

BRIEF DEFINITIVE REPORT

STAT-3-independent production of IL-17 by mouse innate-like $\alpha\beta$ T cells controls ocular infection

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Appropriate regulation of IL-17 production in the host can mean the difference between effective control of pathogens and uncontrolled inflammation that causes tissue damage. Investigation of conventional CD4⁺ T cells (Th17 cells) has yielded invaluable insights into IL-17 function and its regulation. More recently, we and others reported production of IL-17 from innate $\alpha\beta$ + T cell populations, which was shown to occur primarily via IL-23R signaling through the transcription factor STAT-3. In our current study, we identify promyelocytic leukemia zinc finger (PLZF)-expressing iNKT, CD4⁻/CD8⁺, and CD4⁻/CD8⁻ (DN) $\alpha\beta$ + T cells, which produce IL-17 in response to TCR and IL-1 receptor ligation independently of STAT-3 signaling. Notably, this noncanonical pathway of IL-17 production may be important in mucosal defense and is by itself sufficient to control pathogenic *Staphylococcus aureus* infection at the ocular surface.

Introduction

Adaptive IL-17-producing CD4⁺ T cells (Th17) are involved in host defense as well as autoimmunity. Studies of the mechanisms that control IL-17 production in Th17 cells have revealed that IL-6, IL-21, IL-23, TGF- β , and/or IL-1 β , drive differentiation and production of IL-17 through the activation of STAT-3 and the master transcription factor ROR γ t (Ghoreschi et al., 2010).

Recently, attention has expanded to other populations of cells that produce IL-17, which include adaptive CD8⁺ T cells (Fletcher et al., 2010) as well as various innate T cells (Isailovic et al., 2015). Unlike adaptive Th17 cells that require priming and polarization for IL-17 production, innate IL-17-producing cells respond with quick and robust production of the cytokine (Sutton et al., 2009; Takatori et al., 2009; Myles et al., 2013). The ability to produce IL-17 rapidly was shown to be critical during early stages of infection with pathogens such as *Staphylococcus aureus* (Cho et al., 2010), *Klebsiella pneumoniae* (Happel et al., 2003), *Candida albicans* (Gladiator et al., 2013; Conti et al., 2014), and *Toxoplasma gondii* (Passos et al., 2010). Cells that produce innate IL-17 include CD8⁺ T cells (Happel et al., 2003; Fletcher et al., 2010), $\gamma\delta$ TCR⁺ cells, NK1.1⁻ NKT cells (NKT₁₇; Rachitskaya et al., 2008), mucosal-associated invariant T cells (MAIT cells; Dusseaux et al., 2011), CD4⁻CD8⁻ T cells

(Sherlock et al., 2012), natural Th17 cells (nTh17; Marks et al., 2009), lymphoid tissue inducer (LTi) cells, and type 3 innate lymphoid cells (ILC3s; Annunziato et al., 2015). Although different pathways to IL-17 induction have been described (Durant et al., 2010; Ghoreschi et al., 2011), all have reported a critical role for IL-23 and/or STAT-3, with therapeutic strategies to target IL-17 production now based largely around manipulation of these mediators.

In the current study, we report that IL-17 production by innate, promyelocytic leukemia zinc finger (PLZF)-expressing lymphocytes can be driven by TCR ligation and IL-1 β , independently of both STAT-3 and IL-23 signaling, and has in vivo relevance. In particular, we examine three populations of T cells, CD44^{hi} CD4⁻CD8⁺ T cells, CD44^{hi} CD4⁻CD8⁻ double-negative T cells (DNT), and iNKT cells, all of which appear to acquire effector function in the thymus, can use this pathway, and readily produce IL-17, even in mice genetically deficient in STAT-3. Most importantly, we show that in the presence of IL-1 β , these cells produce sufficient levels of IL-17 to prevent the outgrowth of pathogenic *S. aureus* in the conjunctiva, demonstrating the relevance of the STAT-3-independent pathway of IL-17 production in mucosal infection.

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Results and discussion

IL-17-producing T “memory” lymphocytes are present in mice deficient in IL-6, IL-21, and IL-23 signaling, which lack adaptive Th17 cells

IL-1 β provides a critical signal for both conventional Th17 and innate IL-17 responses (Chung et al., 2009; Ikeda et al., 2014). The downstream components of this pathway have not been clearly defined, but one possibility could be that IL-1 β is inducing IL-17 through secondary mediators such as IL-6 and IL-21. To investigate this, we bred mice deficient in IL-6 and in IL-21 receptor (IL-6/21R double knockout [DKO]), which are thought to lack adaptive Th17 cells. To our surprise, $\alpha\beta$ TCR⁺ cells with a memory phenotype (CD44^{hi}CD62L^{lo}), isolated from spleens and lymph nodes of these mice using NKT and $\gamma\delta$ TCR⁺ exclusion gates (Fig. S1), exhibited robust IL-17 production after 72 h of stimulation with anti-CD3 (α CD3) and IL-1 β (Fig. 1 A). IL-17 production after stimulation with IL-1 β or IL-23 did not occur in the absence of CD3 stimulation (not depicted).

We sought to identify the cell types within this population that produce IL-17 independently of both IL-6 and IL-21 signaling. To eliminate potential confounding effects of IL-23, which induces robust IL-17 production in innate cells as well as memory T cells, we additionally generated an IL-6/21R/23R triple-KO (TKO) mouse by crossing the IL-6/21R DKO mouse to the IL-23R GFP mouse, which is a functional IL-23 KO when bred to homozygosity (Awasthi et al., 2009). For both IL-6/21R DKO and IL-6/21R/23R TKO strains, we further fractionated the “Tmem” population into CD4⁺, CD8⁺, and DN $\alpha\beta$ TCR⁺ cells (full scheme in Fig. S1) and stimulated them *in vitro* for 72 h with α CD3 and IL-1 β . NKT₁₇ cells, which we have previously shown to produce IL-17 independently of IL-6 and IL-21 (Rachitskaya et al., 2008), served as comparison and control.

As we expected, adaptive CD44^{hi}CD4⁺ T cells from IL-6/21R DKO mice, which lack conventional Th17 cells, showed minimal IL-17 production after stimulation with α CD3 and IL-1 β , and/or IL-23, and IL-17 was completely absent in CD4⁺ cells from IL-6/21R/23R TKO mice (Fig. 1, B and C, top). Although neither of these strains was expected to harbor conventional memory IL-17-producing cells, we found IL-17-producing cells within the memory phenotype CD8⁺ and DN $\alpha\beta$ TCR⁺ cell populations of DKO and TKO mice (Fig. 1, B and C). Despite a reduction in IL-17 production when the functional IL-23R was also deleted, the memory-phenotype CD8⁺ and DNT cells maintained the ability to produce IL-17 (5% and 20%, respectively). Even iNKT cells, which were previously thought to require IL-23 for IL-17 production (Rachitskaya et al., 2008; Doisne et al., 2011), were unaffected by lack of IL-23R in the presence of IL-1 β .

IL-17-producing memory-like $\alpha\beta$ T cells in TKO mice express the innate-associated transcription factor PLZF and do not phosphorylate STAT-3

The data above suggested that the memory-phenotype CD8⁺ and DNT represent innate-like T cells rather than conventional adaptive lymphocyte populations and that they are able to produce IL-17 independently of the canonical signaling cascades previously described for adaptive memory (IL-6 and/or IL-21 dependent) and innate (IL-23 dependent) IL-17 production. We

therefore set out to examine whether memory-phenotype CD8⁺ and DNT cells expressed PLZF, characteristic of innate lymphocytes (T and non-T; Savage et al., 2008), and whether STAT-3, used by all IL-17 induction pathways described thus far, is phosphorylated by stimuli that lead to IL-17 production in these cells.

Sorted memory-phenotype cells from the lymphoid organs of WT and TKO mice were stimulated with α CD3/IL-1 β for 72 h. IL-17-producing iNKT cells from both WT and TKO mice had increased expression of PLZF, as has already been described by others (Savage et al., 2008) and was absent, as expected, in conventional WT CD4⁺ memory T cells (Fig. 2 A). Notably, enhanced expression of PLZF was observed in IL-17-producing Tmem CD44^{hi}CD8⁺ and DNT cells, supporting their classification as innate lymphocytes; however, PLZF was not a requirement of IL-17 production, as siRNA for PLZF reduced expression of the transcription factor but not IL-17 after stimulation (Fig. 2 B). The development of CD8⁺ and DNT innate-like cells appeared to require MHC class I and/or nonclassical MHC interactions, as they were not present in mice lacking β 2-microglobulin (β 2m; Fig. 2 C). Finally, they appeared to be distinct from MAIT cells, which also produce IL-17, express PLZF, and whose development is β 2m dependent, in that they did not have the conserved TCR V β 6/8 characteristic of MAIT cells (Le Bourhis et al., 2010; Fig. 2 D); however, definitive assessment of STAT-3 signaling in IL-17-producing MAIT cells will require the use of appropriate tetramer reagents.

The cytokines IL-23, IL-6, and IL-21 are all known to induce IL-17 production in CD4⁺ T cells by activation of STAT-3, which is considered to be a critical activating signal for the induction and maintenance of the Th17 lineage (Bettelli et al., 2006; Harris et al., 2007; Huber et al., 2009; Ghoreschi et al., 2010; El-Behi et al., 2011). To rule out the possibility that another cytokine might be circumventing conventional signaling, we examined whether IL-17-producing innate-like TKO cells still phosphorylate STAT-3. CD8⁺ and DNT innate-like cells isolated from TKO or WT mice were pooled within the genotype to obtain sufficient cells for analysis. The innate-like CD8⁺/DNT populations were then stimulated for 18 h with α CD3/IL-1 β to ensure sufficient proliferation within the appropriate cell populations. We assessed STAT-3 phosphorylation by intracellular flow cytometry. Although stimulation with α CD3+IL-1 β led to phosphorylation of STAT-3 in the innate-like cells from WT mice, no detectable phosphorylation occurred in cells from TKO mice (Fig. 2 E), suggesting the existence of an alternative pathway for IL-17 production that does not use STAT-3.

To address the possibility that this phenomenon was a compensatory mechanism unique to the TKO mice, we isolated CD8⁺, DNT, and iNKT cells from WT mice and stimulated them *in vitro* using neutralizing antibodies to the cytokines IL-6, IL-21, and IL-23 and their receptors. IL-17 production not only was maintained after 72 h in culture but also appeared to increase in the presence of the antibody cocktail (Fig. 2 F). Intracellular flow cytometry confirmed that STAT-3 was not being phosphorylated in these cells. (Fig. 2, G and H). Although the increase in IL-17 was not necessarily expected, it is conceivable that the pleiotropic nature of IL-6 and IL-21 could regulate IL-17 production in cells that do not use STAT-3 signaling (Scheller et al., 2011; Croce et al., 2015).

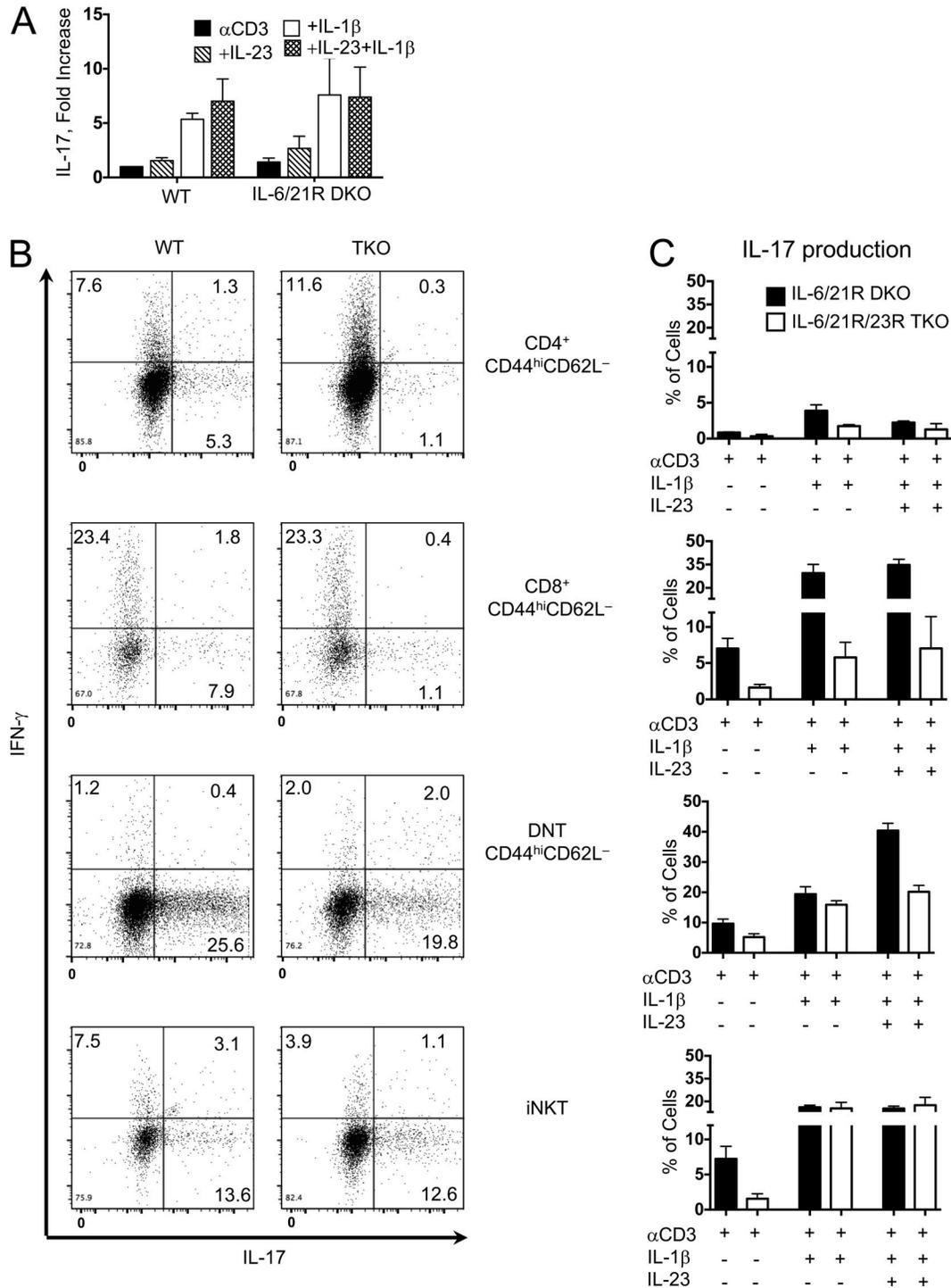


Figure 1. IL-17-producing T “memory” lymphocytes are present in mice that lack adaptive Th17 cells. (A–C) CD4^{hi}CD62L^{low} α β T cells sorted from WT and IL-6/-21R DKO mice (A), WT and IL-6/-21R/-23R TKO (B), or DKO and TKO mice (C) and stimulated for 72 h with α CD3, IL-1 β , and/or IL-23. Brefeldin A was added the last 6 h in culture. Cells were harvested, fixed, and permeabilized and stained for intracellular IL-17 and IFN γ . Flow dot plots represent cytokine production after stimulation between WT and TKO mice, and numbers represent frequencies of cytokine production. (C) Bars in graphs represent the mean frequency \pm SEM of IL-17⁺ cells after culture. *n* = 3 (α CD3), 5 (α CD3+IL-1 β), and 5 (α CD3+IL-1 β +IL-23); each *n* consists of pooled cells from at least three mice.

Innate-like CD8⁺ and DN α β T cells produce IL-17 independently of STAT-3 but require the transcription factor ROR γ T

To directly examine whether STAT-3 is necessary for production of IL-17 by CD8⁺ and DNT cells, we used conditional KO CD4-Cre \times *Stat3^{fl/fl}* (STAT-3 KO) mice, in which all thymically derived T

cells are rendered STAT-3 null during the CD4/CD8 double-positive stage in the thymus (Durant et al., 2010). First, we confirmed that STAT-3 is indeed absent in the innate-like cell populations of STAT-3 KO mice by quantitating STAT-3 genome copy number using RT-PCR (Fig. 3 A). Next, we examined whether

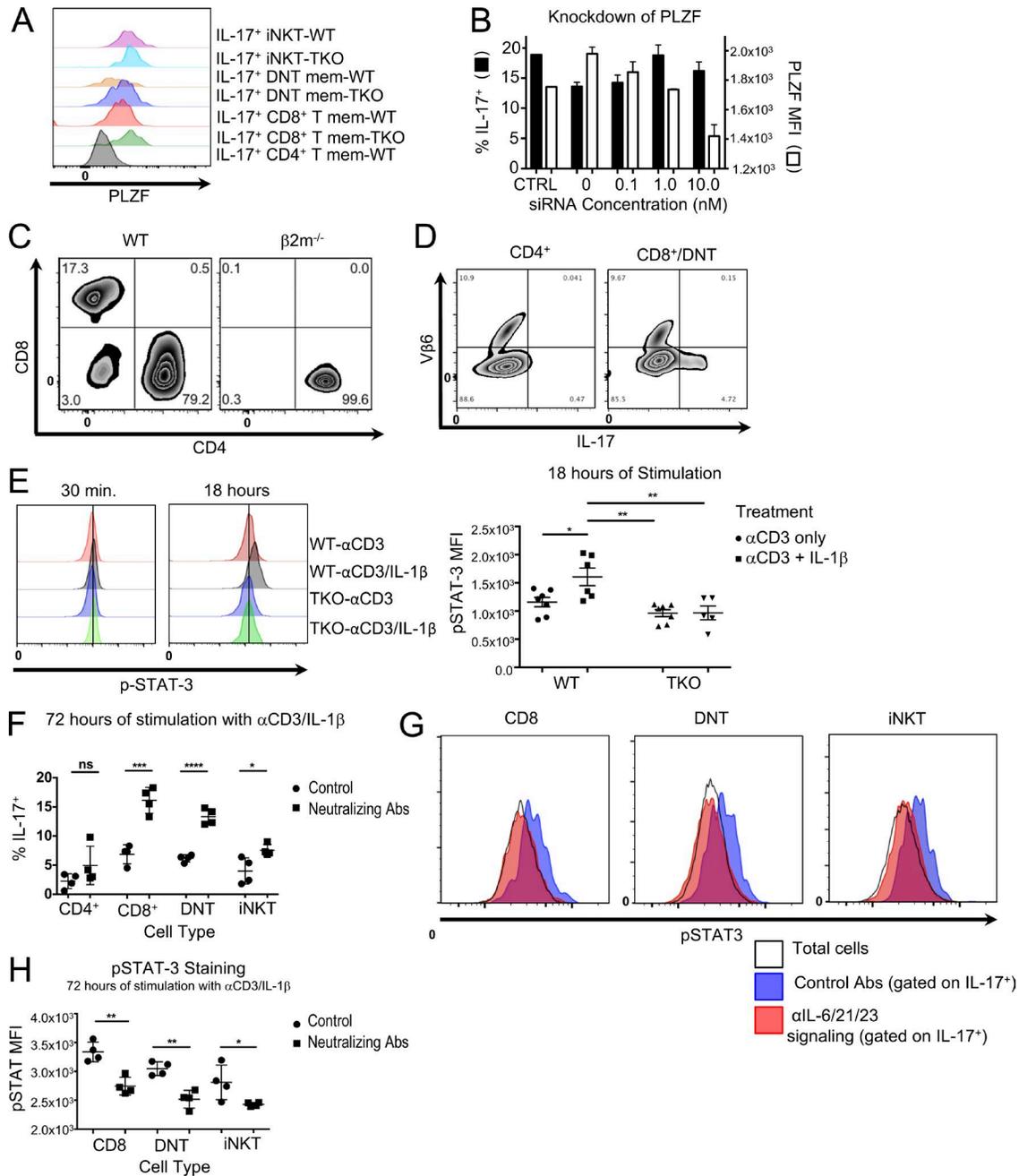


Figure 2. IL-17-producing memory-like $\alpha\beta$ T cells in TKO mice express the innate-associated transcription factor PLZF and do not phosphorylate STAT-3. (A) $CD44^{hi}CD62L^{low} CD4^+, CD8^+, DNT,$ and $iNKT$ cells were sorted, stimulated with IL-1 β and/or α CD3 for 72 h, fixed, and permeabilized. Cells were then stained for intracellular IL-17, PLZF, and lineage-specific markers. The representative histogram (from two independent experiments) depicts the PLZF expression in IL-17 $^+$ cells from the specified populations. (B) $CD44^{hi} CD8^+$ and DNT cells from TKO mice were sorted and stimulated with CD3 and IL-1 β in the presence of siRNA against PLZF for 72 h, and brefeldin A was added the last 6 h of culture. Cells were then stained for intracellular IL-17 and PLZF and analyzed by flow cytometry. Black bars represent IL-17 production, and white bars represent PLZF expression in the IL-17 $^+$ population. (C) $CD4^+$ and $CD8^+ CD44^{hi}CD62L^{low} \alpha\beta TCR^+ \gamma\delta TCR^-$ cells from pooled ($n = 5$) LNs of WT or $\beta 2m^{-/-}$ mice. Data represent two independent experiments. (D) $CD44^{hi}CD62L^{low} CD4^+$ or $CD8^+/DNT$ cells were sorted as stated previously from IL-6/21R/23R TKO and cultured with IL-1 β and α CD3 for 72 h. After stimulation, cells were stained for IL-17 and V $\beta 6$. Data represent two individual experiments. (E) $CD44^{hi}CD62L^{low} CD8^+$ and DNT cells were isolated, combined, and cultured with IL-1 β and/or α CD3 for 30 min and 18 h. After stimulation, cells were harvested, fixed, permeabilized, and stained for phospho-STAT-3. Symbols represent individual mice from two independent experiments. Bars represent mean \pm SEM. MFI of pSTAT-3 in live cells. One-way ANOVA determined significance (*, $P = 0.0295$; ***, $P < 0.0036$). (F–H) WT $CD44^{hi}CD62L^{low} CD8^+, DN,$ and $iNKT$ cells were sorted as described previously. Each population was cultured with either IL-1 β and α CD3 with or without α IL-6/ α IL-6Ra/ α IL-21/ α IL-23p19/ α IL-23R. After 72 h, cells were harvested, fixed, permeabilized, and stained for (F) intracellular IL-17 and (G and H) phospho-STAT-3. Symbols represent technical replicates from a single experiment that consisted of pooling cells from three mice. Bars represent the mean (E) IL-17% or (G) pSTAT-3 MFI \pm SEM, and data are representative of two independent experiments. Student's t test was used to determine significance (for F, *, $P = 0.0274$; ***, $P = 0.0005$; ****, $P < 0.0001$; for G, *, $P = 0.0449$; **, $P < 0.0021$).

innate-like cells that are genetically deficient in STAT-3 are able to produce IL-17 in response to TCR and IL-1 stimulation. Purified CD44^{hi}CD4⁺ T cells, CD44^{hi}CD8⁺ T cells, CD44^{hi}DNT cells, and iNKT cells from STAT-3 KO mice and their WT littermates were stimulated with α CD3 alone or α CD3 plus IL-1 β , and their cytokine production was compared using intracellular cytokine staining and ELISA of culture supernatants. (γ δ T cells, which do not undergo a CD4⁺ stage and hence are not rendered STAT-3 deficient in STAT-3 KO mice, were excluded, as shown in Fig. 1 A.) In contrast to CD44^{hi}CD4⁺ T cells, all three STAT-3-deficient innate-like cell populations maintained IL-17 production in response to TCR ligation, and it was again strongly up-regulated by IL-1 β (Fig. 3, B–D).

To further confirm that our innate-like CD8⁺ and DNT cells are not part of the conventional T memory compartment, we isolated naive CD4 and CD8 T cells from WT and STAT-3 KO mice and primed them in vitro under conventional IL-17-inducing conditions. In contrast to the (originally naive) WT T cells, neither the CD4⁺ nor the CD8⁺ T cells from STAT-3 KO mice gave rise to IL-17 production after 3 d of culture with IL-6, IL-23, and TGF- β (Fig. 3 E), supporting the notion that the populations we have defined are innate T cells that express memory markers rather than conventional memory T cells.

Finally, we wished to examine the ROR γ t dependence of IL-17 production by innate-like T cells. ROR γ t is known to play a role in the maintenance of innate IL-17-producing cells; however, its functional influence on innate IL-17 production is still unclear. Using ROR γ t^{-/-} mice, we found that even under conditions of STAT-3 sufficiency, the innate-like CD8⁺ and DNT cells lacking ROR γ t failed to produce IL-17 after the 72 h with combined TCR and IL-1 stimulation (Fig. 3 F; iNKT cell numbers were too low to isolate for culture). These data show that despite deficiency of STAT-3, ROR γ t is required either for acquisition of function or for the development of IL-17-producing innate-like $\alpha\beta$ T cells.

STAT-3-independent IL-17-producing innate-like $\alpha\beta$ T cells develop in the thymus

We asked whether these cells are thymically derived or develop in the periphery. Within the CD44^{hi} population of thymocytes (gating strategy in Fig. S2), CD8, DN, and iNKT cells all expressed PLZF (Fig. 4 A) and produced IL-17 after stimulation with CD3 plus IL-1 β (Fig. 4 B). We next asked whether peripheral naive STAT-3 KO cells could acquire this phenotype after expansion in a lymphopenic host as observed in a recent study investigating IFN- γ in CD44^{hi} $\alpha\beta$ T cells. After transfer of CD44^{hi} CD8/DNT STAT-3 KO thymocytes or naive CD8/DNT STAT-3 KO cells into Rag2/Il2rg DKO, only the thymus-derived donor cells were able to produce IL-17. Naive peripheral donor cells acquired a memory phenotype (not depicted) and the ability to produce IFN- γ , similar to what was observed in spontaneous memory development in a different model (Kawabe et al., 2017). However in our study, memory cells derived from naive STAT-3 KO did not acquire the ability to produce IL-17 (Fig. 4 C). We conclude that CD44^{hi} expression and effector function by innate-like CD8 and DN $\alpha\beta$ T cells is acquired in the thymus rather than after activation in the periphery.

STAT-3-independent IL-17-producing innate-like $\alpha\beta$ T cells are present in the mucosal tissues of the ocular surface and can protect from *S. aureus* infection

The following experiments primarily used TKO rather than STAT-3 KO mice, because in the latter, STAT-3 is not excised in $\gamma\delta$ T cells, which can also produce IL-17 in the conjunctiva. As shown in Fig. 2 D, STAT-3 was not activated in TKO cells under conditions that lead to IL-17 production. This indicated that CD44^{hi} CD8⁺ and DNT cells from TKO mice were a valid model to assess IL-17 production under STAT-3-insufficient conditions.

We next sought to identify whether TKO mice harbored CD44^{hi} cells within ocular mucosa during steady-state conditions. We assessed the ability of CD44^{hi} cells isolated from ocular mucosa of TKO and WT mice to produce IL-17 (a) directly ex vivo after a PMA/ionomycin pulse, as reflective of their status in situ, and (b) after culture of sorted populations with α CD3 plus IL-1 β , as reflective of their potential after activation. Immunophenotyping combined with intracellular cytokine staining revealed a strikingly different pattern of IL-17 production in conjunctival cells from WT versus TKO mice. Under STAT-3 insufficiency, conjunctival CD4⁺ T cells do not produce IL-17 after PMA/ionomycin stimulation. In contrast, in the same conjunctiva there is an increased frequency of IL-17-producing CD8⁺ T cells (9.8% WT, 33.7% TKO) and a moderately increased frequency of IL-17-producing DNT cells (45.1% WT, 59.8% TKO; Fig. 5 A). These data suggest that rather than abolishing IL-17 production, STAT-3 insufficiency allows for the expansion of other cell populations that can produce IL-17. We next examined the response of purified cell subsets to activation. Because of insufficient numbers of cells in normal conjunctival tissue, we isolated cells from the lacrimal gland, which is also part of the ocular mucosal system. Highly purified CD4⁺, CD8⁺, and DNT cells from lacrimal gland tissue were subjected to α CD3/IL-1 β stimulation. Again, IL-17 was produced by CD4⁺ T cells of WT, but not TKO mice (although TKO cells produced copious amounts of IFN- γ), and again, TKO-CD8⁺ T cells produced more IL-17 than WT controls (Fig. 5 B). We observed a dramatic difference in the pattern of cytokine production between the conjunctiva and lacrimal gland, suggesting that the lacrimal gland may lack a critical signal that drives this nonconventional IL-17 production and/or the tissue may harbor an unknown suppressive factor regulating the production of IL-17.

Despite compelling evidence that populations of innate-like CD8⁺ and DN $\alpha\beta$ T cells could rapidly produce IL-17 independently of STAT3, it was unclear whether these cells, or indeed this pathway, might have relevance in vivo. We noted that IL-17A/IL-17F genetically deficient (A/F DKO) mice maintained in our animal facility spontaneously develop severe conjunctivitis accompanied by swelling of their eyelids. A bacterial nature of this condition was suggested by a published report that IL-17A/F DKO mice are prone to *S. aureus* infections of the lungs and nares (Ishigame et al., 2009). In line with this, we were able to culture *S. aureus* from the eyes of IL-17A/F DKO mice, and the inflammation was cleared by treatment with antibiotics (Fig. 5 C). Because eyes of TKO and STAT-3 KO mice housed under the same conditions remained healthy (Fig. 5 C and not depicted), we hypothesized that STAT-3 KO and TKO mice may harbor innate-like $\alpha\beta$ T cells in

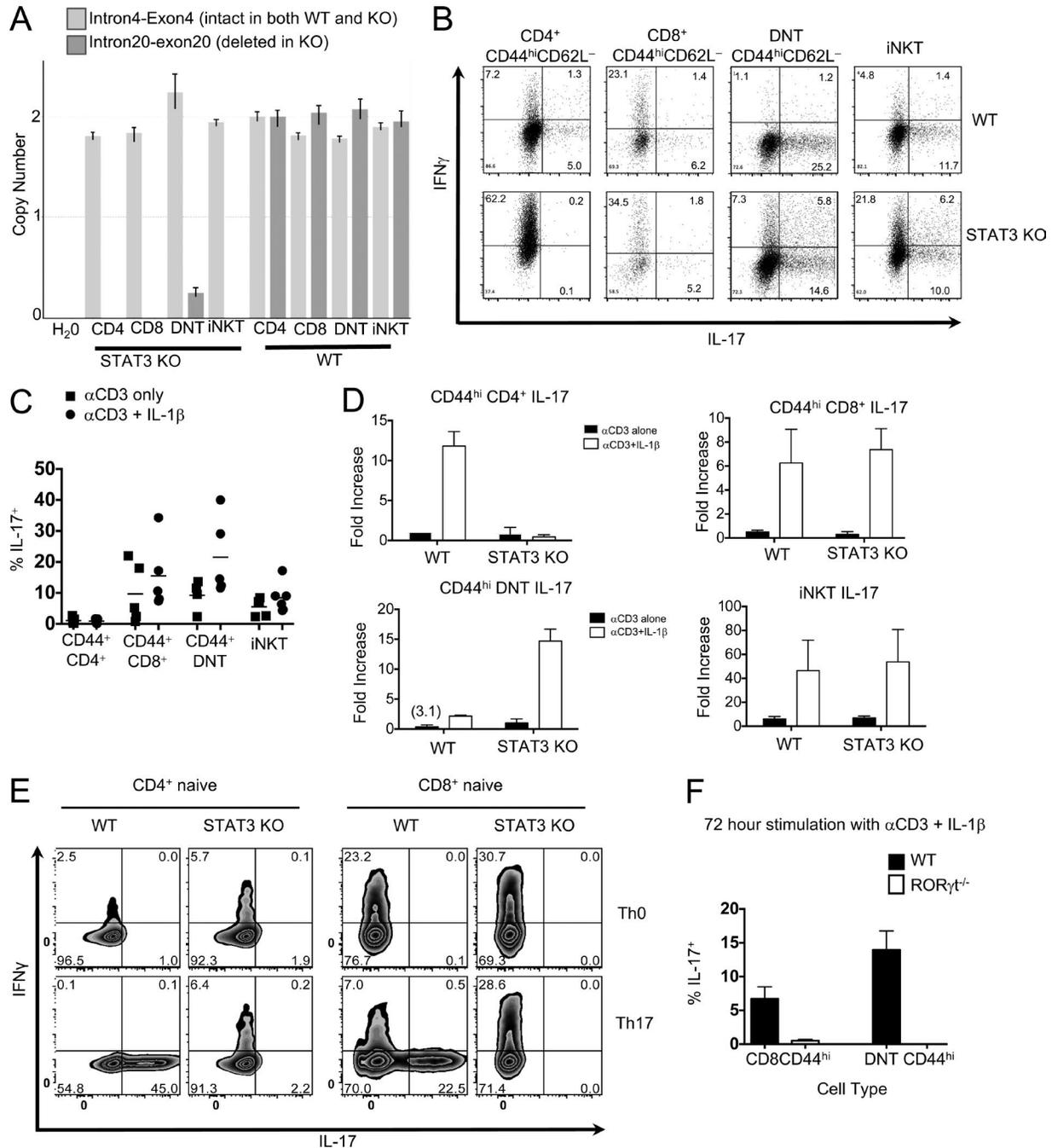


Figure 3. Innate-like CD8⁺ and DN $\alpha\beta$ T cells produce IL-17 independently of STAT-3 but require the transcription factor ROR γ t. (A) DNA was isolated from at least 30,000 CD4⁺, CD8⁺, DN, and iNKT cells from CD4-Cre \times *Stat3^{fl/fl}* (STAT-3 KO) or *Stat3^{fl/fl}* (WT) mice. Data shows copy number using TaqMan primers from ABI spanning intron4–exon4, (intact *Stat3* region control, Mm00369932_cn) and intron20–exon20 (deleted region, Mm00369911_cn). (B–E) CD44^{hi} CD62L^{low} $\alpha\beta$ TCR⁺ $\gamma\delta$ TCR⁻ cells (B–D) or naive T cells (E) were sorted from WT or STAT-3 KO mice and stimulated for 72 h with IL-1 β and/or α CD3. Brefeldin A was added the last 6 h in culture. After incubation, cells were harvested, fixed, permeabilized, and stained for intracellular cytokines, IL-17 and IFN- γ , for flow cytometric analysis. (B) Representative dot plots showing IL-17 and IFN γ production in T memory cells from WT (top) STAT-3 KO (bottom) mice. Frequencies of the cell populations are within the plots. (C) Bars represent the mean frequency of IL-17⁺ cells in T memory cells after culture. Symbols represent individual mice and data are representative of five independent experiments. (D) Bars represent the fold increase \pm SEM in IL-17 protein in supernatants after culture. Background levels ranged from 10 to 50 pg/ml. Total $n = 2$; each n was a single experiment consisting of cells pooled from three mice. (E) Representative flow plots showing IL-17 and IFN- γ production in naive T cells after 72 h of culture using Th0 (top) or Th17 (bottom) skewing condition. Data are representative of at least four independent experiments. (F) CD44^{hi} CD62L^{low} $\alpha\beta$ TCR⁺ $\gamma\delta$ TCR⁻ cells from WT or ROR γ t^{-/-} mice were sorted and stimulated with IL-1 β and α CD3 for 72 h. Brefeldin A was added the last 6 h of culture. Cells were harvested, fixed, stained for intracellular IL-17, and analyzed by flow cytometry. Bars represent the mean frequency \pm SEM of live cells that were IL-17⁺. Symbols represent technical replicates from cells pooled from four mice from a single experiment that was independently performed two times.

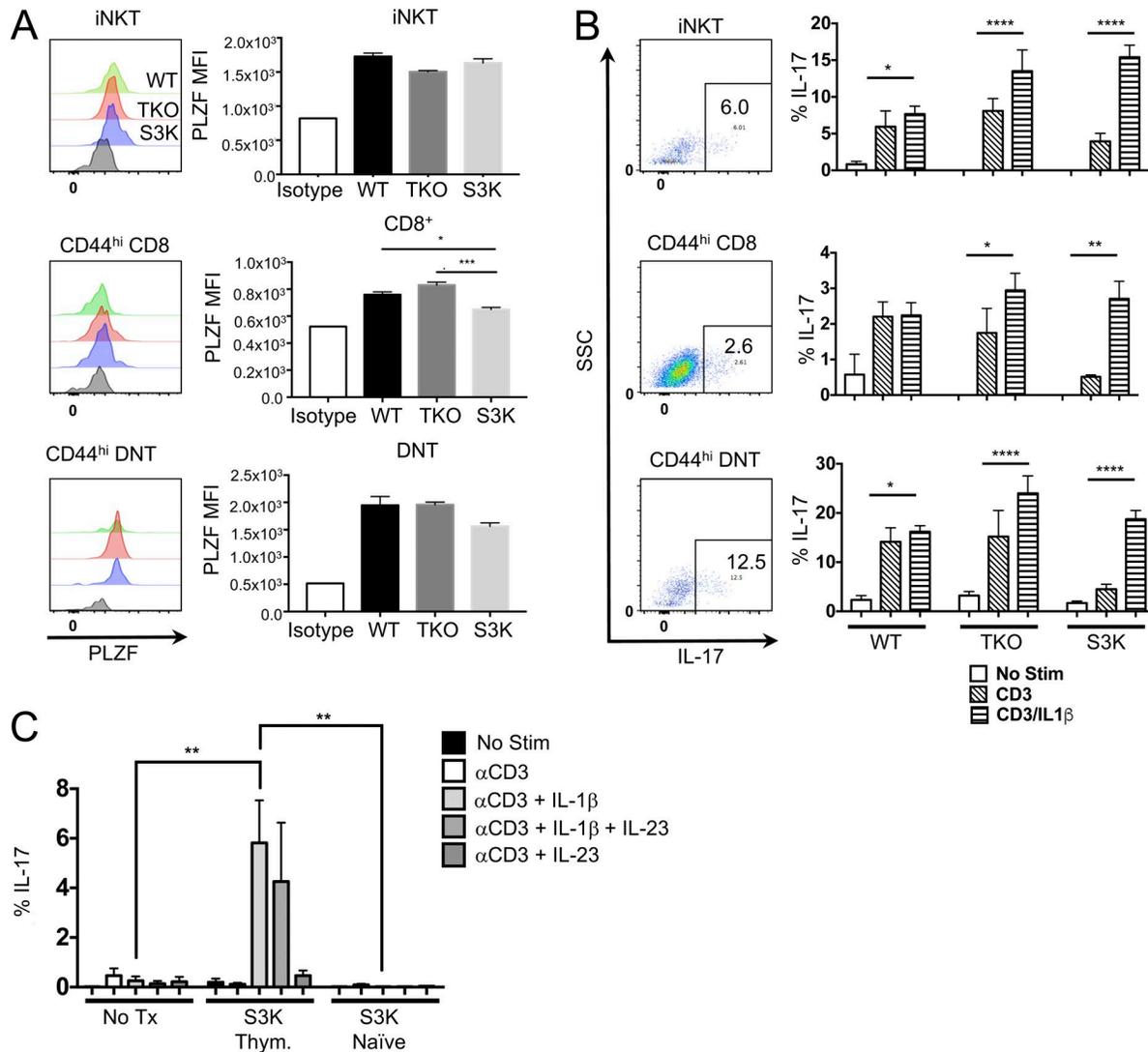


Figure 4. STAT3-independent IL-17-producing innate-like T cells exist in the thymus. (A and B) Single-cell suspensions of thymuses from 6-wk-old WT, TKO, or STAT-3 KO (S3K) mice were either stained for PLZF expression (A) or stimulated with CD3 and IL-1β for 72 h (B). Gating strategies are depicted in Fig. S2 A. Histograms represent the PLZF staining. Flow plots represent staining pattern in the thymus after stimulation with CD3 plus IL-1β. Bars in graphs represent the MFI of PLZF staining ± SEM ($n = 3$ for WT and TKO and $n = 5$ for S3K; *, $P = 0.0130$; ***, $P = 0.0006$). Data represent two independent experiments. **(B)** Flow plots represent the staining pattern after stimulation with either CD3 or CD3 and IL-1β (no stim samples yielded very few cells). Brefeldin A was added the last 6 h of culture, and IL-17 was assessed by flow cytometry. Bars represent the mean frequency ± SEM of cells that were IL-17⁺ ($n = 3$ for WT and TKO and $n = 5$ for S3K; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). Statistical significance was determined using ANOVA. **(C)** 2×10^4 CD44^{hi} CD8/DNT S3K thymocytes or 2×10^5 naive CD44^{hi} CD8/DNT S3K cells were transferred i.v. into Rag2/Il2rg DKO mice. After 4 wk, splenic cells were isolated and stimulated with CD3 and IL-1β and/or IL-23 for 72 h. Brefeldin A was added the last 6 h of culture, and cells were stained for intracellular IL-17. Bars represent the mean frequency ± SEM of IL-17⁺ cells after culture ($n = 3$ for WT and S3K naive, $n = 5$ for S3K Thym.). Data represent two independent experiments. Statistical significance was determined by ANOVA (**, $P = 0.002$).

ocular tissue that produce protective IL-17 under STAT-3-insufficient conditions to prevent outgrowth of the bacteria.

We examined whether IL-17 was required for protection from ocular infection by ocularly challenging TKO mice that were treated with control or IL-17 neutralizing antibody with *S. aureus*. Indeed, IL-17 was required to limit *S. aureus* in ocular tissue as mice treated with αIL-17 threefold more CFUs compared with the control group (Fig. 5 D). Similarly, we next showed that preactivated CD44^{hi} CD8/DNT cells subconjunctivally injected into IL-17A/F could protect mice from local *S. aureus* challenge (Fig. 5 E). Together, these data suggested that

IL-17 produced from CD44^{hi} cells under condition of STAT-3 insufficiently was sufficient to protect ocular mucosa. Finally, we asked if activated STAT-3 KO CD44^{hi} CD8/DNT cells could protect the ocular surface from *S. aureus* challenge. Indeed, after activation in vitro and subconjunctival transfer into IL-17A/F DKO mice, these cells reduced *S. aureus* burden at the ocular surface compared with mice that received unactivated cells (Fig. 5 F), suggesting that cells can protect the ocular surface once activated.

In conclusion, although the requirement for STAT-3 signaling in inducible Th17 development has been clearly demonstrated,

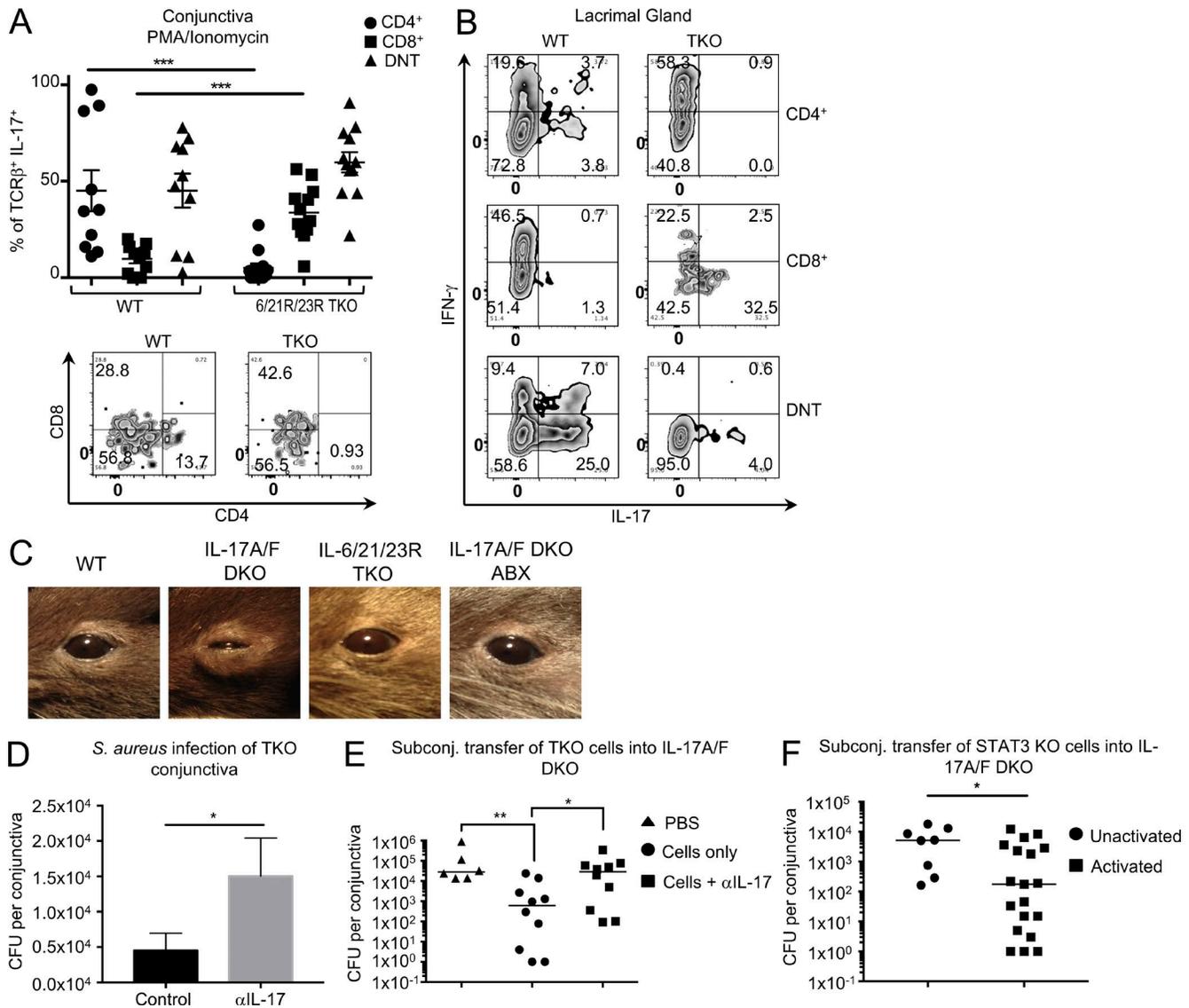


Figure 5. STAT-3-independent IL-17-producing innate-like $\alpha\beta$ T cells are present in mucosal tissue of the ocular surface. (A and B) Conjunctiva (A) or lacrimal glands (B) were harvested from WT or TKO mice, treated with collagenase, and dispersed into single-cell suspensions. Cells were either pulsed for 4 h with PMA/ionomycin in the presence of brefeldin A (A) or stimulated with α CD3/IL-23/IL-1 β for 72 h (B), and brefeldin A was added during the last 6 h of culture. Cells were then stained for TCR β , CD4, and CD8, fixed and permeabilized, and stained for intracellular IL-17. (A) Each symbol represents an individual mouse, and bars represent the mean frequency \pm SEM of TCR β ⁺ that produced IL-17. Zebra plots are gated on IL-17⁺ cells. Data are pooled from three independent experiments. Student's *t* test determined significance (***, *P* < 0.0007; *P* = 0.1532 for DNT). (B) Zebra plots represent cytokine production from lacrimal gland cells from two independent experiments. (C) Representative pictures of mouse eyes from mice treated or not with antibiotics. Disease typically observed in 50% of IL-17A/F DKO mice. (D) TKO mice were given an i.p. injection of either PBS or 500 μ g α IL-17 (DNAX). 2 d later, mice were anesthetized with a ketamine-xylazine mixture, given another i.p. injection of PBS or α IL-17, and conjunctivae were inoculated with a cotton swab soaked in *S. aureus* (USA300). 72 h later, conjunctivae were harvested, and *S. aureus* CFUs were calculated. Bars represent the mean \pm SEM CFUs per conjunctiva. Statistical significance was determined using a Mann-Whitney *U* test (*, *P* = 0.133; *n* = 13 for control, *n* = 16 for α IL-17). (E and F) *S. aureus* was topically applied on days -14 and -7. (E) On day 0, 10,000 activated CD8 and DN memory $\alpha\beta$ T cells from TKO mice were subconjunctivally transferred with or without 5 μ g α IL-17. Mice were sacrificed, and conjunctiva was harvested on day 3. Statistical significance was determined using the Kruskal-Wallis test (*, *P* = 0.040; **, *P* = 0.009). (F) On day 0, 10,000 unactivated or activated CD8 and DN memory $\alpha\beta$ T cells from STAT-3 KO mice were subconjunctivally transferred. Mice were sacrificed and conjunctiva was harvested on day 3. Bars represent the median *S. aureus* CFU counts per conjunctiva. Statistical significance was determined using the Mann-Whitney *U* test (*, *P* = 0.015). Each point represents an individual conjunctiva pooled from two independent experiments.

less attention has been paid to whether the same applies to naturally occurring IL-17-producing $\alpha\beta$ TCR⁺ populations. Although Tanaka et al. (2009) suggested that nTh17 cells develop independently of STAT-3, more recent studies suggest that this population still requires IL-23 signaling for IL-17 production (Conti et

al., 2014), and neither study addressed the role of IL-1 β . Indeed, a lack of appreciation for the role of IL-1 β in IL-17 production led to its omission in early characterization studies (including our own), which instead focused solely on IL-6, IL-21, and IL-23. Our study provides clear evidence that IL-1 β is sufficient to drive IL-17

production independently of both STAT-3 and IL-23 in PLZF-expressing iNKT, CD8⁺, and DNT $\alpha\beta$ TCR⁺ cells. Furthermore, nTh17 cells appear to have distinct migratory patterns compared with the cells described in our study. Whereas nTh17 cells appear to localize to airways and the oral cavity, they appear to be absent in the ocular mucosa of mice with STAT-3 insufficiency (TKO or STAT-3 KO), highlighting a potential role for STAT-3 in the migration pattern of nTh17 cells to ocular mucosa (Oh et al., 2011). Conversely, we observed an enhancement of innate-like CD8⁺ and DNT cells at the ocular surface in mice that cannot use STAT-3 signaling, suggesting that these cells may play a unique biologically relevant role at the ocular surface.

The expression of PLZF in the IL-17-producing cells described in this study confirms their innate-like nature (Savage et al., 2008; Constantinides et al., 2014) and may explain their memory phenotype (Kovalovsky et al., 2010); however, the requirement of PLZF for this phenotype has not been addressed. Our data demonstrate that IL-1 stimulation can induce rapid IL-17 production in a TCR-dependent manner by using signaling pathways distinct from conventional Th17 cells, which may include the usage of transcription factors, NF- κ B and/or interferon regulatory factors (IRFs). Although our study does not address what is the TCR signal in vivo, we speculate that it could originate from the same microorganisms that induce IL-1 β production. Notably, despite the evidence that implicates (adaptive) IL-17 as a pathogenic cytokine in ocular diseases (Chauhan et al., 2009; Suryawanshi et al., 2011; Zaidi et al., 2012), we show a beneficial role of innate IL-17 at the ocular surface and provide evidence that this IL-17 can be produced via a distinct pathway that does not necessarily depend on STAT-3. Importantly, even a small number STAT-3-deficient cells stimulated to produce IL-17 can rapidly and substantially reduce *S. aureus* within ocular mucosa. These data indicate for the first time that STAT-3-independent IL-17 production is sufficient to modulate immunity and prevent infection at the ocular surface, and potentially other sites, without the need for an adaptive Th17 response.

In the aggregate, our data support the interpretation that innate-like production of IL-17 through a noncanonical pathway that does not require IL-23 or activation of STAT-3 may have a physiological role in mucosal host defense. That said, our data do not exclude the possibility that when dysregulated this innate IL-17 could also be deleterious. It is of note that IL-17-producing CD8 and DNT cells have been identified in several human disease states (Blanco et al., 2005; He et al., 2006; Tzartos et al., 2008; Res et al., 2010; Sherlock et al., 2012; Huber et al., 2013). Future studies will hopefully aim to explore potential functional parallels with the murine IL-17-producing CD8⁺ and DNT cells we have described. Although beyond the scope of our current study, such information could be important for therapeutic approaches to T cell-mediated diseases, in which IL-17 production from such cells would be refractory to STAT-3 inhibitors currently under development. While this might lessen the effectiveness of therapy, it could also mitigate increased susceptibility to infections that can result from targeting cytokines that function in host defense, and promote a more favorable immunological balance.

Materials and methods

Mice

C57BL/6 (WT) and IL-6 KO mice were purchased from The Jackson Laboratory. RAG2/IL2rg DKO mice were purchased from Taconic Farms. IL-21R-deficient mice (Ozaki et al., 2002), IL-23R-GFP reporter mice (Awasthi et al., 2009), STAT-3-deficient mice (Lee et al., 2002), and IL-17A/F-deficient mice (Ogura et al., 2008; Ishigame et al., 2009) were generated and provided by the referenced collaborators. Crosses of these various strains were generated and maintained in the animal facilities of the National Institutes of Health (NIH) in Bethesda or Frederick, MD, in full compliance with institutional guidelines. Male and female mice aged 6 to 12 wk were used for analyses. Littermate controls were used for all experiments with STAT-3 KO mice. TKO mice were derived from the respective single KO mice and bred homozygously. Their controls were in-house WT mice. All studies were performed under specific pathogen-free (SPF) conditions in compliance with NIH institutional guidelines, under IACUC-approved protocols (NEI-581 and NIAID/LCID 8E).

Flow sorting and cell surface marker analysis

T cell populations were isolated from spleen and peripheral lymph nodes of naive animals by first enriching for T cells using CD19 and MHCII depletion using MACS separation. After the separation, cell suspensions were stained with fluorescent antibodies against surface antigens, $\alpha\beta$ TCR, $\gamma\delta$ TCR, CD62L, CD44, CD4, CD8, CD1d tetramer binding, NK1.1, and B220. After staining, CD44^{hi} CD4⁺, CD44^{hi} CD8⁺, CD44^{hi} DN, and NKT cell populations were sorted on a FACS ARIA II (BD Biosciences; Fig. 1 A). Typical yields of CD4⁺, CD8⁺, DN, and NKT cells from a single mouse were $\sim 4 \times 10^5$, 10^5 , 2×10^4 , and 10^5 , respectively. For experiments, typically up to three mice were pooled. All antibodies were purchased from BioLegend, eBioscience, or BD Biosciences and used interchangeably, with the exception of B220-V500 (BD Biosciences) and CD62L-eFluor 450 (eBioscience). APC- or PE-labeled CD1d tetramers loaded with the α GalCer analogue, PBS57, were made and provided by the NIH tetramer facility (Emory, GA). We performed pSTAT-3 staining 18 h after cell stimulation to assure that enough proliferation of target cells occurred to provide reliable detection of pSTAT-3.

Culture conditions

HL-1 medium supplemented as described previously (Rachitskaya et al., 2008) plus 4% heat-inactivated fetal calf serum (Hyclone) was used for all cell cultures. FACS-isolated T cell populations were plated between 2 and 5×10^4 cells per well. All cultures were stimulated using plates precoated with α CD3 (3–4 μ g/ml) and where indicated, 10 ng/ml recombinant murine IL-23 or 20 ng/ml IL-1 β was added (R&D Systems). For neutralization experiments, we used α IL-6 (LEAF purified clone MP5-20F3) and α IL-23p19 (LEAF purified clone MMp19B2) from BioLegend at a concentration of 10 μ g/ml. In addition, we used α IL-6R α , α IL-21R, and IL-23R from R&D Systems at a concentration of 10 μ g/ml. Cultures typically were harvested after 72 h.

Detection of cytokine expression

Cytokine levels in culture supernatants were measured either by sandwich ELISA Duosets (R&D Systems) or bead array (Flowcytomix, eBioscience) per the manufacturer's instructions. For intracellular cytokine analysis, brefeldin A (BD Biosciences) was added to culture wells 6 h before harvest. This was followed by fluorescent antibody staining for surface markers followed by fixation, permeabilization, and intracellular staining for IL-17 and IFN- γ . Samples were analyzed using a MACS Quant Analyzer and FlowJo software (Tree Star).

Antibiotic treatment

IL-17A/F DKO mice were given a cocktail of antibiotics in the drinking water for up to 6 wk. Antibiotic cocktail consisted of ampicillin (1g/liter), metronidazole (1g/liter), neomycin sulfate (1g/liter), and vancomycin (0.5g/liter). Only mice with discernible disease were given antibiotic water.

In vitro knockdown of PLZF

Three siRNA constructs targeting PLZF (Zbtb16 ID235320 Trilencer-27 Mouse siRNA, catalog SR41884) were purchased from OriGene. RNAs were reconstituted with OriGene buffer to give a concentration of 20 μ M for a stock solution, and these were further diluted to 5 μ M for the working solution. Working solutions were then diluted in DMEM to reach a final concentration of 10 nM, 1.0 nM, and 0.1 nM when added to 200 μ l CD44^{hi} CD8/DNT cells that were sorted from pooled cells of at least three TKO mice and stimulated with α CD3 and 20 ng/ml IL-1 β .

Subconjunctival injection of cells

CD44^{hi} CD8⁺ and CD44^{hi} DNT cells were activated in vitro for 3 d using plate-bound α CD3 and 20 ng/ml IL-1 β . After 3 d, cells were FACS sorted, resuspended in PBS at 10⁴ cells/5 μ l, and left on ice until transfer. Mice were anesthetized using a mix of ketamine and xylazine. After anesthesia, 5 μ l cells or PBS was injected subconjunctivally using a 5- μ l Hamilton syringe. 3 d after transfer, mice were sacrificed and assessed for bacterial burden and immune cell infiltrate. For inoculation experiments, only mice without discernible disease were used to ensure that prior colonization with *S. aureus* would not cloud results. For CFU quantification, tissue homogenates were assessed by an investigator that was blinded to the treatment groups.

Statistical analysis and data presentation

All experiments were repeated at least twice, and usually three or more times, with reproducible results. Graphs and data analysis were done using GraphPad Prism (GraphPad Software). Experimental procedures determined sample size to ensure adequate power.

Online supplemental material

Fig. S1 shows a gating strategy for isolating CD44^{hi} CD4⁺, CD44^{hi} CD8⁺, CD44^{hi} DNT, and iNKT cells. Fig. S2 shows the gating strategy for Fig. 5.

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