HHS Public Access

Author manuscript

Immunol Cell Biol. Author manuscript; available in PMC 2017 March 22.

Published in final edited form as:

Immunol Cell Biol. 2017 March ; 95(3): 280–286. doi:10.1038/icb.2016.94.

Th17 cells are not required for maintenance of IL-17A producing γδ T cells in vivo

Pawan K. Gupta[1], **Sarah R. Wagner**[1], **Qiang Wu**[1], and **Rebecca A. Shilling**[1]

[1]Division of Pulmonary, Critical Care, Sleep and Allergy, Department of Medicine and Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL

Abstract

γδ T cells producing IL-17A (γδT17) are thought to develop spontaneously in the thymus and to be maintained in the periphery. Previous studies suggested a role for Th17 cells in the maintenance of γδT17 via the expression of TGFβ1. However, we have previously found that Th17 cells were not required for expansion of γδT17 cells after lung transplant in a mouse model. Using mice deficient in STAT3 in $CD4^+$ T cells, which are unable to develop Th17 cells, we investigated the requirement for Th17 cells and TGFβ1 to maintain γδT17 cells in the lung and lymphoid tissues. At steady state, we found no defect in γδT17 cells in the thymus or periphery of these mice. Further, STAT3-deficient $CD4^+$ T cells produced significantly higher levels of TGFβ1 than wild-type $CD4^+$ T cells under Th17 differentiation conditions in vitro. To determine whether STAT3-deficient $CD4^+$ T cells could expand γδT17 cells in vivo, we used $TCR\beta^{-/-}$ mice, which are known to have a defect in γδT17 cells that can be rescued by Th17 cells. However, adoptive transfer of wild-type Th17 cells or bulk $CD4^+$ T cells did not expand γδT17 cells in $TCR\beta^{-/-}$ mice. In contrast, IFN-γ^+ γδ T cells preferentially expanded, particularly in the lungs. Interestingly, we found in vivo and in vitro that TGFβ1 may negatively regulate the pool of γδT17 cells. Our data suggest that Th17 cells and TGFβ1 are not required for the maintenance of γδT17 cells.

Introduction

γδ T cells are innate-like T cells and an important source of IL-17A in mucosal tissues like the lung.[1] The frequency of γδ T cells among lymphocytes circulating in the blood and lymphoid organs is estimated at <5%.[1] However, γδ T cells are more abundant in mucosal tissues, such as the gut, skin and lung.[2, 3, 4, 5] During development, a subset of γδ T cells differentiates in the thymus to produce IL-17A (γδT17).[6] These γδT17 cells are maintained in the secondary lymphoid organs and mucosal tissues.[7, 8] γδT17 cells perform a variety of

Corresponding Author: Rebecca A. Shilling, 909 S. Wolcott, COMRB Rm. 3055, MC719, Chicago, IL 60612, Phone: 312-355-4597, Fax: 312-996-7193, shilling@uic.edu.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Information

The Supplementary Information that accompanies this paper is available at the Immunology and Cell Biology website.

immunologic functions in vivo. They are an early source of IL-17A to recruit neutrophils.[9, 10] In many bacterial and fungal infections, γδ T cells cells play a protective role in controlling infection.[1, 11, 12] Conversely, they have been found to be pathogenic in animal models of autoimmune diseases and in solid organ transplantation.[13, 14, 15, 16] In an orthotopic left lung transplant mouse model, we previously found that γδT17 cells expand in response to lung transplantation and are an important source of IL-17A.[17] However, less is known about the maintenance and expansion of γδT17 cells in the periphery at steady state.

γδT17 cells share a cytokine profile with IL-17A-producing $CD4^+$ T cells (Th17) but have clear distinction in generation and maintenance.[18] Spontaneous development in the thymus and peripheral maintenance of γδT17 cells has been suggested to be dependent on TGFβ1 and does not require IL-6, while Th17 cells differentiate in the periphery after antigen recognition in the presence of TGFβ1 and IL-6, among other cytokines.[8, 19, 20, 21] γδT17 cells require intact Hes1/Notch signaling, and not STAT3, for their development and maintenance.[22] In addition, γδT17 cells may respond earlier than Th17 cells during an immune response.[13] Despite these differences, γδT17 cells and Th17 cells have been found to regulate each other. Previous work suggested Th17 cells promoted the homeostatic maintenance of γδT17 cells in a TGFβ1 dependent manner.[8] Further, γδT17 cells have been found to support the generation and amplification of Th17 cells in vitro and in vivo during inflammation.[13] While these studies have suggested that Th17 and γδ T cells influence the expansion of each other in the periphery, the mechanisms are not clear.

Recently we found that $CD4^+$ T cell depletion after lung transplant decreased the expansion of γδT17 cells in the transplanted lungs compared to controls after transplant.[17] On the other hand, we found that γδT17 cell responses were not diminished in transplanted lungs or secondary lymphoid organs in the absence of Th17 cells after lung transplant.[17] These findings were unexpected given the previous work suggesting that Th17 cells played a role in the maintenance of γδT17.[8] However, the lung transplant model represents a chronic inflammatory state and the regulation of γδT17 cells may be different during homeostatic conditions. In the current study, we investigated the population of γδT17 cells in the absence of Th17 cells under homeostatic conditions and the ability of Th17 cells and TGFβ1 to affect γδT17 cells in the peripheral lymphoid tissues and lung in the absence of inflammation.

Results

γδT17 cells are not affected by the absence of Th17 cells in $STAT3^{CD4-/-}$ mice

STAT3 is a transcription factor required for differentiation of naïve $CD4^+$ T cells into Th17 effector cells.[23] Mice which lack STAT3 in $CD4^+$ T cells ($STAT3^{CD4-/-}$) are not able to generate Th17 cells in vitro or in vivo.[23, 24] We have previously found that the frequency of IL-$17A^+$ γδ T cells among γδ T cells in the transplanted lung and mediastinal lymph nodes (LN) in $STAT3^{CD4-/-}$ recipients is similar to controls (data reproduced in Supplementary Figure 1A and B).[17] These findings suggested Th17 cells were dispensable for the maintenance of peripheral γδT17 cells.[8] However, the transplant model represents an inflammatory condition, which may underlie the differences. Therefore, we investigated the

requirement of Th17 cells in the homeostatic maintenance of γδT17 cells in unmanipulated STAT3$^{CD4-/-}$ mice. Interestingly, we found that the frequency of γδT17 cells was not decreased in unmanipulated STAT3$^{CD4-/-}$ mice and was similar to controls (Figure 1A and B). There was also no significant reduction in the absolute number of γδT17 cells in the lungs, spleen, inguinal LN or thymus of STAT3$^{CD4-/-}$ mice (Figure 1B). As previously reported, we also confirmed that CD4$^+$ T cells in STAT3$^{CD4-/-}$ mice do not differentiate into Th17 cells in vivo (Supplementary Figure 2).[24] Moreover, unmanipulated STAT3$^{CD4-/-}$ mice do not have any defect in the overall frequency and absolute number of total γδ T cells in the lungs, spleen, inguinal LN or thymus (Supplementary Figure 3A and B). These findings suggested that Th17 cells were not absolutely required for the peripheral maintenance of γδT17 cells.

STAT3-deficient CD4 T cells produce TGFβ1 under Th17 polarizing conditions

TGFβ1 plays a key role in the regulation of Th17 and T regulatory cells.[25, 26] In addition, maintenance of γδT17 cells by Th17 cells was previously suggested to be TGFβ1 dependent.[8, 27] We hypothesized that STAT3$^{CD4-/-}$ T cells may be capable of producing TGFβ1 under Th17 differentiation conditions and maintain γδT17 cells in STAT3$^{CD4-/-}$ mice. To test our hypothesis, naïve CD4$^+$ T cells from STAT3$^{CD4-/-}$ mice were stimulated in vitro under Th17 differentiation conditions for 3 days, washed and then restimulated to determine production of TGFβ1. As expected STAT3$^{CD4-/-}$ T cells do not produce IL-17A in vitro (Figure 2A).[23] However, we found that the mRNA expression of TGFβ1 was increased in STAT3-deficient CD4$^+$ T cells as compared to littermate control T cells (Figure 2B). In addition, the culture supernatants from STAT3$^{CD4-/-}$ T cells had significantly higher TGFβ-1 levels as compared to controls (Figure 2C). Thus, STAT3 is not required for the expression of TGFβ1 in CD4$^+$ T cells. Our data suggest STAT3 expression may directly or indirectly negatively regulate TGFβ1 expression during Th17 differentiation.

CD4$^+$ T cells do not restore γδT17 cells but promote expansion of IFN-γ$^+$ γδ T cells in vivo

Based on our findings in Figure 2, it was possible that TGFβ1 produced by STAT3-deficient CD4$^+$ T cells was sufficient for the maintenance of γδT17 cells in the STAT3$^{CD4-/-}$ mice. Previous work in TCRβ$^{-/-}$ mice, which have a defect in γδT17 cells, had found that adoptive transfer of CD4$^+$ T cells or Th17 cells expanded the pool of γδT17 cells and expansion was dependent on TGFβ1.[8] As previously shown, TCRβ$^{-/-}$ mice have a defect in γδT17 cells in the lymphoid tissues but an increase in the overall numbers of γδ T cells in the absence of other T cells (Figure 3A and data not shown).[8] The decrease in the proportion of γδT17 cells was not restricted to lymphoid tissues, as we also found a marked decrease in the lungs (Figure 3A). To determine the ability of Th17 to affect the γδT17 cell population, we adoptively transferred in vitro differentiated Th17 cells from wild-type mice into TCRβ$^{-/-}$ mice. However, we found that the transfer of Th17 cells in our hands did not expand the frequency of γδT17 cells in TCRβ$^{-/-}$ mice and actually decreased their frequency in the lung and lymphoid tissues (Figure 3A and B). As naïve CD4$^+$ T cells have also been found to promote expansion of γδT17 cells, we determined whether these T cells would be more effective. We also did not find any significant increase in the frequency of γδT17 cells with the transfer of naïve CD4 T cells, although there was a small increase in the total number (Figure 3A and B and data not shown). The results were not due to a lack of

Th17 cells homing to secondary lymphoid tissue or the lungs, as IL-17A-producing $CD4^+$ T cells were found after transfer of either Th17 cells or naïve $CD4^+$ T cells (Supplementary Figure 4). Unexpectedly, transfer of Th17 cells or naïve $CD4^+$ T cells into $TCR\beta^{-/-}$ mice preferentially expanded the frequency of IFNγ-producing $\gamma\delta$ T cells (Figure 3A and B). The increase was particularly pronounced in the lungs with a marginal effect on the spleen and inguinal lymph nodes (Figure 3). In addition, the absolute number of lung $\gamma\delta$ T cells was significantly higher after transfer of T cells into $TCR\beta^{-/-}$ mice (Figure 3C). Interestingly, the lymphoid tissues had little expansion of the overall numbers of $\gamma\delta$ T cells with the exception of Th17 cells affecting the numbers of $\gamma\delta$ T cells in the inguinal LN (Figure 3C). This result was correlated with an increase in $CD4^+$ T cells in the inguinal LN when Th17 were transferred as opposed to naïve $CD4^+$ T cells (Supplementary Figure 4). These data suggest that IFN-γ^+ $\gamma\delta$ T cells are preferentially expanding after adoptive transfer of CD4+ T cells into $TCR\beta^{-/-}$ mice with expansion most pronounced in the lung.

TGFβ1 is not necessary for γδT17 cell homeostasis

While our data suggested Th17 cells could not expand the deficient pool of $\gamma\delta$T17 cells in TCR $\beta^{-/-}$ mice, it was still possible that TGFβ1 was important for the maintenance of $\gamma\delta$T17 cells in vivo. Furthermore, the significance of the elevated TGFβ1 found in vitro from $STAT3^{CD4-/-}$ T cells was not clear. To determine the role of TGFβ1 activity in vivo, we administered neutralizing anti-TGFβ antibody twice weekly for 3 weeks to $STAT3^{CD4-/-}$ mice and measured the frequency of $\gamma\delta$T17 cells in the lungs and lymphoid tissues.[28] Interestingly, rather than a decrease in the percentage of $\gamma\delta$T17 cells among $\gamma\delta$ T cells, we found an increase in $\gamma\delta$T17 cells in the lungs and inguinal LN compared to control mice, while the spleen had no difference (Figure 4). There was no increase in the frequency of IFNγ^+ $\gamma\delta$ T cells among the $\gamma\delta$ T cells (Figure 4 and data not shown). These results suggested TGFβ1 suppressed the expansion or differentiation of peripheral $\gamma\delta$T17 cells.

To further investigate these findings, we determined the effects of TGFβ1 on $\gamma\delta$T17 cells enriched in vitro using IL-7. IL-7 has previously been found to promote expansion of $\gamma\delta$T17 cells in vivo and in vitro.[29] In the presence of IL-7, we found that the majority of lymph node-derived $\gamma\delta$ T cells were $\gamma\delta$T17 after 72 hours and their number was significantly increased compared to ex vivo or culturing in media alone (Figure 5). These results are similar to prior work.[29] Unexpectedly, blockade of TGFβ1 with the same neutralizing antibody we used in vivo had minimal effect on the frequency and number of $\gamma\delta$T17 cells expanding in response to IL-7 (Figure 5A and B). It was possible that the concentration of TGFβ1 in the media was lower than the threshold to affect $\gamma\delta$T17 cells or that $\gamma\delta$T17 cells were not expressing TGFβ1 receptors. However, the type II receptor for TGFβ, TGFβRII, was expressed ex vivo and in vitro after expansion with IL-7 (Figure 5C).[30] To investigate further the response of $\gamma\delta$T17 cells to TGFβ1, we cultured LN cells with exogenous TGFβ1 in the presence of IL-7. We found that TGFβ1 at 1ng/ml significantly suppressed the recovery of all $\gamma\delta$ T cells and $\gamma\delta$T17 cells in response to IL-7 (Figure 5D and E). However, the majority of the $\gamma\delta$ T cells in the cultures remained IL-17A producing (Figure F). Increasing concentrations of TGFβ1 did not have any any further effect on decreasing $\gamma\delta$T17 cells (data not shown). Exogenous TGFβ1 also decreased the survival of $\gamma\delta$T17 cells cultured without IL-7 (data not shown). Our data suggest that TGFβ1 may be negatively

regulating peripheral γδT17 cells in vivo and in vitro by directly inhibiting their survival and expansion rather than affecting differentiation.

Discussion

In the current study, we have found that the pool of peripheral γδT17 cells is maintained in mice which have defective Th17 differentiation. The effect was true for secondary lymphoid tissues, as well as the lung. We have also found that Th17 cells generated in vitro produce TGFβ1 and STAT3-deficiency is associated with increased TGFβ1 production. Despite the ability of Th17 cells to be a source of TGFβ1 in vitro, adoptive transfer of these T cells failed to restore γδT17 cell populations in TCRβ$^{-/-}$ mice. Similarly, adoptive transfer of naïve CD4 T cells was also not effective to expand γδT17 cells. Surprisingly, transferred Th17 cells or naïve CD4 T cells preferentially expanded IFN-γ^{+} γδ T cells and the total number of γδ T cells in TCRβ$^{-/-}$ mice. The effect was most pronounced in the lungs suggesting the effect may be tissue-specific. Furthermore, TGFβ1 neutralization in vivo expanded rather than decreased the pool of γδT17 cells in the lungs or lymphoid tissues compared to controls. In support of these findings, exogenous TGFβ1 decreased IL-7-induced γδT17 cell expansion in vitro. γδT17 cells expressed TGFβRII in vitro, which suggests direct inhibition by TGFβ1. Together, our data suggest that Th17 cells are not required to maintain γδT17 cells. Our data also do not support a requirement for TGFβ1 for γδT17 cell homeostasis in the periphery of adult mice and suggest TGFβ1 may negatively regulate γδT17 cells in mucosal tissues.

Our data are consistent with the findings by Haas and colleagues that γδT17 cells develop before birth and are self-renewing.[31] The lack of effect of Th17 cells or naïve CD4^{+} T cells on γδT17 cells expansion, when γδT17 cells development was limited in TCRβ$^{-/-}$ mice, suggests that γδT17 cells may have limited responsiveness to CD4^{+} T cell-derived direct or indirect signals post-natally at steady state. These T cells may still respond under inflammatory conditions. However, we did find that CD4^{+} T cells, regardless of differentiation to Th17 or naïve, affected the pool of IFN-γ^{+} γδ T cells and the effect was most consistent for the lung. Whether IFN-γ^{+} γδ T cells are preferentially expanding or the IFN-γ^{-ve} γδ T cells differentiate into IFN-γ producing cells is not entirely clear. It is likely that there is a preferential expansion of IFN-γ^{+} γδ T cells because the number of IFN-γ^{-ve} γδ T cells did not signficantly change (data not shown). The lung-specific effect may be related to the lung microbiota or mucosal environment influencing T cell trafficking or differentiation. Alternatively, the lung may provide an ideal site for the two T cell subsets to interacte with each other. The mechanisms by which CD4^{+} T cells expand IFN-γ^{+} γδ T cells remain to be determined in future studies.

While our data suggest there are differences in the responsiveness of γδ T cells to homeostatic signals from CD4^{+} T cells, the results contrast with a study suggesting that Th17 cells and naïve CD4^{+} T cells expanded γδT17 cells in TCRβ-deficient mice.[8] We have used mice on the C57Bl/6N background for harvesting of CD4^{+} T cells for adoptive transfer, while the previous study used C57Bl/6J mice. There are several known genetic polymorphisms that exist between these strains.[32] It is also possible that differences in the microbiota may impact IL-17A production from γδ T cells under homeostatic conditions.[33]

As we still found IL-17A producing $CD4^+$ T cells after adoptive transfer, we do not think it was a defect in the survival or persistence of the transferred Th17 cells leading to decreased effectiveness. Nevertheless, we cannot rule out that differences in genetic background or microbiota affected the ability of the transferred T cells to expand the γδT17 cells. Taken together, our data suggest that Th17 cells are variable in the ability to promote expansion of γδT17 cells in vivo.

TGFβ1 had previously been found to be critical for the development of γδT17 cells and was suggested to play a role in the maintenance of γδT17 cells in the periphery.[8, 19] We have found that Th17 cells produced TGFβ1 after differentiation in vitro and STAT3 deficiency was associated with an increase in TGFβ1 production and expression. Deficiency of STAT3 in $Foxp3^+$ T cells was also found to increase the level of TGFβ1 produced by T regulatory cells.[34] Our data suggested in vivo STAT3-deficient $CD4^+$ T cells may be producing increased levels of TGFβ1. However, we did not find an increase in γδT17 cells at baseline in $STAT3^{CD4-/-}$ mice. Further, we found that in vivo TGFβ1 neutralization increased rather than decreased the population of lung and inguinal LN γδT17 cells in $STAT3^{CD4-/-}$ mice, but had no effect in the spleen. The pool of γδT17 cells is small in the spleen and they may not be easily expanded. These results suggest that γδT17 cells are heterogeneous in their response to cytokine stimulation depending on their anatomical location and may be dependent on other signals, such as microbial stimulation. We did not see an enhanced cytokine response from non-γδ T cells with TGFβ1 neutralization to suggest widsepread effects on all T cells. Supporting our in vivo findings, exogenous TGFβ1 reduced the numbers of γδT17 cells expanded in response to IL-7 in vitro. These γδT17 cells expressed TGFβ RII ex vivo and after culture with IL-7 suggesting that TGFβ1 directly inhibited γδT17 cell survival or proliferation. Even in the absence of IL-7, TGFβ1 negatively affected recovery of γδT17 cells in cultures of LN cells when compared to media alone. Overall, these data suggest that TGFβ1 is not required for persistence of γδT17 cells and TGFβ1 may normally restrain the pool of γδT17 cells. Future work will need to understand the relevant sources of TGFβ1 in vivo. From our studies it appears that unstimulated LN $CD4^+$ T cells are not sufficient to produce enough TGFβ1 to suppress γδT17 cells in vitro.

In conclusion, our data suggest that Th17 cells are not necessary for the maintenance of γδT17 cells after development and are not sufficient to expand pre-existing γδT17 cells. Furthermore, in vivo and in vitro γδT17 cell persistence at steady state is not dependent on TGFβ1. In contrast, we found that TGFβ1 inhibited the expansion of γδT17 cells in vitro and may negatively regulate γδT17 cell expansion or survival in the lungs. These data suggest that the factors promoting maintenance of γδT17 cells are distinct from those required for development. Interestingly, in our studies $CD4^+$ T cells expanded IFN-γ^+ γδ T cells while having no effect on γδT17 cells, and preferentially affected the lung rather than lymphoid tissues. $CD4^+$ T cells and TGFβ1 may regulate γδ T cells in a context dependent manner. A better understanding of this regulation may provide effective therapeutic targets for promoting immunity to infections and tumors and for treating autoimmune diseases and transplant rejection.

Methods

Animals

C57BL/6N.*Stat3*$^{fl/fl}$.CD4-Cre (STAT3$^{CD4-/-}$) mice and littermate controls were previously described and bred in house.[23] TCRβ$^{-/-}$, C57BL/10 and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Envigo (Indianapolis, IN, USA). Mice between the ages of 2–3 months were used in the study. Mice were housed under specific pathogen-free conditions in the animal care facility at the University of Illinois at Chicago. All experimental animal protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee of University of Illinois at Chicago.

Tissue preparation

Spleen, inguinal LN and thymus were harvested and dissociated mechanically. Lung tissue was digested with Collagenase I (Life Technologies) for 30 min at 37°C. Single cell suspensions were treated with red blood cell lysis buffer (BioLegend).

FACS analysis

Single cell suspensions were stained with antibodies for CD4 (GK1.5), γδ TCR (GL3), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2) (Biolegend) and TGFβRII (R&D Systems). For intracellular staining, cells were restimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 4h and Brefeldin A (10 μg/ml) was added for the last 3h. Cells were stained with fixable Live/Dead stain (Invitrogen) followed by staining of surface markers, fixed with formaldehyde, permeabilized and stained for intracellular cytokines. Acquisition was performed on a LSR Fortessa (BD Biosciences) and data analysis was performed with Flowjo software (Tree Star).

In vitro Th17 cell differentiation

Naïve CD4$^+$CD62L$^+$ T cells were purified from spleen and lymph nodes using a commercially available kit (Miltenyi Biotec). 1×10^6 naive CD4 T cells per well were stimulated with plate bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of TGFβ1 (2 ng/ml), IL-6 (100 ng/ml), IL-23 (10 ng/ml), IL-1β (100 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) for 3 d. Differentiation of Th17 cells was confirmed by flow cytometry. Antibodies used in Th17 differentiation were purchased from Bio X cell. All the cytokines except IL-23 (R&D Systems) were procured form Peprotech.

TGFβ1 ELISA and Real time PCR

Differentiated CD4 T cells were restimulated with anti-CD3 (2 μg/ml) for an additional 24h in AIM V serum free media (Life Technologies). Culture supernatants were collected and TGFβ1 protein was estimated by ELISA (R&D Systems). Cells were used to extract total RNA using RNA isolation kit (Qiagen). Total RNA was reverse transcribed to cDNA with a qScript™ cDNA Supermix kit (Quanta Biosciences). Real time PCR was performed using PerfeCTa™ SYBR Green (Quanta Biosciences) on a CFX Connect Real-time PCR system (Bio-Rad). The following primer sequences were used: TGFβ1 (F: TGACGTCACTGGAGTTGTACGG, R: GGTTCATGTCATGGATGGTGC); GAPDH (F:

CATGGCCTTCCGTGTTCCTA, R: CCTGCTTCACCACCTTCTTGAT) (Integrated DNA Technologies).

Adoptive transfer

5×10^6 Th17 cells or 3×10^6 naïve ($CD4^+CD62L^+$ T cells) T cells were injected via retro-orbital venous sinus into $TCR\beta^{-/-}$ recipients. At day 14 after transfer, cytokine expression in γδ T cells was investigated in different tissues.

TGFβ neutralization in vivo

Mice were injected i.p. with 500μg anti-mouse TGFβ1 (1D11, Bio X cell) twice per week. The mice were sacrificed after three weeks and the cytokine profile of γδ T cells was investigated by intracellular cytokine staining.

Cell culture

Cells harvested from lymph nodes (inguinal, superficial cervical, axillary and mesenteric) were incubated for 72 hours with IL-7 (20ng/ml, Peprotech). Where indicated, TGFβ1 blocking antibody (50μg/ml) or TGFβ1 (1, 2, 4 or 8ng/ml) were added to the culture. After 72 hours, cells were collected for analysis by flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). Differences were considered significant at $P<0.05$. All data are expressed as mean ± (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Mark Kaplan for providing $STAT3^{CD4-/-}$ mice. We also thank the staff of the Flow Cytometry core facility at University of Illinois at Chicago for their technical help.

Funding: This work was supported by the National Heart, Lung, And Blood Institute of the National Institutes of Health Grant R01HL109310.

References

1. Chien YH, Meyer C, Bonneville M. gammadelta T cells: first line of defense and beyond. Annual review of immunology. 2014; 32:121–155.
2. Itohara S, Farr AG, Lafaille JJ, Bonneville M, Takagaki Y, Haas W, et al. Homing of a Gamma-Delta Thymocyte Subset with Homogeneous T-Cell Receptors to Mucosal Epithelia. Nature. 1990; 343(6260):754–757. [PubMed: 2154700]
3. Rocha B, Vassalli P, Guygrand D. Thymic and Extrathymic Origins of Gut Intraepithelial Lymphocyte Populations in Mice. Journal of Experimental Medicine. 1994; 180(2):681–686. [PubMed: 8046341]
4. Asarnow DM, Kuziel WA, Bonyhadi M, Tigelaar RE, Tucker PW, Allison JP. Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells. Cell. 1988; 55(5):837–847. [PubMed: 2847872]

5. Augustin A, Kubo RT, Sim GK. Resident Pulmonary Lymphocytes Expressing the Gamma-Delta T-Cell Receptor. Nature. 1989; 340(6230):239–241. [PubMed: 2526925]
6. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, et al. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. Immunity. 2008; 29(1):90–100. [PubMed: 18585064]
7. Haas JD, Gonzalez FH, Schmitz S, Chennupati V, Fohse L, Kremmer E, et al. CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. European journal of immunology. 2009; 39(12):3488–3497. [PubMed: 19830744]
8. Do JS, Visperas A, O'Brien RL, Min B. CD4 T cells play important roles in maintaining IL-17-producing gammadelta T-cell subsets in naive animals. Immunology and cell biology. 2012; 90(4): 396–403. [PubMed: 21647171]
9. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. Journal of immunology. 2006; 177(7): 4662–4669.
10. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. Journal of immunology. 2007; 178(7):4466–4472.
11. Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. Nature reviews Immunology. 2002; 2(5):336–345.
12. O'Brien RL, Roark CL, Born WK. IL-17-producing gammadelta T cells. European journal of immunology. 2009; 39(3):662–666. [PubMed: 19283718]
13. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity. 2009; 31(2):331–341. [PubMed: 19682929]
14. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. Journal of immunology. 2007; 179(8):5576–5583.
15. Itoh S, Kimura N, Axtell RC, Velotta JB, Gong Y, Wang X, et al. Interleukin-17 accelerates allograft rejection by suppressing regulatory T cell expansion. Circulation. 2011; 124(11 Suppl):S187–S196. [PubMed: 21911812]
16. Kimura N, Nakae S, Itoh S, Merk DR, Wang X, Gong Y, et al. Potential role of gammadelta T cell-derived IL-17 in acute cardiac allograft rejection. The Annals of thoracic surgery. 2012; 94(2): 542–548. [PubMed: 22560321]
17. Wu Q, Gupta PK, Suzuki H, Wagner SR, Zhang C, Cummings OW, et al. CD4 T Cells but Not Th17 Cells Are Required for Mouse Lung Transplant Obliterative Bronchiolitis. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2015; 15(7):1793–1804.
18. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity. 2009; 31(2):321–330. [PubMed: 19682928]
19. Do JS, Fink PJ, Li L, Spolski R, Robinson J, Leonard WJ, et al. Cutting Edge: Spontaneous Development of IL-17-Producing gamma delta T Cells in the Thymus Occurs via a TGF-beta 1-Dependent Mechanism. Journal of immunology. 2010; 184(4):1675–1679.
20. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, et al. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. The Journal of experimental medicine. 2008; 205(6):1381–1393. [PubMed: 18504307]
21. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annual review of immunology. 2009; 27:485–517.
22. Shibata K, Yamada H, Sato T, Dejima T, Nakamura M, Ikawa T, et al. Notch-Hes1 pathway is required for the development of IL-17-producing gammadelta T cells. Blood. 2011; 118(3):586–593. [PubMed: 21606479]
23. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, et al. Stat3 and Stat4 direct development of IL-17-secreting Th cells. Journal of immunology. 2007; 178(8):4901–4907.

24. Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, et al. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. Journal of immunology. 2007; 179(7):4313–4317.
25. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. The Journal of experimental medicine. 2003; 198(12):1875–1886. [PubMed: 14676299]
26. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007; 26(5):579–591. [PubMed: 17481928]
27. Gutcher I, Donkor MK, Ma Q, Rudensky AY, Flavell RA, Li MO. Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation. Immunity. 2011; 34(3):396–408. [PubMed: 21435587]
28. Dasch JR, Pace DR, Waegell W, Inenaga D, Ellingsworth L. Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. J Immunol. 1989; 142(5):1536–1541. [PubMed: 2537357]
29. Michel ML, Pang DJ, Haque SF, Potocnik AJ, Pennington DJ, Hayday AC. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(43):17549–17554. [PubMed: 23047700]
30. Do JS, Fink PJ, Li L, Spolski R, Robinson J, Leonard WJ, et al. Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism. Journal of immunology. 2010; 184(4):1675–1679.
31. Haas JD, Ravens S, Duber S, Sandrock I, Oberdorfer L, Kashani E, et al. Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. Immunity. 2012; 37(1):48–59. [PubMed: 22770884]
32. Mekada K, Abe K, Murakami A, Nakamura S, Nakata H, Moriwaki K, et al. Genetic Differences among C57BL/6 Substrains. Exp Anim Tokyo. 2009; 58(2):141–149.
33. Duan J, Chung H, Troy E, Kasper DL. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. Cell host & microbe. 2010; 7(2):140–150. [PubMed: 20159619]
34. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science. 2009; 326(5955):986–991. [PubMed: 19797626]

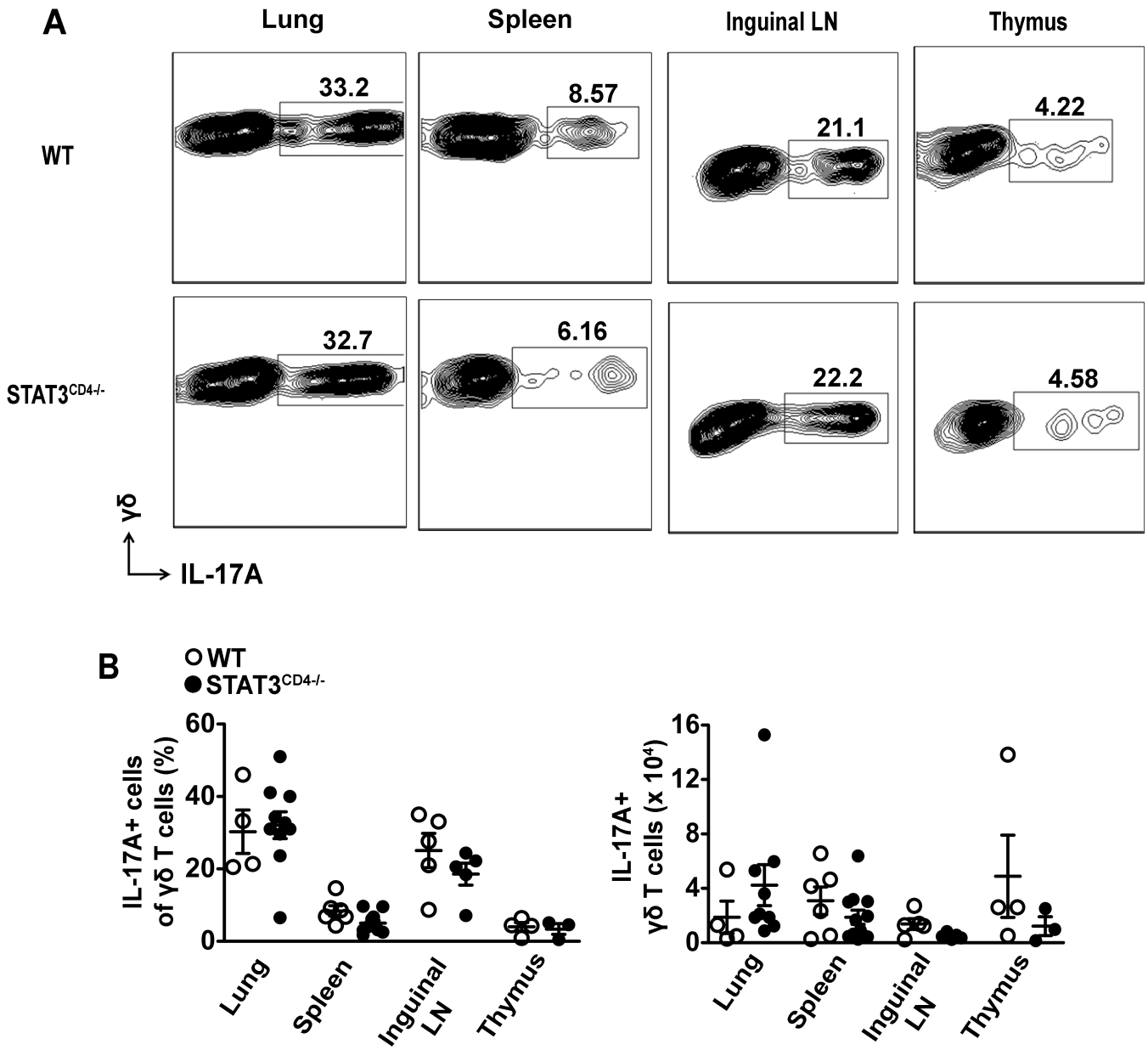

Figure 1. γδT17 cells are not affected by the absence of Th17 cells in STAT3$^{CD4-/-}$ mice

Lung, spleen, inguinal LN and thymus were harvested from unmanipulated STAT3$^{CD4-/-}$ mice or littermate controls and analyzed for γδT17 cells by flow cytometry. (A) Representative contour plots indicate the percentage of IL-17A^{+} cells of γδ T cells. (B) Frequency and absolute number of IL-17A^{+}γδ T cells. For lung, n=4 (control) and n=10 (STAT3$^{CD4-/-}$); spleen, n=6 (control) and n=12 (STAT3$^{CD4-/-}$); inguinal LN, n=5 (control) and n=5 (STAT3$^{CD4-/-}$); thymus, n=4 (control) and n=3 (STAT3$^{CD4-/-}$). Data were analysed using unpaired *t* test.

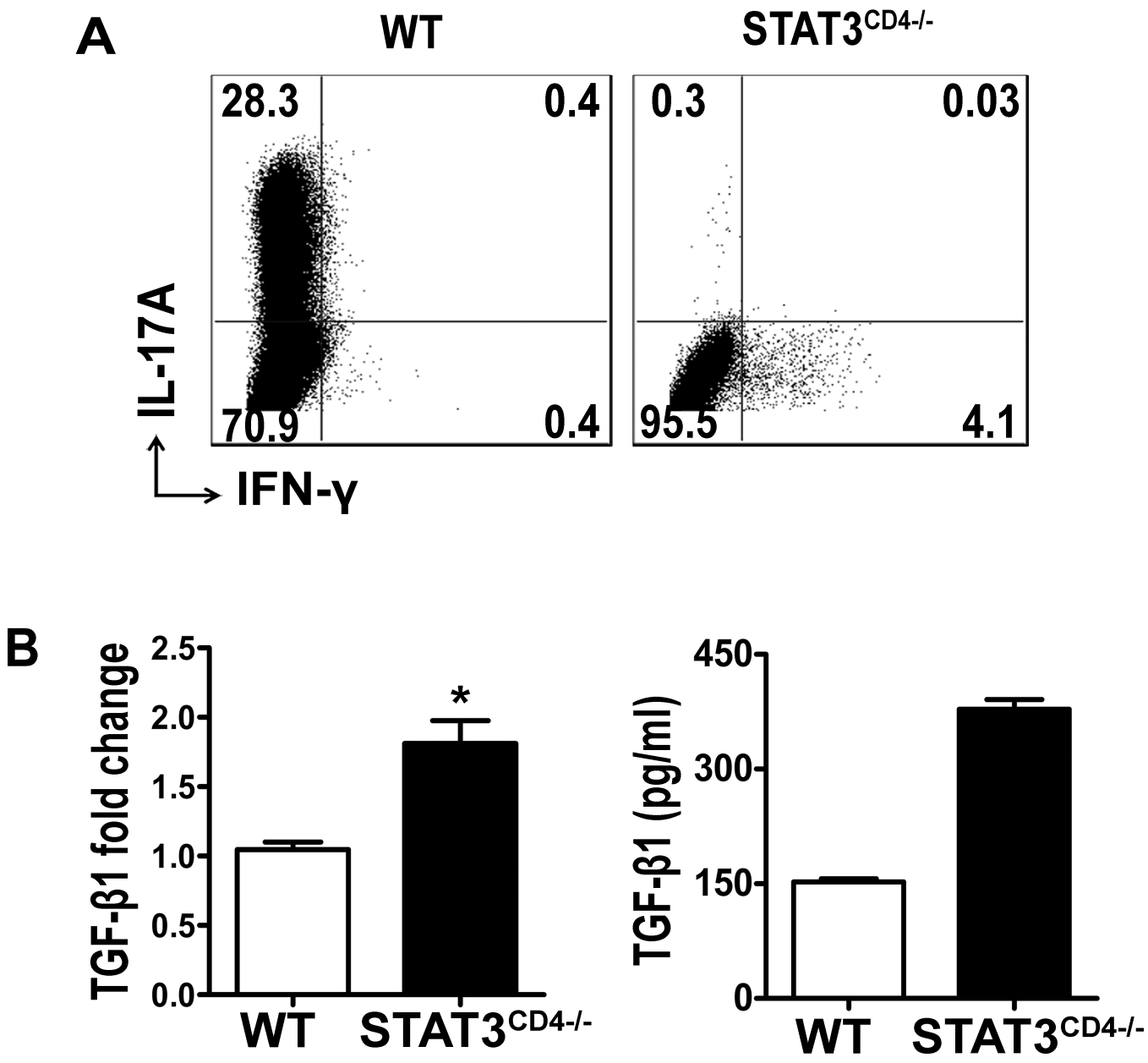

Figure 2. TGFβ1 is increased from STAT3$^{CD4-/-}$ T cells differentiated under Th17 conditions

Naïve STAT3-deficient or littermate control CD4$^+$ T cells were differentiated under Th17 conditions for 3 days as outlined in Methods and then restimulated with anti-CD3 for an additional 24 hours in serum free media. (A) Percentage of IL-17A$^+$ and IFN-γ$^+$ cells of the CD4$^+$ T cells. (B) mRNA expression of TGFβ1 in differentiated CD4$^+$ T cells by Real Time PCR. (C) TGFβ1 in culture supernatants determined by ELISA. Data analysed by unpaired *t* test, **P*<0.05, ***P*<0.005. The experiment was repeated twice and similar results were obtained. Data from one of two experiments are shown and presented as mean of two replicates.

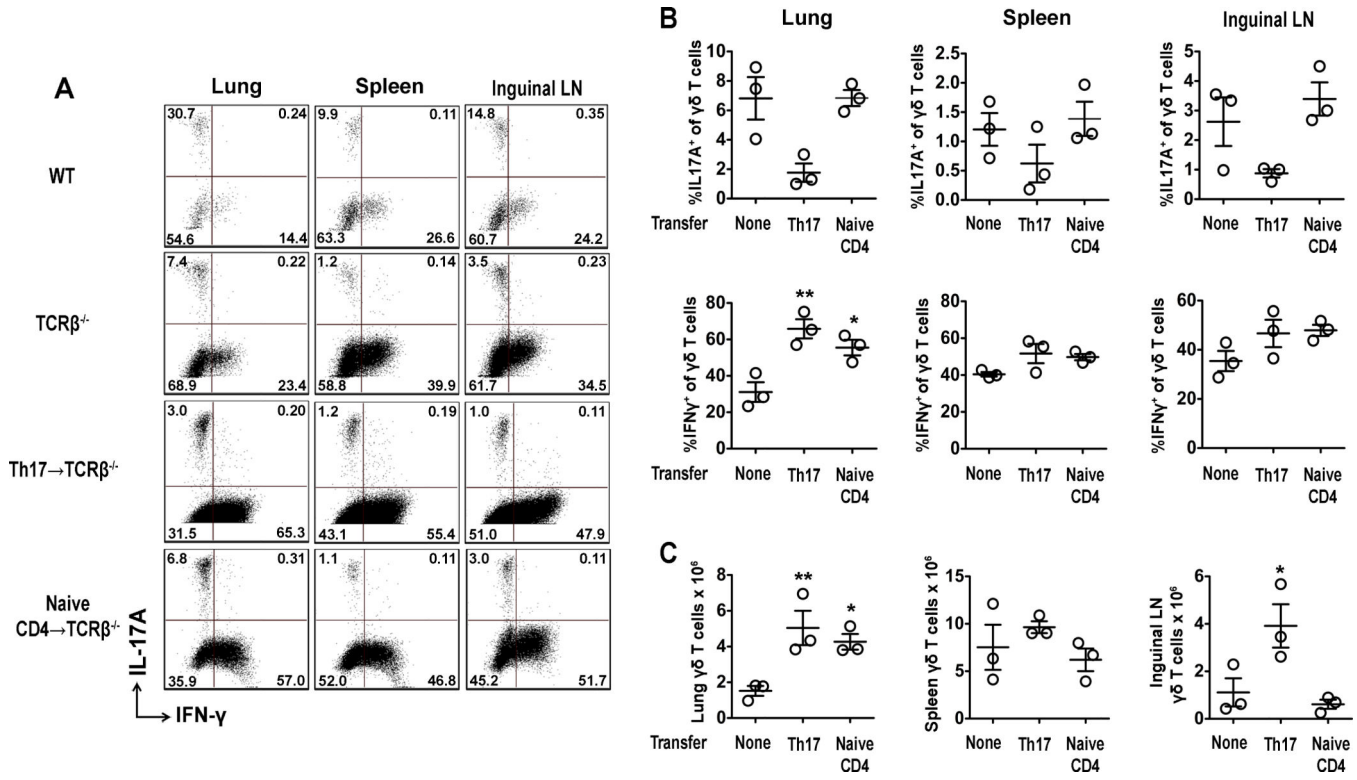

Figure 3. Th17 cells or naïve CD4$^+$ T cells cannot restore γδT17 cells but promote expansion of IFN-γ-producing γδ T cells

Wild-type differentiated Th17 cells or naïve CD4 T cells were adoptively transferred into TCRβ$^{-/-}$ recipients as described in Materials and Methods. (A) Representative dot plots showing frequency of IL-17A$^+$ and IFN-γ$^+$ cells gated on γδ T cells in wild-type mice, TCRβ$^{-/-}$ mice without transfer, and TCRβ$^{-/-}$ recipients which received either Th17 cells or naïve CD4 T cells. (B) The frequency of IL-17A$^+$ and IFN-γ$^+$ cells of γδ T cells in lung, spleen and inguinal LN. (C) The absolute number of overall γδ T cells in indicated tissues. For B and C, **P*<0.05, ***P*<0.005 (One-way ANOVA with Newman-Keuls multiple comparison post-hoc test), compared to non-transferred TCRβ$^{-/-}$ mice, n=3 (non transferred TCRβ$^{-/-}$, Th17→ TCRβ$^{-/-}$ and naïve CD4 T cells→ TCRβ$^{-/-}$).

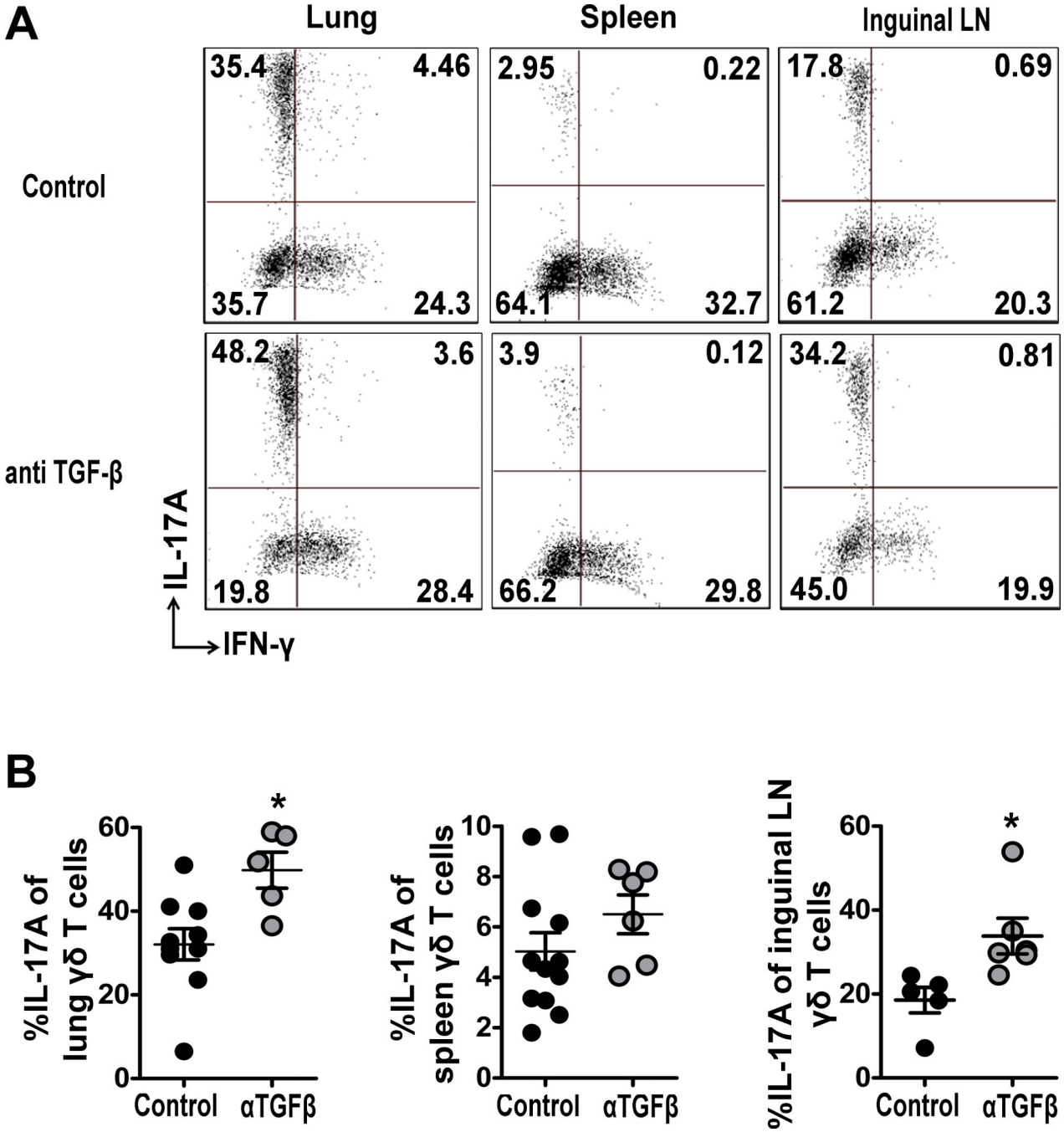

Figure 4. TGFβ1 blockade in vivo expanded peripheral γδT17 cells

TGFβ1 blocking antibody (500μg) was injected i.p. in $STAT3^{CD4-/-}$ mice twice a week. Control $STAT3^{CD4-/-}$ mice did not receive antibody. Lung, spleen and inguinal LN were harvested and analysed for IL-$17A^{+}$γδ T cells after 21 days. (A) Representative dot plots show frequency of IL-$17A^{+}$ and IFN-γ+ cells of γδ T cells. Number in the quadrants indicates percentage. (B) The percentage of IL-$17A^{+}$ cells of γδ T cells in lung, spleen and inguinal LN. *P<0.05 (unpaired t test); for lung, n=10 (control) and n=5 (anti-TGFβ1);

spleen, n=12 (control) and n=6 (anti-TGFβ1); inguinal LN, n=5 (control) and n=6 (anti-TGFβ1).

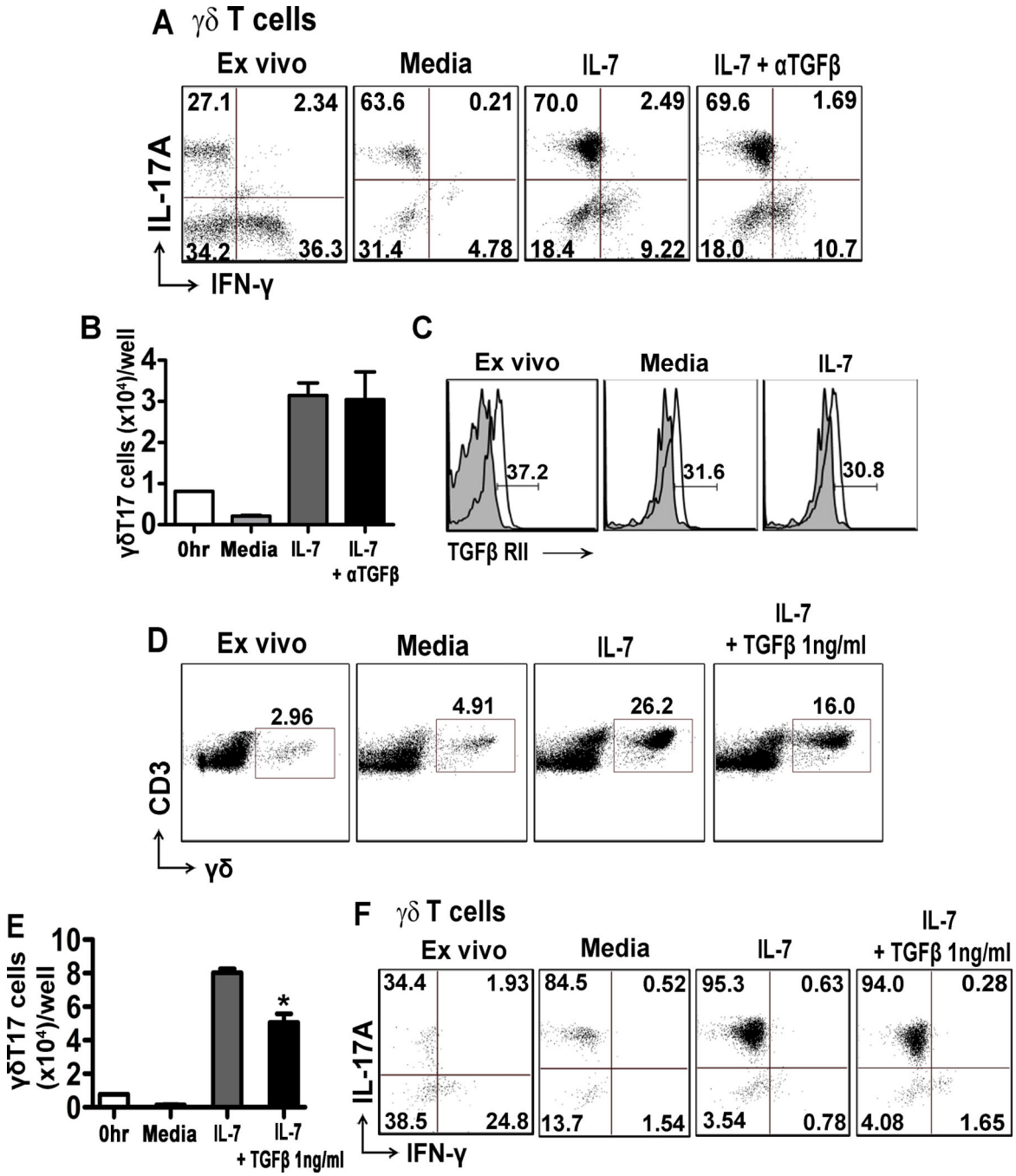

Figure 5. TGFβ1 suppresses expansion of γδT17 cells in vitro

LN cells were cultured in vitro with either TGFβ1 blocking antibody or exogenous TGFβ1 in the presence of IL-7. γδ T cells were analysed for cytokine production after 72 hours compared to ex vivo and media alone. (A) Frequency of IL-17A$^+$ and IFN-γ$^+$ expressing cells of γδ T cells after TGFβ1 blockade are shown by dot plot. (B) Absolute number of γδT17 cells is shown. (C) Expression of TGFβ RII on γδT17 cells (line) compared to unstained cells (gray histogram) is shown ex vivo and after culture in media or with exogenous IL-7. TGFβ RII expression staining was conducted in 4 mice (ex vivo) and in two

different culture experiments (n=2). (D) Dot plots showing frequency of γδ T cells on gated CD3 T cells in presence of exogenous TGFβ1. (E) The absolute number of γδT17 cells. (F) Dot plots with percentage of IL-17A$^+$ and IFN-γ$^+$ cells of γδ T cells in presence of exogenous TGFβ1. Data analysed by unpaired *t* test, **P*<0.05 compared to IL-7 condition. Each experiment was repeated twice and similar results were obtained. Data from one of the two experiments are shown as mean of two or three replicates. Number in dot plots indicates percentage.