complementary approach, we generated bone-marrow chimera mice using CD4cre-HIF-1αfl/fl bone marrow cells. Following vaccination, we harvested spleens to assess the expansion of Tc17 and Tc1 cells. In contrast to Echinomycin treatment, we found a significant reduction in Tc17 cells that lacked HIF-1α intrinsically and a significantly augmented Tc1

Fig 6. Role of Bcl-2 for memory Tc17 cells. IL17aCreR26ReYFP mice were vaccinated and rested for ~90 days. Mice were treated with either vehicle or Bcl-2 inhibitor ABT-199 (20 mg/kg body weight) for 10 days. Lymph nodes (A) and spleens (B) were harvested to analyze memory CD8+ T cells positive for eYFP, IL-17A and IFNγ. Data is representative of 4–5 mice/group. CD4+ T cells were depleted throughout the experiment. *P<0.05 and **P<0.01.

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Fig 7. Impact of HIF-1α on proliferation of effector Tc17 and Tc1 cells. Naïve IL17aCreR26ReYFP or chimera mice were depleted of CD4+ T cells and vaccinated with strain #55. Splenocytes were re-stimulated, surface-stained, and stained for IL-17A, IFNγ, CD8+CD44, and CD8+CD44hi cells.
response (Fig 7B). To reconcile discrepant findings (Fig 7A & 7B), we began Echinomycin treatment before vaccination and continued it afterward to mimic HIF-1α deficient chimeric mice. We found that Tc17 responses were reduced when HIF-1α was blocked both before and after the vaccination, in contrast to results when HIF-1α was blocked only after vaccination (Fig 7C). These results reconcile the disparate findings of HIF-1α inhibitor treatment and HIF-1α−/− chimeric mice (Fig 7A & 7B) and suggest HIF-1α is required for differentiation but not sustenance of effector Tc17 cell responses.

Temporal blockade of HIF-1α shrinks memory Tc17 cells

We showed above that differentiation but not sustenance of effector Tc17 cell responses requires HIF-1α. To our knowledge, the role of HIF-1α during homeostasis of memory Tc17 (or Tc1) cells has not been investigated. We asked here whether HIF-1α is required for memory homeostasis of Tc17 and Tc1 cells. We took two pharmacological approaches. First, we gave Echinomycin for 10 days at day 90 after vaccination (Fig 8A). The proportion of memory Tc17 cells was significantly reduced by inhibitor treatment, whereas the proportion expressing IFN-γ was not affected, indicating that HIF-1α is required for IL-17A expressing memory cells. We further analyzed the requirement for HIF-1α in memory Tc17 cells by gating on CD8+ eYFP+ cells (S8D Fig). The frequency of memory CD8+eYFP+ cells was similar for the vehicle and inhibitor-treated groups (S8C Fig), suggesting that HIF-1α chiefly affects cytokine expression. In a second pharmacological approach, we administered the HIF-1α agonist, Mimosine, for 14 days starting at day 90 after vaccination (Fig 8B). In contrast to the HIF-1α inhibitor, the agonist enhanced memory Tc17 cells, but again did not affect memory Tc1 cells, buttressing evidence for the selective requirement of HIF-1α for memory Tc17 homeostasis.

Above, using chimeric mice that lack HIF-1α (Fig 7B), we observed that T cell intrinsic HIF-1α is required for the differentiation of Tc17 cells. To assess the fate of effector Tc17 cells generated under these conditions, we rested these vaccinated mice for 90 days before analysis of Tc17 memory. Memory Tc17 (and Tc1 cells) were unexpectedly similar in all groups (Fig 8C), suggesting a limited intrinsic role for HIF-1α during memory CD8+ T-cell homeostasis generated in the absence of HIF-1α. Our data together argue that while temporal blockade of HIF-1α negatively impacts memory Tc17 cell homeostasis, some memory Tc17 cells may be generated and persist independent of intrinsic HIF-1α or alternatively extrinsic sources of this factor may compensate for loss of intrinsic HIF-1α.

Discussion

The long term persistence and plasticity of Th17 cells varies in different experimental models. The importance of these features carries particular significance in the setting of vaccine immunity where maintenance and fidelity of the phenotype is often required for durable resistance to infection. Here, we studied vaccine-induced Tc17 cells that are essential for resistance against lethal fungal pneumonia in hosts that lack CD4+ T cells. We report that vaccine-induced antifungal memory Tc17 cells are highly durable over a prolonged period of nearly...
Fig 8. Impact of HIF-1α on homeostasis of memory Tc17 and Tc1 cells. Naïve IL17aCreR26R<sup>Cre<sup>YFP<sup> or chimeric mice were depleted of CD4<sup>+</sup>T cells and vaccinated with strain #55. Mice were rested for at least 90 days. A. Echinomycin was administered for 10 days.
one year in a murine model. The cells stably persist as memory cells, retain the ability to express IL-17A, mediate immunity upon challenge, do not undergo plasticity towards IFN\(\gamma\) (yet do also express other type I cytokines important for fungal immunity), undergo high proliferative renewal, portray phenotypic markers that are consistent with “effector memory” cells, and are dependent on functional HIF-1\(\alpha\) for homeostasis but not on Bcl-2 for survival. Whereas Th17 cells induced by systemic or mucosal bacterial infection are short-lived memory cells characterized by loss of CD27 expression\[15\], we observed long-term persistence of antifungal Tc17 memory cells despite their low expression of CD27. CD27 is a costimulatory molecule known to augment Tc1 cell proliferation and function, but it may be nonessential for Tc17 cell functions and compensated by other co-stimulatory molecules/cytokines such as CD43, TLR2, IL-1\(\beta\), and IL-23\[11,30,53,54\]. Previous studies have reported the persistence of CD27\(^{lo}\) Th17 cells following their transfer into animals after \textit{in vitro} polarization\[12,13,17\].

The anti-fungal memory Tc17 cells investigated herein expressed high levels of TCF-1, which is a marker of a stem cell-like signature\[13\]. It is not clear if mucosal-induced Th17 cells can become \textit{in situ} memory cells in the absence of cognate antigen. Nevertheless, several \textit{in vivo} and \textit{ex vivo} studies in mice and humans have shown that Th17 cells can become long-term memory cells\[12,13,47\]. Taken together, several lines of evidence argue that Th17 cells are able to persist despite their low expression of CD27. We extend these observations to include Tc17 cells.

Studies of antifungal T cell responses have yielded conflicting results concerning persistence and fidelity of Th17 cells. A murine model of oropharyngeal (mucosal) \textit{Candida} infection revealed that Th17 cells persist for weeks\[18\]. In contrast, intradermal acute \textit{Candida} infection in mice induced Th17 cells that quickly lost IL-17A production\[17\]. Herein, we used a vaccination model in mice to track the persistence and fidelity of Tc17 cells for nearly one year. We observed that Tc17 cells were robustly induced and they durably maintained their IL-17A-producing phenotype for the entire period. Th17 cells induced during a cutaneous \textit{Candida} infection might migrate to secondary lymphoid organs, but the transient exposure to antigen and unique microenvironment during acute cutaneous \textit{Candida} infection may not be enough to stabilize a Th17 cell phenotype for memory T cell formation. Nevertheless, our adoptive transfer studies demonstrated that vaccine-induced CD8\(^+\) eYFP\(^+\) T cells persist and produce IL-17A even in the absence of vaccine antigen.

In many models, Th17 cells convert into IFN\(\gamma\) producing cells\[23\]. In the current study, however, we observed little conversion of Tc17 cells into IFN\(\gamma\) producing Tc1 cells, although the cells expressed TNF\(\alpha\) and GM-CSF (Fig 3). The vaccine-induced effector CD8\(^+\) eYFP\(^+\) donor cells that were adoptively transferred produced comparable levels of IL-17A in both WT and immunodeficient TCR\(\alpha\)^\(-/-\) recipients, and little plasticity towards IFN\(\gamma\), implying the importance of a distinct initial microenvironment for phenotype stabilization\[24,55\]. Interestingly, the percentage of antifungal Tc17 cells producing multiple cytokines increased when the cells were exposed to vaccine antigen for an extended period (Fig 3). One possible explanation is that prolonged exposure to fungal ligands and an inflammatory milieu may stabilize the polycytokine-producing phenotype of Tc17 cells \[56\]. Further studies are needed to elucidate how and what microenvironment shapes the stable Tc17 responses following fungal vaccination.

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Memory Th1/Tc1 cells are subdivided into central or effector memory cells distinguished by the surface markers CD62L and CCR7, respectively[10]. Our data showed that antifungal Tc17 cells are effector memory cells as observed in human memory Th17 cells[12]. Notably, antifungal memory Tc17 cells retained expression of the prototypical transcription factor Ror (γ)t, while expressing lower levels of T-bet. Our data also showed that the majority of memory Tc17 cells expressed surface CD103, an integrin marker of tissue resident memory cells, although the significance of this marker for persistent antifungal immunity needs further study.

In general, homeostasis of memory T cells is governed by two key cytokines, IL-7 and IL-15, which are part of the common γc cytokine family. We found that memory Tc17 cells display high levels of CD127 (IL-7Rα chain) and CD122 (IL-2Rβ chain), implying similarity with memory Tc1 cell homeostasis. Nevertheless, we found that memory Tc17 cells belong to the class of “effector memory”, and in contrast to effector memory Tc1 cells, Tc17 cells undergo higher proliferation renewal[5]. The cytokines, IL-7 and IL-15, are required for intermittent proliferation and also promote survival by enhancing anti-apoptotic factors such as Bcl-2 and Bcl-xL, and downregulating apoptotic factors[57,58]. Our studies using a specific Bcl-2 inhibitor revealed that Tc17 cells were unaffected, while central memory Tc1 cells were reduced in the draining lymph nodes and accompanied by increased cell proliferation. Our findings suggest that memory Tc17 cell homeostatic survival is independent of Bcl-2 and that Tc17 cells may depend on the other anti-apoptotic factors for their survival. While central memory Tc1 cells require Bcl-2 [50], the low levels of Bcl-2 in Tc17 cells probably makes them resistant to Bcl-2 inhibition and may promote their high basal proliferative renewal.

HIF-1 family members are transcription factors that regulate survival and function of many cells, including T cells under hypoxic conditions, while also promoting carcinogenesis[59]. Abrogation of HIF-1α inhibits differentiation of Th17 cells, and blockade leads to apoptosis of Th17 cells[12,51]. Ablation of the HIF-1 negative regulator, VHL, boosts cytolytic CD8+ T cell responses, enhancing viral clearance and suppressing tumor growth. Here, we found disparate requirements for HIF-1α during effector and memory Tc17 responses and also between Tc17 and Tc1 cells. After vaccination, during the expansion phase, antifungal Tc1 (IFNγ+) cells required HIF-1α for their sustenance of effector cell responses, a feature consistent with prior work on Tc1 cells exposed to persistent antigen[60]. Antifungal Tc17 cells were different: while differentiation of effector Tc17 cells required HIF-1α, in line with Th17 cells in a prior study[51], sustenance of effector Tc17 during the expansion phase did not require HIF-1α. Of note, genetic ablation of HIF-1α intrinsically in T cells or extended use of an HIF-1α inhibitor begun prior to vaccination enhanced the Tc1 cell responses. This is likely due to a reciprocal increase of Tc1 cells over Tc17 cells[61], partly caused by poor induction of RORγt in HIF-1α−/− T cells[62].

Memory Tc17 cells required HIF-1α for their homeostasis and maintenance of an IL-17A expression phenotype, and this requirement distinguished memory Tc17 and Tc1 cells. This assertion is supported by the results of temporal intervention with Echinomycin in vaccinated mice. Conversely, our bone-marrow chimera studies revealed that a population of effector Tc17 cells lacking HIF-1α−/− intrinsically became memory cells without loss of phenotype or numbers, suggesting either HIF-1α independent generation of memory Tc17 cells or compensation from the extrinsic compartment. We do not know the oxygen level in the microenvironment of antigen presentation and CD8+ T cell expansion in vivo; however, effector Tc17 cells may overcome the requirement of HIF1-α following differentiation while memory Tc17 cells may have a distinct immunometabolism[63,64], accounting for the HIF-1α requirement for maintenance of an active IL-17A locus in addition to survival during the memory phase. Further studies using cell-specific inducible HIF-1α are required to dissect its role in effector and
memory Tc17 homeostasis. It is notable that memory Tc1 cells were relatively unaffected by inhibition of HIF-1α, suggesting that anti-neoplastic drugs such as Echinomycin might be used without affecting some tumor-specific memory CD8+ cell functions.

In conclusion, we have shown that antifungal Tc17 cells display a set of appealing and unexpected features that are relevant to their function in a vaccine setting. These vaccine-induced Tc17 cells persist as long-lasting memory cells without undergoing plasticity towards IFNγ production, while they express other type I cytokines and have high levels of proliferative renewal for homeostasis. Tc17 cells expressed low levels of Bcl-2 compared to Tc1 cells, and Bcl-2 was not required for the maintenance of memory Tc17 cells. Memory Tc17 cells here were uniquely dependent on functional HIF-1α for the expression of the signature cytokine IL-17A. These results may inform the rational design of vaccines that are dependent on long-lived IL-17A producing cells and perhaps guide the development of immune modulators to alleviate immunopathology and autoimmunity, or enhance immunity against cancer.

Methods

Mice

Wild type C57BL/6 and B6.SJL-Ptprc<sup>a</sup>Pepc<sup>b</sup>/BoyCrl mice were obtained from National Cancer Institute/Charles River Laboratories. Breeding pairs of IL17a<sup>tm1.1((icre)Stck)</sup>/J (Stock 016879) and B6.129X1-Gt(ROSA)26Sor<sup>tm1(EYFP)Cos</sup>/J (Stock 006148) were purchased from Jackson Laboratories and were intercrossed to maintain a heterozygous IL-17A<sup>cre</sup> locus in order to analyze both eYFP and functional IL-17A protein expression<sup>[17]</sup>. Homozygous breeding pairs of B6.129S2-TCR<sup>a</sup>tm1Mom/J and B6.PL-Thy1<sup>a</sup>/CyJ also were purchased from Jackson Laboratories. Conditional HIF-1α KO (CD4<sup>Cre</sup>HIF-1α<sup>fl/fl</sup>) mice were a generous gift from Dr. Fan Pan (School of Medicine, John Hopkins University). All mice were 7–8 weeks of age at the time of vaccination. Mice were bred, housed and cared for following strict guidelines of the University of Wisconsin Animal Care Committee, who approved all aspects of this work.

Ethics statement

All animal procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Care was taken to minimize animal suffering. The work was done with the approval of the IACUC of the University of Wisconsin-Madison who approved the relevant animal protocol number MOO969.

Fungi, vaccination and infection

The wild-type virulent strain of Blastomyces dermatitidis was purchased from American Type Culture Collection (ATCC; strain #26199). The isogenic, attenuated mutant lacking BAD1 (strain #55) was used for all vaccinations<sup>[38]</sup>. Isolates of B. dermatitidis were cultured and maintained as yeast on Middlebrook 7H10 agar with oleic acid-albumin complex (Sigma-Aldrich) at 39°C. Mice were vaccinated with B. dermatitidis strain #55 (~5 x 10<sup>5</sup> cfu) subcutaneously at two sites, dorsally and at the base of tail. For challenge studies, the virulent B. dermatitidis strain #26199 (~10<sup>4</sup> cfu) was inoculated intratracheally. To enumerate fungal burden, lung tissue was homogenized and plated on brain heart infusion (BHI; Difco) agar.

Antibodies

All antibodies were purchased from BD Bioscience except antibodies directed against CD43 (clone 1B11), KLRG-1 and CXCR3 obtained from Biolegend; CD44, CD127, IL-22, T-bet, Ror
(γ)t, Eomes, IL-21, FoxP3 from eBioscience; Bcl-2, Bcl-xL, Mcl-1 active Caspase 3, active Caspase 8, TCF-1 from Cell Signaling; HIF-1α from Novus Biologics, and GFP from Life Technologies.

**CD4⁺ T-cell depletion and IL-17A neutralization**

All experiments were done in mice depleted of CD4⁺ T-cells; GK1.5 mAb was given in a weekly dose of 100μg/mouse by the intravenous route. The dose and interval was enough to deplete CD4⁺ T cells with an efficiency of 95%[36]. For *in vivo* IL-17A neutralization, mice were given 300 μg of anti-IL-17A mAb intravenously every other day. Both mAbs were purchased from Bio X Cell, West Lebanon, NH, while control IgG for IL-17A neutralization studies was obtained from Sigma-Aldrich.

**Intracellular cytokine staining**

Single cell suspensions were re-stimulated with anti-CD3 (0.1 μg/ml) and anti-CD28 (1 μg/ml) antibodies in the presence of Golgi Stop at 37°C for 5 hrs. Cells were surface-stained in 2% BSA/PBS buffer, fixed with Fix/Perm buffer (BD Biosciences) and stained for intracellular cytokines in 1X Perm/Wash buffer (BD Biosciences). Cytokine-producing CD8⁺ T cells were analyzed by flow cytometry using BD LSRII and FACS Aria.

**Adoptive transfers**

Single cell suspensions from lymph nodes and spleens were subjected to a CD8⁺ T-cell magnetic bead-enrichment kit (BD Bioscience) to purify CD8⁺ T cells. Numbers of CD8⁺ eYFP⁺ cells were enumerated by flow cytometry, and equal numbers of CD8⁺ eYFP⁺ cells were adoptively transferred into naïve WT and TCRα⁻/⁻ recipient mice by the intravenous route.

**Transcription factors staining**

Single cell suspensions were re-stimulated, surface-stained and fixed with Phosflow Lyse/Fix buffer and Phosflow Perm/Wash buffer (BD Biosciences). Cells were then stained for intracellular cytokines and transcription factors (TCF-1, Ror(γ)t and T-bet) simultaneously. Staining of FoxP3, HIF-1α and EOMES was done using a FoxP3 buffer kit (eBioscience). In some experiments, cells were first fixed with Perm/Fix buffer (BD Bioscience) followed by addition of antibodies to stain cytokines and eYFP (anti-GFP antibody), then subjected to transcription factor staining.

**Apoptosis factor staining**

Cells were first surface-stained followed by intracellular staining for apoptosis factors using the BD Perm/Fix buffer kit. In some experiments, cells were subjected to intracellular staining for cytokines along with apoptosis factors following *ex vivo* re-stimulation.

**Assessment of CD8⁺ T cell proliferation**

We performed BrdU pulse treatment (0.8mg/ml drinking water, DW) for twelve days. Cells were surface-stained followed by intracellular staining for cytokines using the BD Perm/Fix kit. Later, cells were subjected to anti-BrdU antibody staining using a BrdU kit according to manufacturer’s instructions (BD Pharmingen). BrdU incorporation in DNA of proliferating cells was analyzed by flow cytometry.
Bcl-2 inhibitor treatment

Bcl-2 specific inhibitor, ABT-199, was purchased from APEXBio (Houston, TX) and was re-suspended in 60% Phosal 50G, 30% Polyethylene Glycol 400 and 10% ethanol as described [65]. The inhibitor was administered (20 mg/kg body weight) daily by oral gavage for 10 days. On day 11, tissues were harvested and CD8⁺ T cells were analyzed by flow cytometry.

HIF-1α inhibitor and HIF-1α agonist treatment

HIF-1α inhibitor, Echinomycin, was purchased from Cayman Chemical Company and re-suspended in 100% methanol. Echinomycin was administered intraperitoneally at the concentration of 20–30 µg/kg body weight in sterile 1X PBS every other day. A total of 5 doses were administered during the expansion and memory phases over 10 days, unless indicated. The HIF-1α agonist, Mimosine, was purchased from Sigma Aldrich and used at the concentration of 70 mg/kg body weight by s/c route every other day for 14 days during memory phase.

Bone marrow chimera experiments

Bone marrow cells from congenic donor mice (WT/Thy1.1⁺ and CD4Cre-HIF-1αfl/fl/Ly5.2⁺) were transferred into lethally irradiated Ly5.1⁺ recipient mice. For generation of mixed-bone marrow chimera mice, the donor cells were mixed in 1:1 ratio and were co-transferred into Ly5.1⁺ recipient mice. After 2 months rest, mice were vaccinated to assess the role of HIF-1α in CD8⁺ T cells for expansion and generation of memory Tc17 cells.

Statistical analysis

All statistical analysis was performed using a two-tailed unpaired Student t test except for analysis of fungal CFUs, which was measured by the non-parametric Kruskall-Wallis (one-way ANOVA). Prism 5 (GraphPad Software, Inc.) software was used to analyze all statistics. A two-tailed P value of ≤0.05 was considered statistically significant.

Supporting information

S1 Fig. Long-term persistence of memory Tc17 cells. Naïve IL17aCreR26REYFP mice were vaccinated and draining LNs (dLNs) and spleens collected. Cells were stained and analyzed by flow cytometry. Percent of IL-17A producing cells among CD8⁺ eYFP⁺ T cells is shown on day 19 post-vaccination. (B & C) IL17aCreR26REYFP mice were vaccinated and rested as in Fig 1. On indicated days, dLNs were harvested to enumerate CD8⁺ eYFP⁺ cells in vaccinated mice (B) or in donor T-cell recipient mice (C). N = 3–5 mice per group. Data is representative of at least two independent experiments. Mice were injected with GK1.5 throughout the experiment to deplete CD4⁺ T cells.

S2 Fig. In vitro plasticity of memory Tc17 cells. Splenocytes from vaccinated IL17aCreR26REYFP mice (D162 post-vaccination) were incubated with IL-12/IFNγ (10ng/ml) for 18 hrs in the presence of IL-2 (10ng/ml). Cells were washed and re-stimulated with anti-CD3/CD28 antibodies for 5 hrs before intracellular cytokine staining. A. Percent IFNγ cytokine-producing cells among activated Tc1 cells. B. Percent IL-17A and IFNγ cytokine-producing cells among activated eYFP⁺ Tc17 cells. Each respective colored line represents data from a single mouse. * p ≤ 0.05.

S3 Fig. In vivo plasticity of memory Tc17 cells. Naïve IL17aCreR26REYFP mice were vaccinated and rested for at least 46 days. Spleens were harvested and surface-stained for CD8⁺ T-
cell markers along with PD-1 (A), intracellularly stained for FoxP3 and IL-22 (B) and stained for surface IL-1R1 and IL-23R followed by intracellular Stat3 (C). Frequency of IL-1R1 and IL-21 CD8+ T cells (D). Numbers represent frequencies among CD8+ eYFP+/eYFP- T cells. Histogram values represent mean florescence intensity. N = 4–5 mice. Data is representative of two independent experiments.

(TIF)

**S4 Fig. Phenotypic attributes of memory Tc17 cells.** Naïve IL17aCreR26R eYFP mice were vaccinated and rested as described in Fig 6. Spleens were harvested and surface-stained for phenotypic markers on CD8+ eYFP+ T cells. Numbers represent frequencies (mean ± SD) among CD8+ T cells. N = 5 mice/group. *P<0.05.

(TIF)

**S5 Fig. Proliferative renewal of Tc17 cells.** Naïve IL17aCreR26R eYFP mice were vaccinated, rested and pulsed with BrdU as in Fig 7. dLN cells were harvested on indicated days. Cells were surface-stained, intracellularly stained for cytokines, and stained with anti-BrdU. Numbers represent percent ± SD of BrdU+ cells among CD8+ CD44hi T cells. N = 4–5 mice/group. **P<0.01 and ****P<0.0001.

(TIF)

**S6 Fig. Apoptosis of memory Tc17 cells.** Naïve IL17aCreR26R eYFP mice were vaccinated and rested for 76 days as described in Fig 7B. Splenocytes were re-stimulated with anti-CD3 and -CD28 antibodies followed by staining for surface markers and intracellular staining for active-Caspase 3 and 8 molecules. Data represent dot plots gated on CD8+ T cells (top panels). Isotype control staining is shown (bottom).

(TIF)

**S7 Fig. Role of Bcl-2 for memory Tc17 cells.** IL17aCreR26R eYFP mice were vaccinated, rested, treated with Bcl-2 inhibitor ABT-199 and tissues were harvested for analysis as described in Fig 7. (A) Frequency and total numbers of CD8+ T cells, activated and naïve CD8+ T cells in the tissues. (B) To assess proliferation, cells were stained with anti-Ki-67 mAb intracellularly following intracellular cytokine staining, and the frequencies of Ki-67+ cells were analyzed by flow cytometry. N = 4–5 mice/group. CD4+ T cells were depleted throughout the experiment. *P<0.05 and **P<0.01.

(TIF)

**S8 Fig. Impact of HIF-1α on memory Tc17 and Tc1 cells.** Naïve IL17aCreR26R eYFP mice were vaccinated and rested as described in Fig 8. Splenocytes were harvested and surface-stained followed by intracellular staining for HIF-1α either directly ex vivo (A) or after re-stimulation with anti-CD3 and -CD28 antibodies (B). Histograms represent the mean florescence intensity of HIF-1α on different populations along with isotype control. (C) Mice were vaccinated, rested, and treated with either Echinomycin or vehicle as described in Fig 7. (D) Percent cytokine-producing cells among CD8+ CD44hi eYFP+ T cells. Numbers are percent ± SD of eYFP+ among total splenocytes or CD8+ T cells (parenthesis). N = 4–5 mice/group.

(TIF)

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