

Egr3 Induces a Th17 Response by Promoting the Development of $\gamma\delta$ T Cells

Rose M. Parkinson¹, Samuel L. Collins², Maureen R. Horton², Jonathan D. Powell^{1*}

1 The Sidney-Kimmel Cancer Research Center, The Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, **2** Division of Pulmonary Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America

Abstract

The transcription factor Early Growth Response 3 (Egr3) has been shown to play an important role in negatively regulating T cell activation and promoting T cell anergy in Th1 cells. However, its role in regulating other T helper subsets has yet to be described. We sought to determine the role of Egr3 in a Th17 response using transgenic mice that overexpress Egr3 in T cells (Egr3 TG). Splenocytes from Egr3 TG mice demonstrated more robust generation of Th17 cells even under non-Th17 skewing conditions. We found that while Egr3 TG T cells were not intrinsically more likely to become Th17 cells, the environment encountered by these cells was more conducive to Th17 development. Further analysis revealed a considerable increase in the number of $\gamma\delta$ T cells in both the peripheral lymphoid organs and mucosal tissues of Egr3 TG mice, a cell type which normally accounts for only a small fraction of peripheral lymphocytes. Consistent with this marked increase in peripheral $\gamma\delta$ T cells, thymocytes from Egr3 TG mice also appear biased toward $\gamma\delta$ T cell development. Coculture of these Egr3-induced $\gamma\delta$ T cells with wildtype CD4⁺ T cells increases Th17 differentiation, and Egr3 TG mice are more susceptible to bleomycin-induced lung inflammation. Overall our findings strengthen the role for Egr3 in promoting $\gamma\delta$ T cell development and show that Egr3-induced $\gamma\delta$ T cells are both functional and capable of altering the adaptive immune response in a Th17-biased manner. Our data also demonstrates that the role played by Egr3 in T cell activation and differentiation is more complex than previously thought.

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* E-mail: poweljo@jhmi.edu

Introduction

Early growth response 3 (Egr3) is an immediate early zinc finger transcription factor activated in response to a variety of mitogenic signals [1]. Within the lymphocyte compartment, both mature and developing T cells highly express Egr3 shortly after TCR or pre-TCR engagement in a Ca²⁺ and NFAT-dependent manner [2,3]. Our group has previously demonstrated a role for Egr3 in negative regulation of mature CD4⁺ T cell function. *In vitro* anergized and self-antigen tolerized CD4⁺ T cells express significantly higher levels of Egr3 than cells fully activated in the presence of costimulation [4,5]. Additionally, the overexpression of Egr3 in T cells promotes an anergic phenotype, inhibiting both proliferation and IL-2 production, while T cells lacking Egr3 are hyper-responsive to activation and fail to become tolerized in *in vivo* models of anergy [4,5]. Egr3 negatively regulates T cell activation by inhibiting genes that induce IL-2 transcription [6] and by promoting transcription of FasL [7] and E3 ubiquitin ligases, which target proteins involved in TCR signaling for degradation [4,8,9]. Although its importance in T cell anergy has been established, these experiments were focused on Th1 effector cells. The influence of Egr3 on the development of other T helper subsets has not been previously investigated.

Th17 cells are critical in the defense against certain bacterial and fungal infections but also contribute to the pathogenesis of a

number of autoimmune diseases. These cells produce the proinflammatory cytokines IL-17, IL-21, and IL-22 and their differentiation relies on the activation and expression of the transcription factors STAT3 and ROR γ t [10–12]. The precise mechanism by which Th17 T cells are produced *in vivo* remains controversial, but can involve IL-6 and TGF β , IL-23 and IL-1, or some combination of these cytokines, with IL-23 believed to help maintain the Th17 phenotype or perhaps promote Th17 pathogenicity [13–16]. Many cell types can contribute to the Th17-biasing cytokine milieu, including nonlymphoid epithelial cells, innate immune cells like macrophages, DCs, and neutrophils, or even other activated T cells [17–19]. In addition to the downregulation of T cell activation machinery, Egr3 is thought to function in T cell development in part by inhibiting the function of ROR γ t in thymocytes [20], suggesting Egr3 might inhibit both Th1 and Th17 differentiation.

We sought to determine the role of Egr3 in the Th17 response using mice that transgenically overexpress Egr3 in T cells (Egr3 TG). Surprisingly, we found an increased proportion of CD4⁺ T cells from these mice produced IL-17 when stimulated under non-skewing conditions. This increase in Th17 cells does not appear to be a direct result of Egr3 overexpression, but instead correlates with an increase $\gamma\delta$ T cell production in Egr3 TG mice. Our data highlight the ability of peripheral $\gamma\delta$ T cells to markedly influence the adaptive helper T cell response. We show here that these Egr3-

induced $\gamma\delta$ T cells function similarly to wildtype $\gamma\delta$ T cells, in that they produce Th17-polarizing cytokines, are capable of skewing CD4+ T cells to a Th17 phenotype, and can promote inflammatory disease in a model of pulmonary fibrosis. We also show that, contrary to findings in thymocyte populations, Egr3 expression does not inhibit Th17 activation in the periphery.

Materials and Methods

Mice

Egr3 TG mice on the B6.AKR background were obtained from G. Kersh, Emory University School of Medicine, Atlanta, Georgia [21]. The Egr3 TG mice were backcrossed onto C57 Bl/6 mice from Jackson Laboratories for at least five generations. This study was carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Cells, Abs, and reagents

Stimulatory anti-CD3 (2C11) and anti-CD28 (37.51) were isolated by affinity chromatography from hybridoma supernatants. Cytokines were purchased from Peprotech. PMA and ionomycin were purchased from Sigma. CD3-PE, CD4-APC, CD4-PE, CD8a-PE, CD8a-APC, CD8b-PE, CD8a-PerCP, $\gamma\delta$ TCR-FITC, (GL3), V γ 4-FITC (UC310A6), CD25-PE, CD44-FITC, CD44-PerCP Cy5.5, CD62L-APC, CD90.1-PE, IFN γ -APC, and IL-17-PerCP Cy5.5 antibodies were purchased from BD biosciences and used for flow cytometry. Vg1-FITC (2.11) and Vg7-FITC (F2.67) antibodies were generously donated by the Pereira lab. Lymphocytes were isolated from lung as described under the "Pulmonary Fibrosis Model." To isolate lymphocytes from small intestine and colon, tissues were harvested, sliced open longitudinally, washed several times in saline, and cut into small pieces. These pieces were then incubated with 0.1 mM EDTA, washed, then incubated in 1 mg/ml collagenase. Supernatant from these steps were collected, strained, and lymphocytes were separated from remaining epithelial cells using a 40% 60% Percoll gradient centrifugation step. CD4+ T cells were isolated from pooled spleen and lymph nodes of Egr3 TG mice using the CD4+ T cell negative isolation kit (Miltenyi Biotec). $\gamma\delta$ T cells were isolated by enriching splenocytes using a T cell negative isolation kit (Miltenyi Biotec) and positively selecting for $\gamma\delta$ T cells using a biotinylated $\gamma\delta$ TCR Ab (BD biosciences), streptavidin coated beads, and magnetic columns (Miltenyi Biotec). CD4+ CD44- CD62L+ naive T cells were collected by FACS sorting. The Heilig and Tonegawa nomenclature is used when referring to specific subsets of $\gamma\delta$ T cells.

T Cell Activation

For activation of T cells with APC costimulation, 10^7 whole splenocytes were cultured in a 6 well plate with soluble anti-CD3 at 2 μ g/ml in 3 ml of media. After 48 h of stimulation, cells were collected, then added to 15 ml fresh media and cultured an additional 4 days. For CD4+ activation, 5×10^6 purified CD4+ T cells or 5×10^6 naive CD4+ T cells were stimulated in 1 ml in a 12 well dish with 2 μ g/ml plate-bound anti-CD3 and 2 μ g/ml soluble anti-CD28. After 48 hours, CD4+ T cells were expanded into 5 ml fresh media in a 6 well dish. All Th17 skewing was done by including IL-6 (10 ng/ml), TGF β (5 ng/ml), and IL-23 (10 ng/ml) in the initial stimulation media. No exogenous cytokines were added to Th0 stimulation media or $\gamma\delta$ coculture media. For $\gamma\delta$ skewing experiments, purified $\gamma\delta$ T cells were added to stimulation

reaction at 1:1 ratio with CD4+ T cells, keeping the total T cell number constant. All cells were restimulated on day 6 for 4-6 hours with PMA (50 ng/ml) and ionomycin (500 ng/ml) prior to intracellular cytokine staining. For RT-PCR of splenocytes, cells were collected and lysed prior to stimulation (day 0), 24 hours post-stimulation (day 1), or after 48hour of stimulation and 4 days of rest followed by a 6 hour restimulation with 1 μ g/ml plate-bound anti-CD3 and 2 μ g/ml soluble anti-CD28 (day 6). For RT-PCR of purified T cells, freshly isolated CD4+ T cells and $\gamma\delta$ T cells were stimulated 16 hours with 1 μ g/ml plate-bound anti-CD3 and 2 μ g/ml soluble anti-CD28 prior to cells lysis for RT-PCR.

Quantitative Real Time PCR

Total RNA was isolated from cultured T cells using TRIzol reagent (Invitrogen). Reverse transcriptase was performed on 1 μ g of total RNA with the SuperScript III reverse transcriptase kit (Invitrogen). Real-time PCR was performed using ready-made primer/probe sets for Egr3, Id3, IL-17A, IL-17F, IL-6, IL-21, IL23, IL-23R, ROR γ t, 18S endogenous control, and 2X PCR Master Mix (Applied Biosystems) on the ABI 7500 machine. Data were analyzed using SDS 1.5 software (Applied Biosystems). RNA abundance was calculated using the $\Delta\Delta C_t$ method normalized to control 18S RNA and is plotted as $2^{-\Delta\Delta C_t}$ of isolated sample or time point divided by an unstimulated wildtype control (fold increase over baseline) unless otherwise indicated.

Pulmonary Fibrosis Model

Mice were anesthetized using ketamine and xylazine, and 0.025 U of bleomycin was instilled intratracheally (i.t.). Mice were then monitored for weight loss and survival. To assess lymphocyte infiltration into the lungs, bleomycin was administered as above and mice were killed with a lethal injection of sodium pentathol (Ampro Pharmaceutical, Arcadia, CA). Bronchoalveolar lavage (BAL) was performed by cannulating the trachea then instilling and retrieving 1 ml of sterile normal saline. The entire lavage volume was centrifuged, and the cell pellet was resuspended in RPMI 1640 supplemented with FBS and antibiotics. Following cardiac perfusion, lungs were harvested, chopped in a petri dish with a sterile razor blade, then lightly crushed and washed with RPMI over a 10um cell strainer. Recovered lung-infiltrating lymphocytes were stimulated 5 hours for intracellular cytokine staining.

Results

Increased Th17 differentiation in Egr3 TG mice is cell extrinsic

While the ability of Egr3 overexpression to inhibit T cell activation has been demonstrated [4,5], this inhibition was measured through decreases in IL-2 production and proliferation, and the effects of Egr3 on T-helper differentiation have not been addressed. We hypothesized that Egr3 would inhibit Th17 differentiation in a similar fashion. To test this, we stimulated splenocytes from mice transgenically overexpressing Egr3 in T cells (Egr3 TG) or their wildtype counterparts (Egr3 WT) with or without the Th17 skewing cytokines IL-6, TGF β , and IL-23. After two days of stimulation, cells were expanded into fresh media, rested, and then restimulated with PMA and Ionomycin on day 6 for intracellular cytokine staining. Both Egr3 WT and Egr3 TG CD4+ T cells produce IL-17 when splenocytes were stimulated under skewing conditions (Th17), and surprisingly, a large fraction of Egr3 TG CD4+ T cells also produced IL-17 under non-skewing (Th0) conditions (Figure 1a). To further explore this observation,

we measured mRNA expression of various markers of the Th17 lineage by Egr3 WT and Egr3 TG splenocytes stimulated under non-skewing conditions. ROR γ t, IL-6 and IL-21 expression was higher in Egr3 TG splenocytes, even prior to stimulation (day 0). After only 24 hours of stimulation (day 1), IL-17A and IL-17 F expression was also higher in Egr3 TG splenocytes. However, the greatest differences between Egr3 WT and Egr3 TG splenocytes was found after splenocyte reactivation (day 6), when IL-17A, IL-17F, ROR γ t, IL-6, IL-21 and IL-23 mRNA levels were all significantly higher in Egr3 TG samples (Figure 1b). Such findings are consistent with the generation of Th17 cells and suggest that in the absence of exogenous skewing cytokines, the splenic environment in Egr3 TG mice is conducive to Th17 skewing. Many types of cells can contribute to the cytokine milieu in the spleen; therefore, to assess the intrinsic ability of Egr3 TG CD4+ T cells to produce IL-17, naive CD4+ T cells were sorted from Egr3 WT or TG splenocytes and activated in the absence (Th0) or presence (Th17) of skewing cytokines. Not surprisingly, isolated CD4+ T cells make less IL-17 than those stimulated in the presence of other splenocytes. More importantly, we also found that naive CD4+ T cells from both Egr3 WT and Egr3 TG mice have a similar capacity to become Th17 cells (Figure 1c). Though we have previously shown that Egr3 TG CD4+ T cells are hyporesponsive in their ability to proliferate and produce IL-2 [4,5], our results here suggest that constitutive expression of Egr3 by T cells neither inhibits nor directly promotes Th17 generation in peripheral CD4+ lymphocytes.

Egr3 promotes the development of $\gamma\delta$ T cells

Though Egr3 TG mice demonstrated an increase in splenic Th17 cells, the lymphoid organs of these mice contained a smaller proportion of total T cells (Figure 2a, left) as well as a reduced percentage of CD4+ and CD8+ T cells (Figure 2a, middle). This decrease in peripheral CD4+ and CD8+ T cells corresponds with a 4-8 fold increase in the percentage and absolute number of CD3+ T cells which lack expression of both CD4 or CD8 (Figure 2a, middle and Figure 2b), hereafter referred to as double negative T cells (DN) T cells. Upon further analysis, the majority of both the DN and CD8+ T cells from the spleens of Egr3 TG mice express a $\gamma\delta$ TCR (Figure 2a, right). These DN $\gamma\delta$ T cells and CD8+ $\gamma\delta$ T cells together comprise nearly 35% of the T cell pool in Egr3 TG mice (Figure 2b, middle), a more than 5-fold increase in the number of $\gamma\delta$ T cells (Figure 2b, right). Interestingly, the mean fluorescence intensity of CD8 is consistently half a log lower in Egr3 TG T cells (Figure 2a, middle), and closer inspection reveals that while very few CD8+ $\alpha\beta$ T cells from either genotype express the CD8 $\alpha\alpha$ homodimer, there was a significantly higher proportion of CD8 $\alpha\alpha$ -expressing $\gamma\delta$ T cells in Egr3 TG mice compared to their wildtype counterparts (Figure S1). The increase in $\gamma\delta$ T cell numbers in these mice is not limited to the lymphoid organs, as similar increases were also detected in the lung and intestinal mucosa of Egr3 TG mice (Figure 2c). We subsequently analyzed the subsets of $\gamma\delta$ T cells by their V γ chain expression and found increases in all subsets that were assessed. The V γ 1 and V γ 4 subsets made up the largest fractions of $\gamma\delta$ T cells in the lymphoid organs of Egr3 TG mice, while the majority of $\gamma\delta$ T cells in the gut mucosa were either V γ 1 or V γ 7 subsets (Figure 2d).

The Egr transcription factors are known to play a role in thymocyte development and β -selection [21–24] and increasing evidence suggests that this transcription factor also plays a role in determining $\gamma\delta$ T cells fate [25–27]. Developing $\gamma\delta$ T cells express higher levels of Egr1, Egr2 and Egr3 as well as the transcriptional regulator Id3 [26], and ectopic expression of any of the Egrs can promote $\gamma\delta$ TCR rearrangement *in vitro* [25,27]. It has also been

shown that mice that transgenically overexpress Egr1 have increased numbers of thymic $\gamma\delta$ T cells. However, these studies did not address the functional properties of Egr1-induced $\gamma\delta$ T cells in the periphery or the ability of Egr3 to promote development of $\gamma\delta$ T cells that function in the periphery. Therefore, we assessed $\gamma\delta$ T cell development in Egr3 TG mice in order to determine whether our mouse model aligned with these findings.

To this end we harvested thymi from age-matched WT and Egr3 TG littermates and surface stained for the thymocyte subset markers CD4, CD8, CD44, and CD25. A decrease in thymic cellularity (not shown) and a robust increase in the percentage of CD4- CD8- (DN) thymocytes at the expense of the CD4+ CD8+ (DP) population in Egr3 TG mice (Figure 3a, left) is consistent with previous findings that Egr3 functions in survival and proliferation, during the DN to DP transition of developing $\alpha\beta$ thymocytes [21,22,24]. Further investigation revealed a slight increase in the proportion of the CD44-, CD25- (DN4) thymocyte subset (Figure 3a, right) and an increase in both the percentage and absolute number of $\gamma\delta$ T cells in the thymus (Figure 3b and 3c). It has been proposed that a strong TCR stimulus during thymic development, mediated by Id3, can promote $\gamma\delta$ development over $\alpha\beta$ selection [27]. To determine whether Egr3 might promote $\gamma\delta$ development through modulation of Id3, we sorted freshly isolated CD4- CD8- thymocytes into DN1 through DN4 subsets and compared the mRNA expression of Id3 in Egr3 WT and Egr3 TG samples by qRT-PCR. Id3 expression was increased in the DN2 through DN4 subsets from Egr3 TG mice (Figure 3d). These results, along with the increased absolute number of peripheral $\gamma\delta$ T cells in Egr3 TG mice (Figure 2b, right), support a role for Egr3 in promoting $\gamma\delta$ T cell selection and inhibiting $\alpha\beta$ T cell development in the thymus.

$\gamma\delta$ T cells from Egr3 TG mice are capable of skewing CD4+ T cells to Th17

Having demonstrated a role for Egr3 in promoting the generation of $\gamma\delta$ T cells and determining that these $\gamma\delta$ T cells are capable of populating the peripheral lymphoid organs, we wanted to determine whether $\gamma\delta$ T cells in the lymphoid compartment of Egr3 TG mice were functionally similar to those from Egr3 WT mice. When splenocytes from Egr3 WT and Egr3 TG littermate mice were activated, DN $\gamma\delta$ T cells from both genotypes produced significantly more IL-17 than CD8+ $\gamma\delta$ T cells, and the fraction of $\gamma\delta$ T cells producing IL-17 was equivalent between genotypes (Figure 4a). Yet due to differences in their proportions within the spleen, there was a greater than 5-fold increase in absolute number of IL-17-producing $\gamma\delta$ T cells in the Egr3 TG sample (Figure 4a, bottom bar graph). Early mRNA expression of IL-17A, IL-17F, IL-6, and IL-23R was also found to be comparable between Egr3 WT and Egr3 TG $\gamma\delta$ T cells (Figure 4b). It is thought that cytokine expression by $\gamma\delta$ T cells may segregate in part according to different V γ subsets, with V γ 4 T cells being a common producer of IL-17 [28–30]. To assess this in our model, we measured IL-17 production by the two $\gamma\delta$ T cell subsets with the largest increase in Egr3 TG mice. Not surprisingly, V γ 4 T cells from both Egr3 WT and Egr3 TG mice produced more IL-17 than V γ 1 T cells (Figure 4c). Though IFN γ production by these $\gamma\delta$ subsets appears lower than expected, we confirmed IFN γ staining using stimulated $\alpha\beta$ T cells from the same Egr3 WT mice as a control (Figure S2).

Our findings that both CD4+ T cells and $\gamma\delta$ T cells from Egr3 TG mice are capable of expressing IL-17 were surprising, given that Egr3 is thought to inhibit both ROR γ t expression and function in thymocyte and $\gamma\delta$ T cell models [20,31]. While we

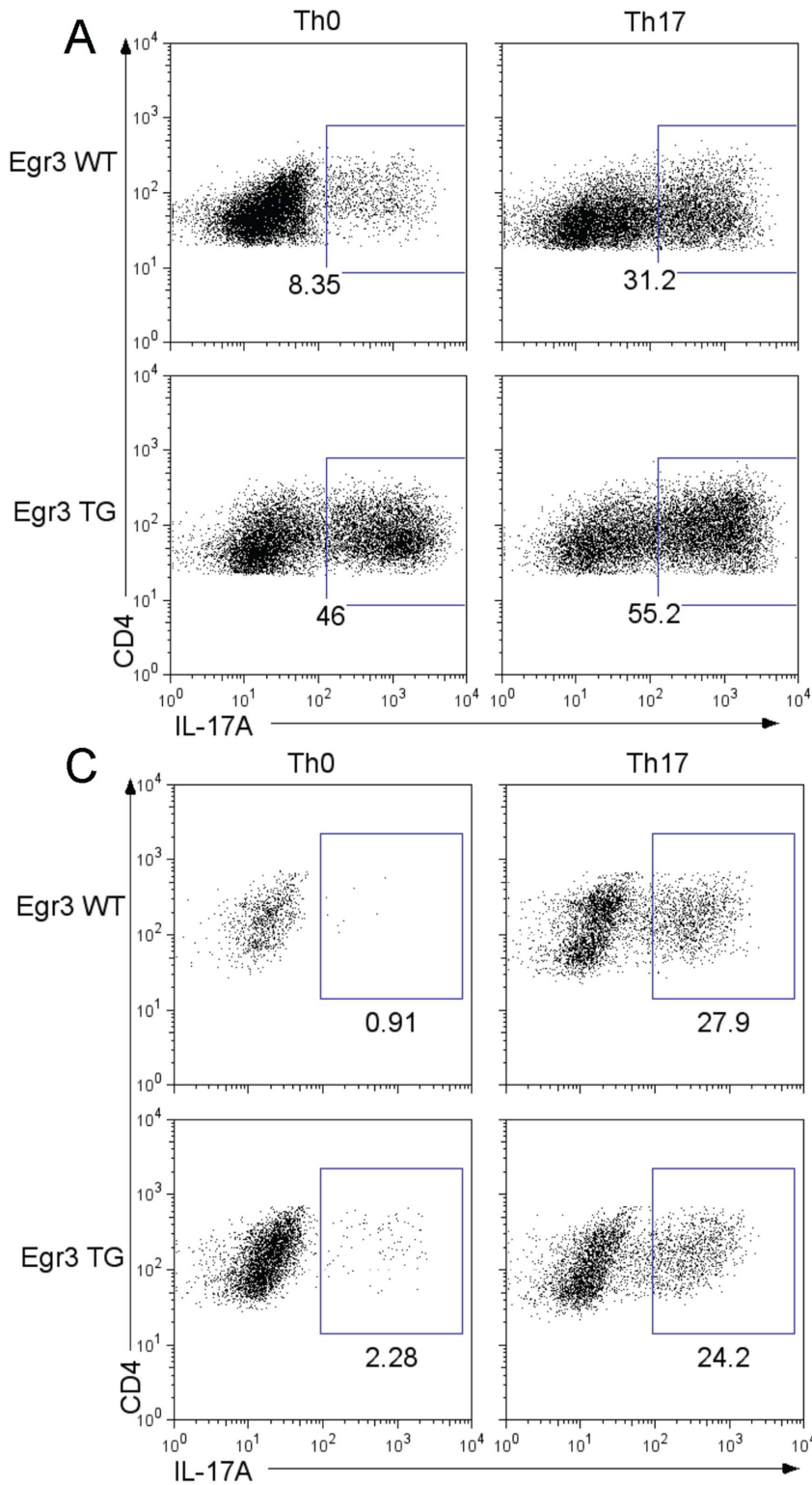


Figure 1. CD4+ T cells from Egr3 TG mice are biased toward Th17 differentiation. A-B) Splenocytes from 10-12 week old Egr3 WT and Egr3 TG mice were stimulated for 2 days with soluble anti-CD3 either with (Th17) or without (Th0) the addition of skewing cytokines. A) IL-17 production by Egr3 WT and Egr3 TG CD4+ T cells within the splenic population after restimulation. B) IL-17A, IL-17F, IL-21, ROR γ t, IL-23, and IL-6 mRNA expression in unstimulated (day 0), overnight stimulated (day 1) or previously activated and restimulated (day 6) splenocytes from Egr3 WT and Egr3 TG mice. Cells stimulated for mRNA were not skewed and mRNA expression was determined by qRT-PCR done in triplicate and normalized to 18s expression. C) Naive CD4+ T cells from Egr3 WT and Egr3 TG mice were stimulated with anti-CD3 and anti-CD28 for 2 days with or without skewing cytokines as in A, then restimulated and assessed for IL-17 production. Data are representative of 3 independent experiments, each using 2 mice per genotype. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$
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observed that constitutive expression of Egr3 did not reduce ROR γ t expression at either early or late time points, our splenic activation model shows that ROR γ t expression was briefly inhibited after 24 hours of stimulation (Figure 1b and Figure S3). Our data support a model whereby activation-induced levels of Egr3 inhibit ROR γ t function temporarily, and that this inhibition is relieved after Egr3 expression decreases. In fact, this model has already been proposed to explain the role of Egr3 in $\gamma\delta$ T cell development [20]. Taken together, our findings suggest that Egr3-induced $\gamma\delta$ T cells are similar to wildtype $\gamma\delta$ T cells in their ability to produce cytokine. The clearest difference between these genotypes is the increase in the absolute number of $\gamma\delta$ T cells (Figure 2b), particularly the V γ 4+ $\gamma\delta$ T cells, a subset shown to produce IL-17 and other cytokines involved in Th17 skewing and stabilization (Figure 2d and Figure 4).

It has been suggested that $\gamma\delta$ T cells have the ability to directly skew CD4+ T cells through the production of IL-17 [18]. Our findings suggest that the increased numbers of $\gamma\delta$ T cells found in Egr3 TG mice are able to contribute to this skewing milieu, in part through the expression of both IL-17 and IL-6. To directly test whether Egr3-induced $\gamma\delta$ T cells can skew CD4+ T cells, we isolated CD4+ T cells from Thy1.1- Egr3 WT mice and stimulated them alone, in the presence of Thy1.1+ Egr3TG $\gamma\delta$ T cells, or with Th17-skewing conditions. Note that the “readout” cells in this assay are wildtype CD4+ T cells, thus eliminating a cell-intrinsic role for Egr3 in regulating Th17 production. CD4 and Thy1.1 were used to differentiate cell types within the co-culture, as it otherwise may have been difficult to separate $\gamma\delta$ T cells from CD4 T cells based on post-activation TCR expression (Figure 4d, top row). Plotting these two gates together (Figure 4d, top right) shows both that this gating strategy was successful and that large numbers of Egr3 TG $\gamma\delta$ T cells in this co-culture experiment produce IL-17. The addition of $\gamma\delta$ T cells to the stimulation consistently increased production of IL-17 by CD4+ T cells more than 2-fold (Figure 4d, bottom row and bar graph). This co-culture demonstrates the influence of $\gamma\delta$ T cells during CD4+ T cell activation and expansion, suggesting that prolonged exposure of CD4+ T cells to Egr3-induced $\gamma\delta$ T cells may promote the strong Th17 skewing seen in Egr3TG mice.

$\gamma\delta$ T cells from Egr3 TG mice promote inflammation in a model of pulmonary fibrosis

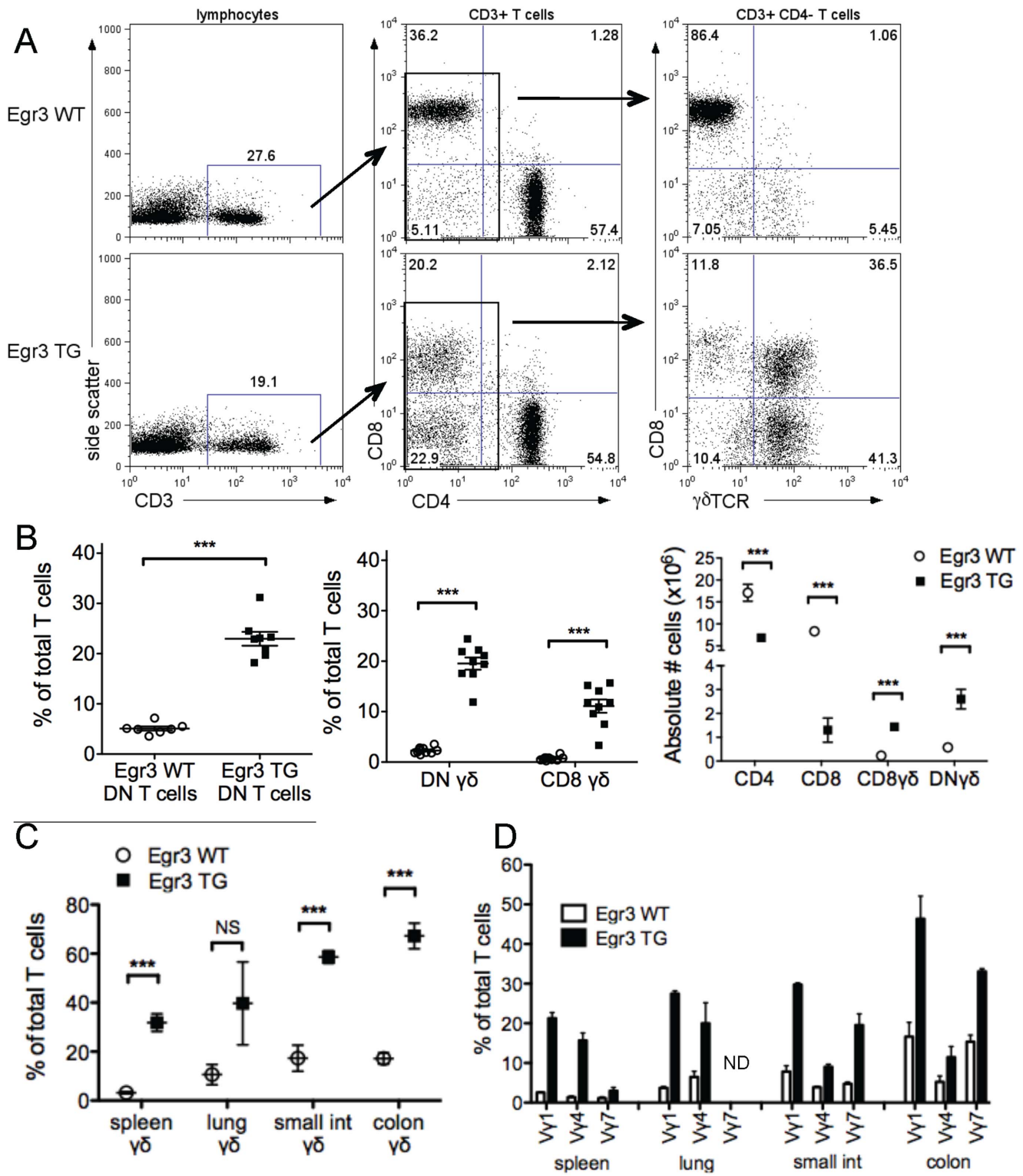
We next wanted to examine whether Egr3-induced $\gamma\delta$ T cells could promote an *in vivo* inflammatory response. To this end, we utilized Egr3WT and Egr3TG mice in a model of pulmonary fibrosis, a disease in which both $\gamma\delta$ T cells and IL-17 play a role [32,33]. When mice were challenged with intratracheal (i.t.) bleomycin to induce inflammation and fibrosis, weight loss occurred faster and to a greater extent in Egr3TG mice compared to their Egr3 WT littermates (Figure 5a). In addition, a larger fraction of Egr3TG mice died prior to day 25 (Figure 5b). In light of these observations, we wanted to examine the inflammatory responses in the lungs of the Egr3WT and Egr3TG mice. Mice were given i.t. bleomycin and sacrificed 5 days later to determine

the levels of cell infiltration and cytokine production within the lungs. Both the BAL and lungs of bleomycin-treated Egr3 TG mice contained 2-3 fold more total $\gamma\delta$ T cells and a significantly higher number of IL-17-producing $\gamma\delta$ T cells than in wildtype mice (Figure 5c). We also observed an increase in both total CD4+ T cell numbers and IL-17+ CD4+ T cell numbers in the BAL of Egr3 TG compared with Egr3 WT mice (Figure 5c). This finding is particularly striking considering Egr3 TG mice have impaired $\alpha\beta$ T cell development and lower circulating numbers of CD4+ T cells (Figure 2). These data are consistent with the idea that Egr3-induced $\gamma\delta$ T cells are capable of inducing inflammation and disease either directly or through their influence on CD4+ T cells.

Discussion

Egr3 has been shown to be a negative regulator of CD4+ T cell responses and we initially undertook these experiments to examine the role of Egr3 in Th17 development. Surprisingly, we found that not only do Egr3 TG CD4+ T cells produce robust amounts of IL-17 but they skewed to become Th17 cells even under non skewing conditions. In pursuing this finding we revealed that this increase in Th17 differentiation in the Egr3 TG mice was not cell intrinsic. Rather, we demonstrate a role for Egr3 in the development of functional $\gamma\delta$ T cells that can influence the adaptive immune response and promote inflammatory disease. These Egr3-induced $\gamma\delta$ T cells reside in peripheral lymphoid organs as well as in circulation and in epithelial tissues, and are capable of producing cytokines at levels similar to wildtype $\gamma\delta$ T cells. We found that though CD4+ T cells from Egr3 TG mice are similar to those from Egr3 WT mice in their capacity to secrete IL-17, they are skewed toward a Th17 fate, in part by the increased ratio of $\gamma\delta$ T cells to CD4+ T cells in Egr3 TG mice. While we would have liked to compare the skewing ability of Egr3 WT and Egr3 TG $\gamma\delta$ T cells in our co-culture experiment (Figure 4d), we were unable to isolate large enough numbers of Egr3 WT $\gamma\delta$ T cell to do this. It is therefore not our intent to suggest that Egr3-induced $\gamma\delta$ T cells are better at promoting a Th17 phenotype than wildtype $\gamma\delta$ T cells. It has been shown that wildtype $\gamma\delta$ T cells are capable of direct Th17 skewing [18], and our data highlight that Egr3-induced $\gamma\delta$ T cells also possess this capability. Notably, our finding that Egr3-induced $\gamma\delta$ T cells possess the functionality of wildtype $\gamma\delta$ T cells, yet are present in larger numbers in the periphery, make them a potential model for further study of $\gamma\delta$ T cells and their ability to influence the adaptive immune response.

While we demonstrate the ability of the $\gamma\delta$ T cells to directly affect the skewing of CD4+ T cells, $\gamma\delta$ T cells may also influence APCs and other cells in the lymphoid compartment to aid in Th17 skewing and maintenance. Our finding that IL-23 mRNA is elevated in Egr3 TG splenocytes (Figure 1b) supports this idea. This would also explain the greater difference in IL-17 production between genotypes in the presence of antigen presenting cells (APCs) and other splenocytes which could contribute to the skewing milieu (Figure 1a compared to Figure 4d). Our data support previous findings that innate-like $\gamma\delta$ T cells can profoundly influence T helper cell differentiation and adaptive



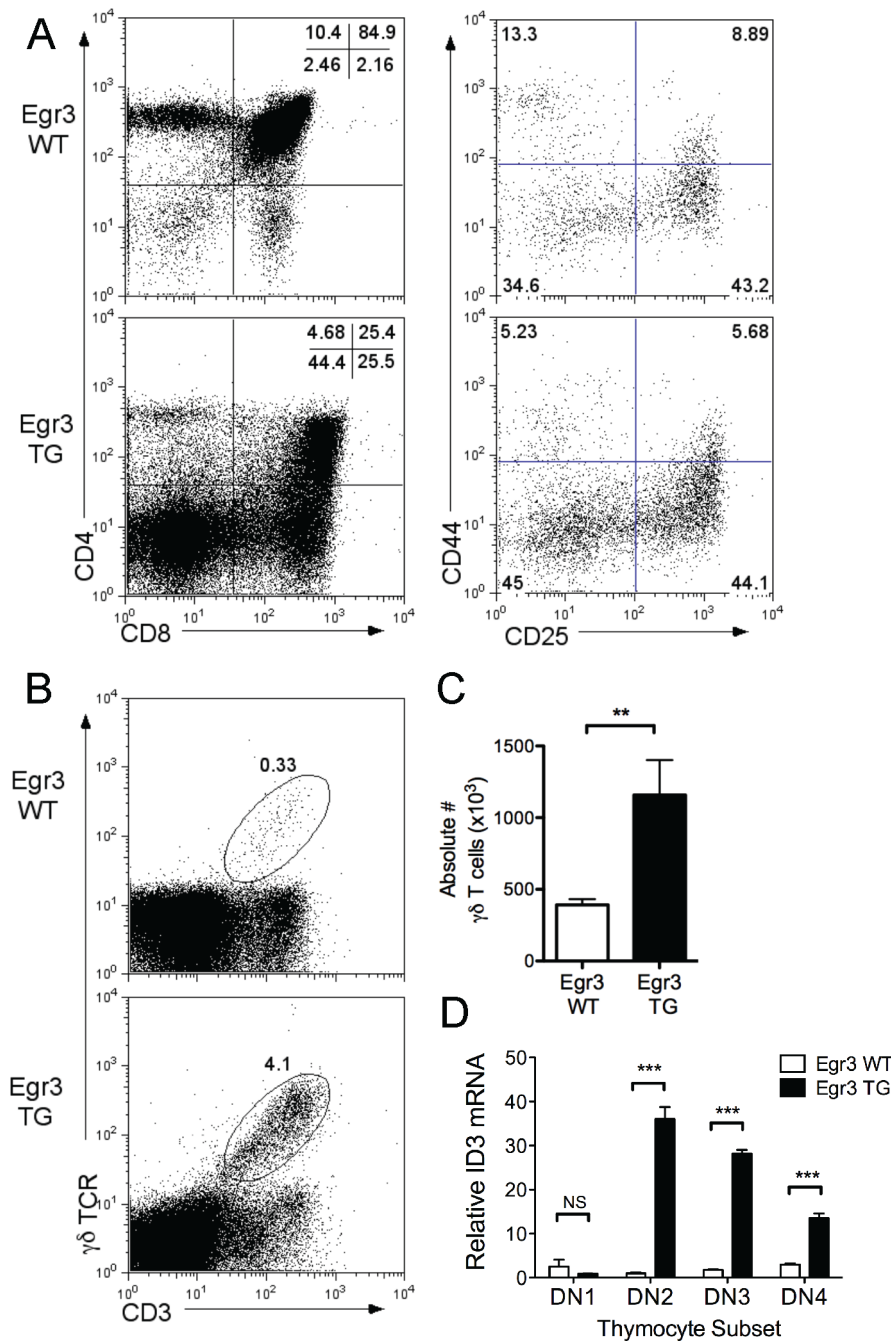


Figure 3. Thymocyte selection in Egr3 TG mice favors $\gamma\delta$ T cells. A-B) Thymocytes harvested from littermate Egr3 WT and Egr3 TG mice were stained for surface marker expression or lysed for mRNA quantification. A) CD4 vs. CD8 expression was measured to determine double negative, double positive, and single positive subsets (left panels). DN1, DN2, DN3, and DN4 subsets shown as a percentage of the double negative fraction (right panels). B) $\gamma\delta$ T cells shown as a percentage of thymocytes (left panels) and C) $\gamma\delta$ T cells shown as an absolute number. D) Expression of Id3 mRNA in DN1, DN2, DN3, DN4 thymocytes assessed by qRT-PCR and normalized to 18s expression. Data in A-D are representative of 3 independent experiments, each using 2 mice per genotype. ** $p \leq 0.01$, *** $p \leq 0.005$ doi:10.1371/journal.pone.0087265.g003

immune responses [18,34,35]. Our work also demonstrates an indirect role for Egr3 in promoting Th17 development, in which Egr3 promotes the development of $\gamma\delta$ T cells which can in turn drive Th17 differentiation.

$\gamma\delta$ T cells primarily produce either IFN γ or IL-17 and these populations can be segregated by V γ chain usage or by surface marker expression. V γ 4+ cells are considered one of the most

common IL-17 producers in the periphery [28,30], and while IFN γ -producing $\gamma\delta$ T cells are largely CD27+ NK1.1+, IL-17-producing $\gamma\delta$ T cells are most often CD27- and CCR6+ [36,37]. Though there were increases in all measured subsets of $\gamma\delta$ T cells in Egr3 TG mice, one of the largest increases we found in the peripheral lymphoid organs was in this IL-17-associated V γ 4+ subset. This is not surprising, as we believe Egr3 influences $\gamma\delta$ T

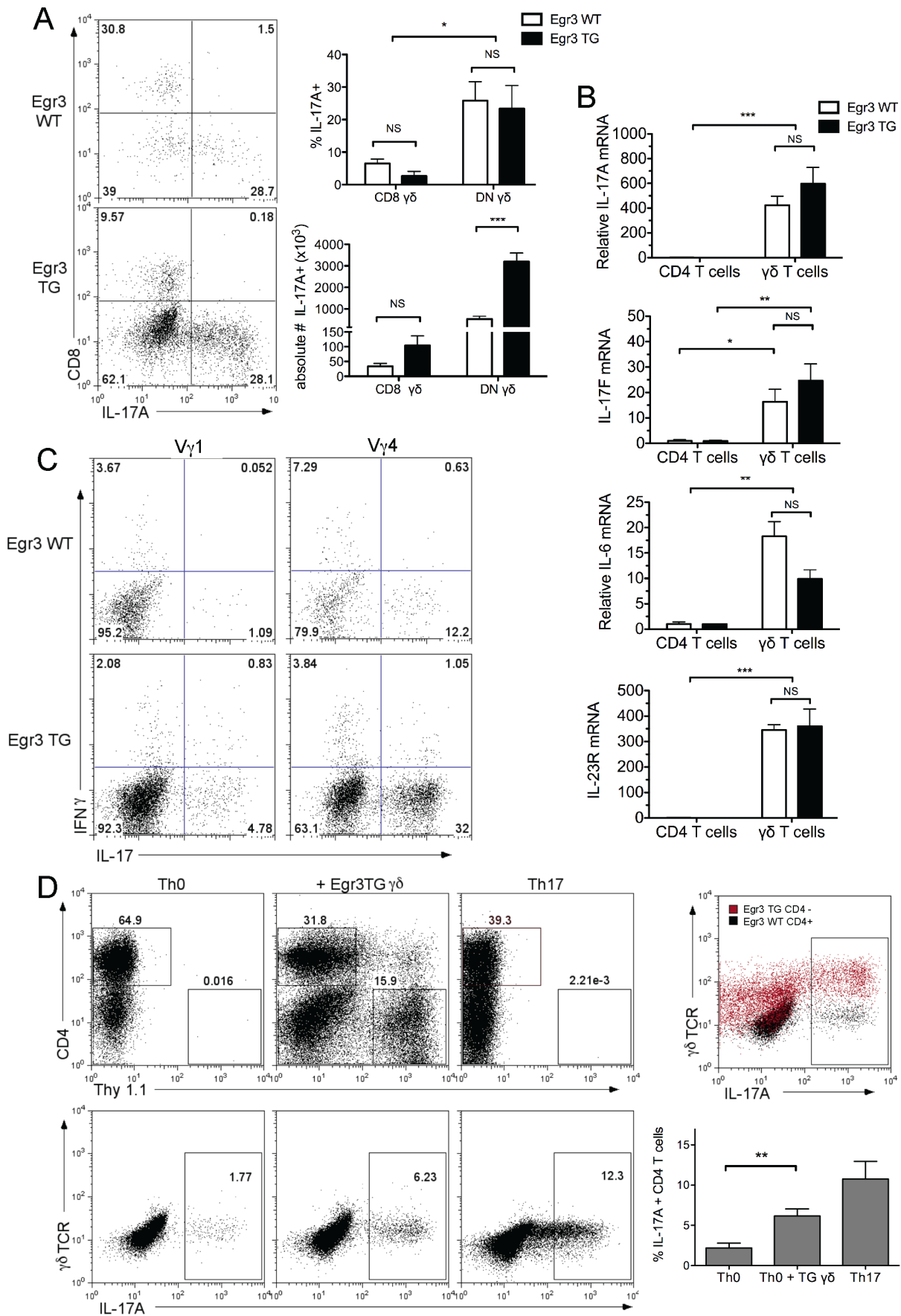


Figure 4. Egr3 TG $\gamma\delta$ T cells produce IL-17 and skew CD4+ T cells to a Th17 phenotype. A) Splenocytes were stimulated and rested, and IL-17 production by CD8 $\gamma\delta$ and DN $\gamma\delta$ T cells was measured on day 6 after short restimulation (flow plots). The percentage of each subset producing IL-17 (top bar graph) and absolute number of IL-17 producing cells of each subset (bottom bar graph) are shown as an average of 4 independent experiments, each using 2 mice per genotype. B) Purified CD4+ T cells or $\gamma\delta$ T cells were stimulated directly *ex vivo* with plate bound anti-CD3 and soluble anti-CD28 for 16 hours. IL-17A, IL-17F, IL-6, and IL-23R mRNA expression was measured by qRT-PCR done in triplicate and normalized to 18s expression. Data are representative of 3 independent experiments, each using 2 mice per genotype. C) IL-17 production measured as a percentage of either the V γ 1-expressing subset (left panels) or the V γ 4-expressing subset (right panels) of $\gamma\delta$ T cells in splenocyte stimulation. Data are representative of 1 experiment using 5 mice per genotype. D) Egr3 WT CD4+ T cells were stimulated for 2 days with plate bound anti-CD3 and soluble anti-CD28 either alone (Th0), with the skewing cytokines TGF β , IL-6, and IL-23 (Th17), or with equal numbers of Egr3 TG $\gamma\delta$ T cells. IL-17 production by was assessed on day 6 after restimulation. CD4 and Thy1.1 expression shows the gating strategy used in this experiment (top row). IL-17 expression by the CD4+ Thy1.1- gated Egr3 WT T cells (bottom row). The overlay flow plot (top row, right) shows both the Egr3 WT CD4+ Thy1.1- T cells and Egr3 TG Thy1.1+ gates from the co-culture. Plots are representative of 3 independent experiments, which are averaged in the bar graph (bottom row, right). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$
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cell development through promoting expression of Id3, and it has been reported that Id3 $^{-/-}$ mice have a severe reduction of this particular subset of $\gamma\delta$ T cells in the spleen [27]. Preliminary work by our group suggests that while NK1.1 and CCR6 expression is similar between Egr3 WT and Egr3 TG $\gamma\delta$ T cells, IFN γ producing $\gamma\delta$ T cells from Egr3 TG mice may be less likely to express CD27 than those from Egr3 WT mice (not shown). Future studies will more precisely define these findings.

It has also been suggested that IL-17 is produced by antigen-naïve $\gamma\delta$ T cells, mediated by innate immune signals, while IFN γ production is induced by TCR stimuli [38–40]. The idea that TCR activation inhibits IL-17 production is supported by work showing that developing thymocytes which engage Skint-1 upregulate Egr3, which in turn suppresses ROR γ t expression and thus IL-17 production [31]. Though this would seem to conflict with our findings, most of the work by this group was done

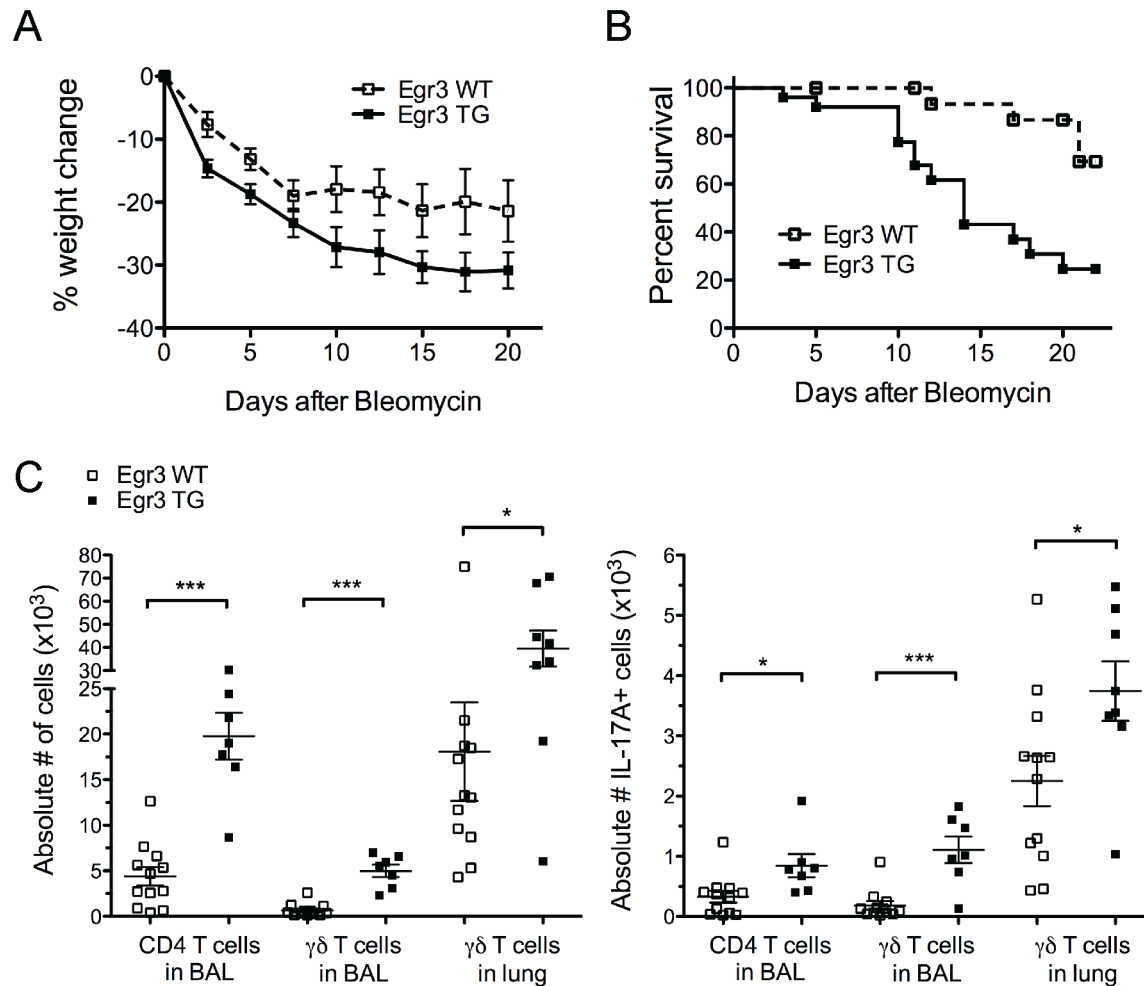


Figure 5. Egr3 TG mice are more susceptible in a model of pulmonary fibrosis. Egr3 WT or Egr3 TG mice were challenged with intratracheal bleomycin at day 0 and monitored for A) weight loss and B) survival. Data in A and B are compiled from 4 independent experiments. C) Separate mice were challenged as in A and sacrificed at day 5 to determine lymphocyte infiltration in the bronchoalveolar lavage (BAL) and lung as well as the absolute number of IL-17-secreting cell infiltrates in each compartment. Data in C are representative of 3 independent experiments. * $p \leq 0.05$, *** $p \leq 0.005$
doi:10.1371/journal.pone.0087265.g005

in thymocyte cultures, comparing V γ 5 and V γ 6 subsets of $\gamma\delta$ T cells which normally populate the skin and reproductive mucosa. The *in vivo* skint-1 knock-out models show little to no increase in IL-17 production by peripheral $\gamma\delta$ T cells [31], while our IL-17 producing Egr3-induced $\gamma\delta$ T cells are both a different subset (V γ 4) and from peripheral lymphoid organs which lack Skint-1 expression.

Though the idea that $\gamma\delta$ T cells can promote Th17 skewing is not new, we were initially surprised that CD4⁺ T cells and $\gamma\delta$ T cells which overexpressed Egr3 were both capable of producing IL-17. Egr3 has been established as a mediator of T cell anergy, and its overexpression is thought to inhibit T cell activation [4,5]. One possible explanation is that the level of Egr3 necessary for complete ROR γ t inhibition is not maintained in Egr3 TG mice. While Egr3 expression is higher in T cells from Egr3 TG mice both prior to- and at all time points after activation, the Egr3 transgene is expressed separately from the endogenous gene and the kinetics of Egr3 expression mimic that of the Egr3 WT mice, increasing to peak expression shortly after TCR engagement and returning to baseline levels just as quickly [21] (Figure S3). Egr3 has been shown to inhibit both ROR γ t expression and function during thymocyte development, though this inhibition is similarly transient; ROR γ t expression and function are restored once Egr3 expression drops [20]. With these latter points in mind, another possible explanation for increased IL-17 by Egr3-overexpressing CD4⁺ T cells is that decreased IL-2 production by Egr3 TG T cells [5] balances any IL-17 inhibition by Egr3, as IL-2 plays a known role in inhibiting IL-17 expression [16,41]. Overall, the finding that Egr3 TG CD4⁺ T cells are able to produce IL-17 has forced us to reconsider our definition of T cell anergy. Previous results led us to conclude that CD4⁺ T cells from Egr3 TG mice were anergic [4,5], but a cell that proliferates more slowly and does not produce IL-2 or IFN γ is anergic only in the context of Th1 activation. We have shown here that transgenic overexpression of Egr3 does not inhibit Th17 differentiation in CD4⁺ T cells or IL-17 production by peripheral $\gamma\delta$ T cells. While this is a negative finding, it shows that the role of Egr3 in IL-17 production is more complex than we originally believed.

It is widely accepted that the cytokine milieu experienced by a CD4⁺ T cell during primary activation is responsible for its differentiation into one of the many T helper subsets [42] and there is growing evidence to suggest that $\gamma\delta$ T cells can contribute to this instructive environment [34,35]. CD4⁺ T cells activated either in the presence of IL-6 and TGF- β , or IL-6, IL-1 and IL-23 both produce IL-17, but those skewed with TGF- β appear to be less inflammatory and fail to transfer susceptibility to experimental autoimmune encephalomyelitis (EAE) [15,16]. Similarly, $\gamma\delta$ T cells cultured *in vitro* with IL-23 and IL-1 produce IL-17 and have been linked to amplification of a Th17 response and exacerbation of inflammatory disease [18]. We have demonstrated here for the first time that Egr3 TG mice have an abundance of IL-17-producing $\gamma\delta$ T cells, and that these Egr3-induced $\gamma\delta$ T cells are capable of skewing CD4⁺ T cells *in vitro* without the addition of recombinant cytokines. Our data support not only the idea that $\gamma\delta$ T cells shape the adaptive immune response, but also support a role for Egr3 in the development of this influential population of cells.

Though normally comprising 1-2% of circulating lymphocytes, $\gamma\delta$ T cells are found more abundantly in epithelial and mucosal

tissues. These cells are capable of responding rapidly to stress, tissue damage, and infection and can modulate the adaptive immune response in either a tolerogenic or inflammatory manner [43–48]. Recent work on the role of $\gamma\delta$ T cells and IL-17 in pulmonary fibrosis suggests that $\gamma\delta$ T cells amplify a Th17 response and promote inflammation, but differ on whether they amplify collagen deposition [32], help to control fibrosis via production of CXCL10 or IL-22 [32,49–51] or have no effect on fibrotic disease [33]. In this report we demonstrate an increase in morbidity and mortality in Egr3 TG mice using a model of lung fibrosis. Previous work by our lab have shown that Egr3 TG mice are less susceptible to autoimmune pneumonitis [5], which at first seems to conflict with our current findings. However, while these experiments indicate less Th1 inflammation as measured by IFN γ production, total numbers lung infiltrates were similar and IL-17 was not measured. Along these lines, it remains to be determined if in the autoimmune pneumonitis model, the presence of increased Egr3 $\gamma\delta$ T cells could actually be protective. Overall our findings suggest a role for Egr3 in promoting peripheral IL-17-producing $\gamma\delta$ T cells, reveal a selective role for Egr3 $\gamma\delta$ T cells in promoting tissue pathology, and offer a potential model for studying this population of cells.

Supporting Information

Figure S1 Higher percentage of Egr3 TG $\gamma\delta$ T cells express CD8 α . Egr3 WT and Egr3 TG splenocytes were gated on $\gamma\delta$ TCR⁺ T cells and $\gamma\delta$ TCR⁻ T cells ($\alpha\beta$ T cells) and expression of CD8 α and CD8 β chains were compared. Plots are representative of two experiments using 4 mice per genotype. Results are averaged in the bar graph. (TIFF)

Figure S2 IL-17 and IFN γ intracellular cytokine staining controls. Egr3 WT splenocytes from the experiment shown in Figure 4c were gated on CD3⁺ $\gamma\delta$ TCR⁻ cells ($\alpha\beta$ T cells) and were either left unstimulated as a negative control (left panel) or stimulated as a positive control (right panel). (TIFF)

Figure S3 Egr3 TG T cells express higher levels of Egr3 and ROR γ t. Purified $\gamma\delta$ T cells were stimulated directly *ex vivo* for the times specified, then ROR γ t (A) or Egr3 (B) mRNA expression was measured by qRT-PCR done in triplicate and normalized to 18s expression. Data is from a single experiment using cells from 3 mice per genotype. (TIFF)

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Author Contributions

Conceived and designed the experiments: RP JP. Performed the experiments: RP SC. Analyzed the data: RP. Contributed reagents/materials/analysis tools: JP MH. Wrote the paper: RP.

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