

CD3^{bright} signals on $\gamma\delta$ T cells identify IL-17A-producing V γ 6V δ 1⁺ T cells

C Paget^{1,2,3,4,15}, M T Chow^{1,2,5}, N A Gherardin^{1,2,6}, P A Beavis^{1,2}, A P Uldrich⁶, H Duret^{1,2}, M Hassane^{3,4}, F Souza-Fonseca-Guimaraes⁵, D A Mogilenko^{4,7,8}, D Staumont-Sallé^{4,7,8,9}, N K Escalante¹⁰, G R Hill^{5,11}, P Neeson^{1,2}, D S Ritchie^{1,2}, D Dombrowicz^{4,7,8}, T Mallevaey¹⁰, F Trottein^{3,4}, G T Belz¹², D I Godfrey^{6,13} and M J Smyth^{1,2,5,14,15}

¹Cancer Immunology Program, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria 3002, Australia.

²Sir Peter MacCallum Department of Oncology and Department of Pathology, University of Melbourne, Parkville 3010, Australia.

³INSERM U1019, Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, 59000 Lille, France.

⁴University of Lille 2, Lille, France.

⁵QIMR Berghofer Medical Research Institute, Herston, Queensland 4006, Australia.

⁶Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, VIC 3010, Australia.

⁷INSERM U1011, Institut Pasteur de Lille, 59000 Lille, France.

⁸European Genomic Institute of Diabetes, Lille, France.

⁹Department of Dermatology, Claude Huriez Hospital, Lille, France.

¹⁰Department of Immunology, University of Toronto, Toronto, ON M5S 1A8, Canada.

¹¹Department of Bone Marrow Transplantation, Royal Brisbane Hospital, Herston, Australia.

¹²Division of Molecular Immunology, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, 3052, Australia.

¹³Australian Research Council Centre of Excellence in Advanced Medical Imaging at University of Melbourne.

¹⁴School of Medicine, University of Queensland, Herston, Queensland 4006, Australia.

¹⁵Correspondence: christophe.paget@inserm.fr or mark.smyth@qimrberghofer.edu.au

The authors have no financial conflicts of interest.

Running title: CD3 as a marker of V γ 6V δ 1⁺ T cells

Keywords: $\gamma\delta$ T cells, IL-17, IL-23, IL-1 β , invariant TCR, microbiome, ROR γ t, alpha-galactosylceramide, lipopolysaccharide, psoriasis, pneumococcus, innate immunity, neutrophils, mucosal immunity.

Abbreviations: α -GalCer, α -galactosylceramide; CBA, cytometric bead array; NLRP3, NOD-like receptor family, pyrin domain containing 3; TCR, T cell receptor; WT, wildtype.

Abstract

Interleukin-17A is a pro-inflammatory cytokine that plays an important role at mucosal sites in a wide range of immune responses including infection, allergy and auto-immunity. $\gamma\delta$ T cells are recognized as IL-17 producers, but based on the level of CD3 expression we now define the remarkable ability of a CD3^{bright} $\gamma\delta$ T cell subset with an effector memory phenotype to rapidly produce IL-17A, but not IFN- γ . CD3^{bright} $\gamma\delta$ T cells uniformly express the canonical germline encoded V γ 6/V δ 1⁺ TCR. They are widely distributed with a preferential representation in the lungs and skin and do not develop properly in the absence of ROR γ t expression or endogenous flora. This population responded rapidly to various stimuli in a mechanism involving IL-23 and NLRP3-inflammasome-dependent IL-1 β . Finally, we demonstrated that IL-17-producing CD3^{bright} $\gamma\delta$ T cells responded promptly and strongly to pneumococcal infection and during skin inflammation. Here, we propose a new way to specifically analyse IL-17-producing V γ 6/V δ 1⁺ T cells based on the level of CD3 signals. Using this gating strategy, our data reinforce the crucial role of this $\gamma\delta$ T cell subset in respiratory and skin disorders.

Introduction

$\gamma\delta$ T cells have diverse functional specializations and promptly produce copious amounts of immunoregulatory cytokines, conferring on them a pivotal role in a broad range of pathologies including infection, allergy, auto-immunity and cancer¹. In particular, mouse $\gamma\delta$ T cells are known as major early IL-17 producers². In this context, IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ T17) may be either considered beneficial or harmful to the host depending upon to the disease. For example, IL-17 is an important anti-microbial cytokine predominantly due to its ability to induce chemokine release, growth factors, and adhesion molecules; and to indirectly increase neutrophilia through epithelial and stromal cell-dependent soluble factors^{3, 4}. This feature is of particular interest during mucosal infections in which IL-17 has been demonstrated to be critical in the control of tuberculosis, candidiasis, *Escherichia coli* and *Staphylococcus aureus* infections^{5, 6}. In these models, $\gamma\delta$ T cells have been reported to be a major source of this cytokine^{5, 7, 8, 9}. On the other hand, IL-17 has been proposed to participate in the development of a diversity of inflammatory or auto-immune disorders including arthritis, asthma, psoriasis and EAE⁶ and similarly, $\gamma\delta$ T17 cells play an important part in these processes¹⁰. Finally, the role of IL-17 in tumorigenesis or control of tumor progression has recently been studied but remains controversial¹¹. However, $\gamma\delta$ T17 cells appear to be important in eliciting a positive outcome during immunogenic anti-cancer chemotherapy¹². In humans, little is known about the real functional relevance of $\gamma\delta$ T17 cells in IL-17-dependent pathologies¹³ despite the fact that IL-17-production by $\gamma\delta$ T cells has been detected during infections and chronic inflammation^{14, 15, 16, 17}. However, characterization, isolation and activation of these cells in humans remain extremely challenging¹⁴.

Numerous recent studies have also focused on the factors influencing $\gamma\delta$ T17 cell differentiation, homeostasis and activation^{10, 18}. $\gamma\delta$ T17 cells express a fairly restricted TCR repertoire (V γ 4⁺ and V γ 6⁺ chain predominantly)^{9, 19, 20, 21} and originate from fetal thymus without evidence of TCR-dependent positive selection^{19, 22}. Within the thymus, $\gamma\delta$ T17 cells have a pre-programmed IL-17-producing phenotype, although these cells do not require the transcription factors ROR γ t or STAT3 to develop²³, even though STAT3 remains mandatory for their activation in the periphery. The strength of engagement with cognate antigen(s) (Ag) by $\gamma\delta$ TCRs in the thymus seems to be the key factor influencing the commitment towards IFN- γ - or IL-17-producing $\gamma\delta$ T cell lineages^{19, 22, 24}. In this context, the absence of (or weak) TCR ligation seems to determine a transcriptional program leading to the development of $\gamma\delta$ T17 cells^{19, 22, 24}. In addition, Notch/Hes1 signaling pathways are involved in the development and homeostasis of $\gamma\delta$ T17 cells²³.

Here, we demonstrate in mice that the level of cell surface CD3 expression on $\gamma\delta$ T cells reveals the V γ 6/V δ 1⁺ $\gamma\delta$ T cells, a population associated with a great ability to secrete IL-17. We confirm and refine some key homeostatic factors and activation mechanisms for this population *in vitro* and *in vivo*. This population participates in the early phase of the immune response against respiratory bacterial infections and skin inflammation by increasing in absolute number and producing high levels of IL-17 compared to other previously reported innate IL-17-producing cells. Given the versatile role of IL-17 at mucosal sites, our data reinforce that the prevalence of IL-17-producing V γ 6V δ 1⁺ T cells in lungs and skin of adult mice is consistent with a pivotal role of this cell subset in respiratory and dermal disorders.

Results

Detection of a $\gamma\delta$ T cell subset harboring an intense expression of CD3

We have recently demonstrated that $\gamma\delta$ T cells are an important early source of cytokines (IFN- γ and IL-17A) in response to α -GalCer²⁵. During our investigations, we have consistently observed in our control mice at steady-state, a minor population of hepatic $\gamma\delta$ T cells displaying a brighter expression of CD3 compared with the remainder of the $\gamma\delta$ T cell population (Figure 1a). As shown in Figure 1b this population is widely distributed although their frequency as a proportion of the entire $\gamma\delta$ T cell population differs in each organ tested. Thus, although this subset was preferentially represented in lungs and skin, it was also detected in thymus and various peripheral organs. However, the population was virtually absent in the blood and the intestinal tract as well as associated lymphoid tissues (Peyer's patches and mesenteric lymph nodes) (Figure 1b-d and not shown). Importantly, we were also able to detect these cells in BALB/c mice with a similar pattern of distribution to that observed in C57BL/6 (Supplementary Figure 1). Moreover, their morphology was similar to normal lymphocytes (Supplementary Figure 1) and this population was absent in TCR $\delta^{-/-}$ mice (Supplementary Figure 1), supporting that these are true $\gamma\delta$ T cells. Of note, we are still able to discriminate both $\gamma\delta$ T cell subsets after culture of total lung mononuclear cells in cytokine-free media based on CD3 signals suggesting that this phenotype is not due to a recent activation of this particular population during cell preparation (not shown). To examine if our observation was specifically due to the use of a particular clone of anti-CD3, we have compared staining profiles with 2C11 and 17A2 clones that recognize CD3 ϵ and CD3 $\epsilon\gamma$ dimers respectively. Interestingly, both clones allow a clear discrimination of the two $\gamma\delta$ T cell subsets based on CD3 signals suggesting that we can rule out the possibility that our observation

was solely due to a difference in epitope accessibility (Supplementary Figure 1). Together our data define a population of $\gamma\delta$ T cells with a unique phenotype characterized by a bright expression of the CD3 molecule.

CD3^{bright} $\gamma\delta$ T cells display a phenotype of $\gamma\delta$ T17 cells

Since no studies have yet reported a population of $\gamma\delta$ T cells with such a phenotype, we investigated the markers expressed by these cells in comparison to the conventional $\gamma\delta$ T cells. CD27 expression segregates $\gamma\delta$ T cell population into IFN- γ -producing (CD27⁺) and IL-17-producing (CD27⁻) $\gamma\delta$ T cells²⁶ and so represents a critical marker to define $\gamma\delta$ T cell functions. Strikingly, the CD3^{bright} cells uniformly lacked CD27 and NK1.1 expression, a phenotype of $\gamma\delta$ T17 cells (Figure 2a). A more extensive investigation of these cells determined that this population had an activated (CD69⁺) (Figure 2b) and effector memory phenotype (CD62L⁻ CD44^{high}) (Figure 2c) and was well equipped to produce T_H17-related cytokines (IL-1R1⁺ IL-23R⁺ IL-18R⁺) (Figure 2d). Interestingly, we also detected the expression of receptors for cytokines involved in leukocyte survival including IL-2 and IL-7, but not IL-15 (Figure 2e). It is noteworthy that CD127 (IL-7R) and CD25 (IL-2R) expression on CD3^{bright} $\gamma\delta$ T cells was higher compared to conventional $\gamma\delta$ T cells. CD3^{bright} $\gamma\delta$ T cells also expressed the orphan nuclear receptor ROR γ t, a key transcription factor for IL-17 production^{27, 28} (Figure 2f) and the common marker of $\gamma\delta$ T cell-mediated cytotoxicity, NKG2D (Figure 2g). Collectively, these data suggest that these cells have a phenotype of $\gamma\delta$ T17 cells.

CD3^{bright} $\gamma\delta$ T cells express the canonical germline encoded V γ 6/V δ 1 TCR

The nature of the TCR expressed by $\gamma\delta$ T cells has also been shown to correlate with their functions^{29, 30}. Here we have used the Heilig & Tonegawa nomenclature³¹ and Elliott's nomenclature³² to define the $V\gamma$ and $V\delta$ chains respectively. Thus, usage of a $V\gamma 1^+$ TCR is associated with a T_H1 -profile whereas $V\gamma 4^+$ and $V\gamma 6^+$ TCRs are mainly associated with a T_H17 phenotype¹. Given the limitation of commercially available monoclonal antibodies for specific γ and δ chain detection, we have used a single-cell RT-PCR multiplex system containing a set of primers for each gene (Table 1) encoding the different γ and δ chains (Figure 3a). To date, all the pulmonary $CD3^{\text{bright}}$ $\gamma\delta$ T cells were found to express a unique TCR δ rearrangement TRDV4-TRDD2-TRDJ2 encoding for a $V\delta 1$ -D $\delta 2$ -J $\delta 2$ TCR (Figure 3b). Of interest, the PCR products indicated that all $V\delta 1^+$ sequences included J $\delta 2$ and had a similar CDR3 δ amino acid sequence with no N diversity. The TCR γ repertoire also demonstrated a unique rearrangement TRGV6-TRGJ1 encoding for a $V\gamma 6$ -J $\gamma 1$ TCR (Figure 3b) with a similar CDR3 γ . By contrast, sequencing of the PCR products obtained with the conventional population indicated that this population expressed a more diverse TCR repertoire (Figure 3b). Consistent with this, we observed by flow cytometry that $CD3^{\text{bright}}$ $\gamma\delta$ T cells were negatively stained using anti- $V\gamma 1$, anti- $V\gamma 4$ or anti- $V\delta 6.3$ mAbs whereas conventional $\gamma\delta$ T cells did (Figure 3c). Together, these results suggest that pulmonary $CD3^{\text{bright}}$ $\gamma\delta$ T cells uniformly express the canonical fully germline encoded $V\gamma 6/V\delta 1$ TCR, a subset of $\gamma\delta$ T cells already associated with a T_H17 -like phenotype^{30, 33}.

$CD3^{\text{bright}}$ $\gamma\delta$ T cells are influenced by the endogenous flora and are ROR γ t-dependent.

According to their relative variable frequency between mice and their preferential distribution in mucosal sites, a place where immune cells are largely influenced by the microbiome, we have investigated the potential involvement of the commensal flora on the homeostasis of this

particular subset of $\gamma\delta$ T cells. In line with our hypothesis, C57BL/6 mice bred in germ-free (GF) conditions had a selective reduction in their CD3^{bright} $\gamma\delta$ T cell compartment in the lungs, thymus, spleen, and liver compared to mice bred in SPF conditions (Figure 4a). Moreover, mice chronically exposed to antibiotics (neomycin) also displayed a reduced frequency of CD3^{bright} $\gamma\delta$ T cells in the periphery (Supplementary Figure 2). By contrast, the frequency of conventional $\gamma\delta$ T cells was not modulated under these conditions. Interestingly the relationship to the microbiota seems to occur in a TLR-independent manner as MyD88^{-/-}TRIF^{-/-} mice show a normal CD3^{bright} $\gamma\delta$ T cell compartment (Supplementary Figure 2).

Expression of ROR γ t is mandatory in the development of T_H17 cells^{27, 28} but appears dispensable for the development and maintenance of $\gamma\delta$ T17 cells⁹. However, using ROR γ t-deficient mice, we determined that CD3^{bright} $\gamma\delta$ T cells were fully dependent on the expression of ROR γ t (Figure 4b). The level of CD3 surface expression on these cells suggests they might express an atypical CD3/TCR complex structure³⁴, an observation already made for some $\gamma\delta$ T cell subsets³⁵. This could be due to an alternate structure with a biased expression of particular CD3 components such as the substitution of a CD3 ζ subunit by an FcR γ ^{36, 37}. However, using FcR γ -deficient mice, we observed no modulation in the prevalence of CD3^{bright} $\gamma\delta$ T cells compared to control mice (Supplementary Figure 2).

CD3^{bright} $\gamma\delta$ T cell subset preferentially produces IL-17A in response to IL-23 and NLRP3 inflammasome-dependent IL-1 β

Having established that CD3^{bright} V γ 6/V δ 1⁺ T cell population displayed a phenotype of IL-17-producing cells, we next checked whether indeed these cells produced IL-17. To investigate this, we purified $\gamma\delta$ T cells from lungs according to their CD3 expression and stimulated them with

PMA/ionomycin. As depicted in Figure 5a, this resulted in extensive secretion of cytokines by $\gamma\delta$ T cell subsets including IFN- γ and IL-17A. Interestingly, the two subsets displayed a clear dichotomy in their cytokine profile. The conventional $\gamma\delta$ T cells showed a preferential T_H1-bias while the CD3^{bright} subset was only capable of secreting IL-17A. Of note, this was observed using both hepatic and splenic CD3^{bright} $\gamma\delta$ T cells (Supplementary Figure 3). We compared the ability of CD3^{bright} $\gamma\delta$ T cells to produce IL-17 to other $\gamma\delta$ T cell subpopulations reported to secrete high amounts of this cytokine. Recently, CD27 has been demonstrated to be a thymic determinant for $\gamma\delta$ T cell differentiation where the CD27⁻ subset was associated with a T_H17 profile²⁶. Therefore we investigated in our *in vitro* assay whether the CD3^{bright} $\gamma\delta$ T cell subset (CD27⁻ NK1.1⁻) had a distinct ability to secrete IL-17A compared to the CD27⁻ subset represented in the conventional population of $\gamma\delta$ T cells (Supplementary Figure 3). In line with the study from Ribot et al.²⁶, we observed that CD27⁺ $\gamma\delta$ T cells produced IFN- γ , but no IL-17A, whereas CD27⁻ $\gamma\delta$ T cells secreted IL-17A, but no IFN- γ . Importantly however, comparison of conventional CD27⁻ $\gamma\delta$ T cells vs CD3^{bright} $\gamma\delta$ T cells indicated that the latter were capable of producing a lot more IL-17A (Supplementary Figure 3). We also performed a similar analysis to compare CD3^{bright} $\gamma\delta$ T cells and V γ 4⁺ $\gamma\delta$ T cells, another population strongly associated with IL-17 production³⁰. Again, CD3^{bright} $\gamma\delta$ T cells produced more IL-17A than V γ 4⁺ $\gamma\delta$ T cells in this setting (Supplementary Figure 3). Furthermore, despite a clear ability to produce IL-17, V γ 4⁺ $\gamma\delta$ T cells were also able to secrete a substantial amount of IFN- γ . Similarly, activation of CD3^{bright} $\gamma\delta$ T cells by means of anti-CD3 stimulation also resulted in IL-17A production, but not IFN- γ (Figure 5b). Together, these data demonstrate that the CD3^{bright} $\gamma\delta$ T cells are intrinsically

skewed towards an IL-17A-producing cell profile and have a higher ability to produce this cytokine compared to other $\gamma\delta$ T cells.

We have recently demonstrated that α -GalCer injection resulted in a rapid production of IL-17A by $\gamma\delta$ T cells²⁵. According to the hallmarks of CD3^{bright} $\gamma\delta$ T cells, this population represented an interesting candidate to be a preferential IL-17-producing $\gamma\delta$ T cell subset in response to α -GalCer. In line with our hypothesis, we observed that hepatic CD3^{bright} subsets of $\gamma\delta$ T cells rapidly produced IL-17A after α -GalCer administration (Figure 5c). In stark contrast, only few conventional $\gamma\delta$ T cells (despite a significant proportion of CD27⁻ $\gamma\delta$ T cells) produced IL-17A (Figure 5c). Interestingly, we found that a large proportion of CD3^{bright} $\gamma\delta$ T cells (~ 50%) produced IL-17A only 2 hrs post- α -GalCer injection and partially sustained this secretion for another 10 hrs. This subset did not secrete IFN- γ at any time studied, but consistent with our recent report²⁵, non-CD3^{bright} $\gamma\delta$ T cells were capable of secreting a substantial amount of IFN- γ (Figure 5c). We next investigated whether the CD3^{bright} $\gamma\delta$ T cells were also capable of producing IL-17A in an immune response to LPS. As expected, $\gamma\delta$ T cells were an important source of IL-17A³⁸, but only ~11% of conventional $\gamma\delta$ T cells produced IL-17A in response to LPS, whereas 83% CD3^{bright} $\gamma\delta$ T cells secreted this cytokine (Figure 5d).

Recent studies have suggested that IL-1 β and IL-23 drive IL-17A secretion by $\gamma\delta$ T cells in the absence of TCR ligation^{39, 40}. To test this hypothesis in the context of CD3^{bright} $\gamma\delta$ T cells, we have used IL-1R1^{-/-} mice or WT mice pre-treated with an anti-IL-23p19 mAb. Interestingly, we observed that IL-17A production by hepatic CD3^{bright} $\gamma\delta$ T cells was significantly impaired in both settings following α -GalCer treatment (Figure 5e). As expected, the employment of gene-targeted mice for NLRP3-inflammasome components (NLRP3 and caspase-1) indicated that IL-

1 β -dependent IL-17A production was dependent on the NLRP3/caspase-1 activation cascade in this setting (Supplementary Figure 4). Experiments conducted with LPS indicated a similar mechanism of activation (Figure 5f). To further confirm these findings, we checked whether these cytokines were sufficient to induce IL-17A production by highly purified $\gamma\delta$ T cells. In agreement with our recent report and others^{25, 39, 40}, combination of IL-12 and IL-18 led to IFN- γ production by conventional $\gamma\delta$ T cells whereas IL-23 plus IL-1 β resulted in IL-17A secretion (Supplementary Figure 4). The same combinations incubated with CD3^{bright} $\gamma\delta$ T cells also resulted in the secretion of IL-17A (IL-23 + IL-1 β), but not IFN- γ (IL-12 + IL-18) (Supplementary Figure 4). Notably, at the doses tested, individual recombinant cytokines failed to promote cytokine production. Together these data indicate that IL-23 and IL-1 β are important for IL-17A production by CD3^{bright} $\gamma\delta$ T cells in response to α -GalCer and suggest that these cytokines are sufficient to induce IL-17A production by purified CD3^{bright} $\gamma\delta$ T cells.

CD3^{bright} $\gamma\delta$ T cells display a feature of specialized cells in skin and pulmonary immunity.

With respect to the preferential localization in skin and lungs and to the ability of CD3^{bright} $\gamma\delta$ T cells to rapidly produce large amount of IL-17A, we next investigated their potential biological activity in both organs. Firstly, dermal IL-17-producing $\gamma\delta$ T cells have been shown to play a detrimental role in skin inflammation using an IL-23-induced psoriasis-like model⁴¹. Using another mouse model of psoriasis⁴², we have observed that topical application of imiquimod (IMQ) led to the development of psoriasiform manifestations (Supplementary Figure 5). Interestingly, we demonstrated that IMQ treatment led to a significant increase frequency of CD3^{bright}, but not conventional, $\gamma\delta$ T cells in the immune compartment of the inflamed skin (Figure 6a). Of note, despite sharing an identical V δ 1 chain with V γ 6/V δ 1⁺ T cells³³, dendritic

epidermal T cells ($V\gamma 5/V\delta 1^+$) did not display a $CD3^{\text{bright}}$ phenotype and were included in the conventional population (Supplementary Figure 5). Moreover, the percentage of $CD3^{\text{bright}}$, but not conventional, $\gamma\delta$ T cells in the inflamed skin of mice positively correlated with the epidermal thickness (Figure 6b). Finally, in line with our hypothesis, $CD3^{\text{bright}}$ $\gamma\delta$ T cells from psoriasitic lesions produced IL-17A upon restimulation (Figure 6c), while conventional $\gamma\delta$ T cells failed to do so (Figure 6c). This indicates that, in our setting, $V\gamma 5/V\delta 1^+$ T cells were not able to produce IL-17 (Figure 6c) unlike recent reports in other models of skin inflammation^{43, 44}. Furthermore, the frequency of IL-17-producing $CD3^{\text{bright}}$ $\gamma\delta$ T cells within $CD45^+$ cells positively correlated with epidermal thickness (Spearman's test: $r = 0.639$, $p = 0.016$) whilst the same analysis for $CD3^{\text{dim}}$ $\gamma\delta$ T cells was not significant (Spearman's test: $r = -0.024$, $p = 0.939$). This supports our hypothesis that the $CD3^{\text{bright}}$ $\gamma\delta$ T cells are responsible for the pathology of skin inflammation in this setting.

In a different model, intranasal injection of α -GalCer, the prototypical type I NKT cell activator induces airway neutrophilia in an IL-17-dependent mechanism^{45, 46, 47} and its activity as a mucosal adjuvant is now widely accepted as an inducer of protective immunity against pathogens^{45, 48, 49}. Intranasal instillation of α -GalCer resulted in IL-17A and IFN- γ production by $\gamma\delta$ T and type I NKT cells (Figure 7a and Supplementary Figure 6). Notably however, the $CD3^{\text{bright}}$ $\gamma\delta$ T cells were prominent producers of IL-17A on a per cell basis compared to conventional $\gamma\delta$ T cells and type I NKT cells (Figure 7a). As expected, this was accompanied by airway neutrophilia 8 hrs after α -GalCer instillation (Figure 7b); an observation largely dependent on $\gamma\delta$ T cells (Figure 7b) suggesting that the $CD3^{\text{bright}}$ $\gamma\delta$ T cells might participate in this effect. As observed in the liver after systemic administration of α -GalCer, the pool of

CD3^{bright} $\gamma\delta$ T cells in lungs expanded by ~ 10 fold within 24 hrs of i.n. administration of α -GalCer (Figure 7c). Using a relevant model of respiratory bacterial infection, we observed that CD3^{bright} $\gamma\delta$ T cells were early and major sources of IL-17 following intranasal *Streptococcus pneumoniae* infection (Figure 7d). As expected, unlike the conventional subset, CD3^{bright} $\gamma\delta$ T cells failed to secrete IFN- γ (Supplementary Figure 6). Recently expression of CCR6 has been correlated with $\gamma\delta$ T17 cells⁵⁰. Although IL-17 production was restricted to the CCR6⁺ subset in conventional $\gamma\delta$ T cells, CD3^{bright} $\gamma\delta$ T cells could produce IL-17 independently from CCR6 expression (Supplementary Figure 7). Moreover, similar to the α -GalCer model, the frequency and number of these cells selectively increased during the early course of infection (Figure 7e). Altogether, these data indicate that CD3^{bright} $\gamma\delta$ T cells may play a critical role in skin inflammation and mucosal immunity against respiratory pathogens.

Discussion

The detection and separation of immune cells with specialized functions is important as this offers more refined targets for immunotherapeutic purposes. Here, we have reported a subset of $\gamma\delta$ T cells with a heightened ability to produce IL-17A based on their high expression of CD3. CD3^{bright} $\gamma\delta$ T cells are a homogenous population predominantly represented in lungs and skin that display a phenotype of effector memory IL-17-producing cells and in different mouse backgrounds (C57BL/6 and BALB/c). Pulmonary CD3^{bright} $\gamma\delta$ T cells use a canonical germline encoded V γ 6/V δ 1 TCR. All previous reports on V γ 6/V δ 1 $\gamma\delta$ T cells have used PCR-based strategies or a monoclonal Ab recognizing all V δ 1⁺ $\gamma\delta$ T cells (including V γ 5/V δ 1 $\gamma\delta$ T cells) but not commercially available^{34, 51}. Here we describe a simple gating strategy to directly and specifically analyze V γ 6/V δ 1 $\gamma\delta$ T cells using level of CD3 surface expression. This new strategy allowed us to determine and refine several features about their phenotype and factors regulating their development/homeostasis.

Previous reports indicated that V γ 6/V δ 1 T cells are predominantly represented in mucosal sites including female genital tract and tongue³³. Consistent with the possibility that our strategy allows for a specific gating of V γ 6/V δ 1⁺ T cells, we observed that CD3^{bright} $\gamma\delta$ T cells were virtually undetectable in the gut and associated lymphoid structures under steady-state conditions as recently proposed⁵². The reason for this discrepancy with other mucosal surfaces is currently unknown but might depend on a difference in microbial flora richness and diversity in these different tissues.

Of interest, breeding mice in a germ-free environment profoundly affected the CD3^{bright} $\gamma\delta$ T cell compartment in the periphery indicating that this subset is tightly regulated by commensal bacteria. Interestingly, this phenotype is recapitulated after prolonged exposure of SPF adult mice

to antibiotics suggesting that the microbiome plays a role on expansion/maintenance of these cells rather than their development. This finding is in concert with a recent study demonstrating that some specific commensals are required to maintain IL-1R1⁺ $\gamma\delta$ T cells through a signaling pathway involving the proto-oncogene Vav1⁵³. Given the potential role of this population in antimicrobial immunity^{9, 21, 51}, this observation is in line with the consensus that chronic exposure to antibiotics results in transient immunodeficiency and increased susceptibility to (super)-infections⁵⁴.

How microbiota impacts on CD3^{bright} $\gamma\delta$ T cells remains unclear, although these organisms appear to promote IL-7 production under homeostatic conditions⁵⁵, a factor important to maintain ROR γ t expression in innate lymphocytes⁵⁶. Interestingly, we have demonstrated that CD3^{bright} $\gamma\delta$ T cells are completely dependent on ROR γ t expression for their development and maintenance in the periphery. Moreover, these cells have a high expression of IL-7R compared to other leukocytes and, in line with a recent report⁵⁷, this population promptly responds to IL-7, which may be a selective growth factor for $\gamma\delta$ T17 cells⁵⁷. Since Notch1 signaling triggers CD127 expression and IL-7 signaling^{58, 59}, the intense expression of this receptor may result from the involvement of Notch1 during the development of $\gamma\delta$ T17 cells⁹ and therefore, based on our findings, CD3^{bright} $\gamma\delta$ T cells. However in the same study, the authors demonstrated that ROR γ t expression was dispensable for the development of $\gamma\delta$ T17 cells. The reason for this discrepancy is likely to be due to the fact that we focused on the CD3^{bright} $\gamma\delta$ T subset while the previous report analyzed the entire pool of $\gamma\delta$ T17 cells that may contain both ROR γ t-dependent (innate pre-programmed V γ 6⁺ cells) and -independent (acquired V γ 4⁺ cells) $\gamma\delta$ T cells. Consistent with this idea, $\gamma\delta$ T17 cells (CD27) from the conventional population were only minimally affected by the absence of ROR γ t expression. Our data indicate that the interplay between endogenous flora and CD3^{bright} $\gamma\delta$ T cells

is independent from TLR signaling. Therefore it is possible that others pattern recognition receptors are involved in this phenomenon.

Their level of CD3 signals is also intriguing and might simply be explained by a differential accessibility to the epitope by the anti-CD3 mAb. However the use of 2C11 and 17A2 clones, two clones that recognize CD3 ϵ and CD3 $\gamma\epsilon$ dimers respectively allow a similar separation of the two populations based on CD3 signal intensity. Thus this phenotype could also be explained by other hypotheses including the fact that CD3^{bright} $\gamma\delta$ T cells may have a unique CD3/TCR complex structure. Consistent with this, a previous report had demonstrated that the 17D1 mAb, which primarily detects V γ 5/V δ 1 TCR, can cross-react with V γ 6/V δ 1 TCR if the structure is first stabilized with an anti-C δ mAb suggesting an alternate structure for this CD3/TCR complex³⁴.

We observed that dendritic epidermal (V γ 5/V δ 1⁺) T cells have a similar CD3/TCR surface ratio compared to conventional $\gamma\delta$ T cells. Thus, the differential CD3 expression between the V γ 6/V δ 1 TCR and the other $\gamma\delta$ TCRs could explain this observation. Further biochemical analysis will help to examine the precise structure of the CD3 complex and investigate if this feature could partially explain their particular biology. Nevertheless, an alternate structure involving FcR γ ^{36, 37} cannot be considered as CD3^{bright} $\gamma\delta$ T cells from mice deficient for this signaling chain have a normal distribution pattern. Furthermore, the canonical germline encoded V γ 6/V δ 1 TCR expressed with a high level of CD3 suggests that this TCR does not engage any recombination processes during ontogeny. This observation is important regarding the current controversial hypotheses concerning thymic development of $\gamma\delta$ T17 cells. While studies suggest that $\gamma\delta$ T17 arise from thymus during embryonic life and persist in adults through self-renewal process^{9, 19, 26}, the requirement of TCR ligation in their development is still debated^{22, 24, 33}. A recent study elegantly demonstrated that $\gamma\delta$ T17 can produce IL-17 in the embryonic thymus prior to TCR

rearrangement, suggesting that these cells may be selected in a TCR-independent manner¹⁹. By contrast, a previous report suggested that $\gamma\delta$ T17 selection might require a weak TCR engagement²⁴. Overall, our data mainly support the first hypothesis and emphasize the innate imprint of the CD3^{bright} $\gamma\delta$ T cells.

There remains a critical question about the actual function of the TCRs expressed by $\gamma\delta$ T17 cells, especially on V γ 6/V δ 1 T cells⁶⁰. It seems clear that TCR engagement by anti-CD3 antibody resulted in cytokine release, indicating that this TCR is functional. While soluble factors like IL-1 β and IL-23 appear sufficient to induce IL-17A production by highly purified V γ 6/V δ 1 T cells, we cannot exclude the possibility that the anti-TCR δ and anti-CD3 mAbs used to sort these cells may contribute to their cytokine production via TCR engagement-mediated activation. It is also possible that their TCR engages antigens expressed by the same adjacent cells in the culture. Interestingly, using the same number of cells, CD3^{bright} $\gamma\delta$ T cells were more sensitive to CD3 triggering compared to conventional $\gamma\delta$ and $\alpha\beta$ T cells. While the higher level of CD3 expressed by this subset is the simplest explanation for this hyper-reactivity, it may also reflect their memory phenotype that may render them more sensitive to the TCR signaling pathway⁶¹. However, further studies will be required to understand whether this TCR should be considered as a conventional TCR that recognize Ag/Ag-presenting molecule complex or more as a pattern recognition receptor, a homing receptor, or a vestigial structure that may be simply required for the development/expansion of these cells.

Using various activation strategies (TCR-dependent and -independent), we consistently observed that CD3^{bright} $\gamma\delta$ T cells could only produce T_H17-related cytokines, reinforcing the concept that these cells are fully differentiated with a pre-programmed capacity to produce this cytokine. This specialized function suggests a selection pressure to maintain this very population of innate cells

throughout evolution and is in line with their memory phenotype. To the best of our knowledge, it is the first time that an entire population of cells carrying a canonical TCR produces such a conserved spectrum of cytokines to various stimuli. This pool of cells can either be rapidly expanded and/or recruited at sites of inflammation, which could compensate for their low number at steady-state. The cytotoxic capacity of these cells has not been directly investigated in our study, but it is noteworthy that CD3^{bright} $\gamma\delta$ T cells displayed a high expression of NKG2D at steady-state, which might imply potential capacity to mediate cytotoxicity on transformed or virus-infected cells.

Interestingly, we have observed in human a population displaying a similar bright expression of CD3 and phenotypically associated with a T_H17-like cell signature (not shown). However further studies are required to understand whether these populations can be somehow related. Such a classification across species would represent a significant step in understanding the functional dichotomy of $\gamma\delta$ T cell subsets. Of note, a subset of human $\gamma\delta$ T cells showing a bright expression of CD3 has already been demonstrated in patients with tuberculous pleurisy⁶². In line with our hypothesis, these cells were predominantly V δ 2⁺⁶², a subset of human $\gamma\delta$ T cells with high capacity in IL-17 production⁵⁷.

The presence of V γ 6/V δ 1 T cells in various organs has been demonstrated during early life³⁴. Thus it is possible that V γ 6/V δ 1 T cells play a major role during early life, at a time the adaptive immune system remains not fully educated. In any case, the clonality of this population at this stage of life has never been studied and the preferential persistence of the CD3^{bright} $\gamma\delta$ T cells in particular niches in adults might be due to the presence of a favorable local environment (e.g. self-Ag, cytokines, and particular homing receptors) for these cells.

Nonetheless, the selection pressure which conserved this population in different backgrounds may also imply specialized functions in some organs. In line with this, the rapid response of CD3^{bright} $\gamma\delta$ T cells, in various pathological situations (eg. *S. pneumoniae* infection and psoriasis-like model), is astounding and indicates that this population is likely to be involved in immune regulation especially at the interface with the external environment. Accordingly, we have highlighted a new simple strategy to detect V γ 6/V δ 1⁺ T cells with potent ability to produce IL-17 and with putative key functions in a broad range of IL-17-driven pathologies.

Methods

Mice

Male C57BL/6J wild-type mice were purchased from the Walter and Eliza Hall Institute for Medical Research (WEHI). C57BL/6 TCR δ -deficient (TCR δ ^{-/-}) mice, C57BL/6 IL-1R1-deficient (IL-1R1^{-/-}), C57BL/6 NLRP3-deficient (NLRP3^{-/-}) mice, C57BL/6 caspase-1-deficient (ICE^{-/-}) mice, and C57BL/6 IL-23R^{+/-}-GFP knock-in (IL-23R-GFP reporter) mice (kindly provided by Vijay Kuchroo, Harvard Medical School, Boston, USA) were bred in house at the Peter MacCallum Cancer Centre (Peter Mac). C57BL/6 ROR γ t^{+/-}-GFP knock-in mice, ROR γ t^{-/-}-GFP knock in (ROR γ t-GFP reporter) mice, Germ-free mice and SPF controls were obtained from Walter and Eliza Hall Institute (WEHI). C57BL/6 MyD88/TRIF-deficient (MyD88^{-/-}TRIF^{-/-}) mice (kindly provided by Dr. Geoff Hill, QIMR Berghofer) and C57BL/6 Fc ϵ RI γ -deficient (Fc ϵ RI γ ^{-/-}) mice (kindly provided by Dr. Mark Hogarth) were bred in house at the QIMR Berghofer Medical Research Institute (QIMR Berghofer). All mice were backcrossed to C57BL/6J at least ten times. Sex-matched mice were used at the ages of 8–10 weeks. All experiments were performed in accordance with the animal ethics guidelines ascribed by the National Health and Medical Research Council of Australia. All experiments were approved by the Peter Mac and QIMR Berghofer Animal Ethics Committees.

Reagents and Abs

α -galactosylceramide (α -GalCer) was from Axxora Life Sciences (San Diego, CA, USA). Lipopolysaccharide (LPS) from E. coli O111:B4 was from Sigma-Aldrich (St. Louis, MO USA). Monoclonal Abs against mouse CD3 (17A2 and 2C11: PB- and AF647-conjugated respectively), NK1.1 (PK136: PECy7- or PE-conjugated), anti-C δ (GL3: APC-, PERCP-Cy5.5- or FITC-

conjugated), CD69 (FN50: FITC-conjugated), IFN- γ (XMG1.2: PE-conjugated), IL-17A (TC11-18H10: PE-conjugated), CD27 (LG.7F9: PECy7-conjugated), TCR β (H57-597: PE-conjugated), V γ 1 (2.11: FITC-conjugated), V γ 4 (UC3-10A6: PECy7-conjugated), V δ 6.3/2 (8F4H7B7: PE-conjugated), CD44 (IM7: FITC-conjugated), CD62L (MEL-14: PE-conjugated), CD127 (A7R34: FITC-conjugated), CD122 (TM- β 1: FITC-conjugated), NKG2D (C7: APC-conjugated), IL-18R α (112614: APC-conjugated), CD121a (35F5: PE-conjugated), CCR6 (29-2L17: AF647-conjugated) and isotype controls were purchased from BD Biosciences (San Diego, CA, USA), BioLegend (San Diego, CA, USA), R&D system (Minneapolis, MN USA), Immunostep (Salamanca, Spain) or eBiosciences (San Diego, CA, USA). Recombinant mouse IL-23, IL-1 β , IL-12p70 and IL-18 were from R&D Systems. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma-Aldrich.

Infection with S. pneumoniae

S. pneumoniae serotype 1 clinical isolate E1586 sequence type ST304 has been described elsewhere⁴⁵. Mice were anesthetized and administered i.n. with 2×10^6 live bacteria.

IMQ-induced psoriasis-like model

IMQ-induced psoriasis-like model was described previously⁴². C57BL/6 wild-type mice were treated with a daily topical dose (62.5 mg) of commercially available IMQ cream (Aldara 5%, Meda AB) or control “Lanette” cream (Fagron, ref. 1289-511) on the shaved abdominal skin for 5 days. PASI score (Psoriasis Area and Severity Index, based on the intensity of “redness”, “thickness” and “scaling” clinical parameters) was estimated at the 1st to 5th days during treatment with IMQ cream or control “Lanette” cream. Mice were sacrificed 5 days after the first

application of the cream and the skin samples were embedded in the ImmunoHistoWax for Giemsa staining or used for cell isolation followed by flow cytometry analysis. Images were acquired by Eclipse Ti Nikon microscope system.

Preparation of immune cells from various organs

Splenic, pulmonary and hepatic mononuclear cells from control, LPS- or α -GalCer-treated mice were prepared as previously described⁶³. Briefly, lungs and livers were perfused with PBS, excised and finely minced, followed by enzymatic digestion for 30 minutes at 37°C in PBS containing 1 mg/ml collagenase type IV and 1 μ g/ml DNase type I (Roche). After washing, homogenates were resuspended in a 35% Percoll™ gradient, carefully layered onto 70% Percoll™ and centrifuged at 2300 (liver) or 2600 (lungs) rpm without brake at 22°C for 30 min. The layer at the interface between the two Percoll™ concentrations was carefully aspirated and washed in PBS 2% FCS. Red blood cells were removed with Ammonium-Chloride-Potassium (ACK) lysis buffer.

LPLs and IELs were isolated as previously described⁶⁴. Briefly, small intestines and colons were excised, washed and the Peyer's patches removed. Tissues were sectioned and incubated in PBS with 5 mM EDTA, 1 mM DTT, 1% FBS and penicillin/streptomycin for removal of IELs. For LPL isolation, stripped tissues were digested twice in RPMI with 2 mg/mL collagenase D (Roche) and 20 μ g/mL DNase I (Sigma) and then mashed through a cell strainer.

Mouse abdominal skin was digested in RPMI containing 0.05% collagenase D (Roche) and 0.05% DNase I (Sigma) at 37°C for 30 min, passed through 18-G needle followed by centrifugation at 400 g for 10 min. Cell pellets were washed 2 times, suspended in cold PBS and filtered through a sieve (70 μ m).

Flow cytometry and cytospin

Mice were injected with PBS, α -GalCer (2 μ g/mouse i.p. or 500 ng/mouse intranasal (i.n.) route) or *Escherichia coli* LPS (0.5 mg/mouse). Livers, lungs and spleens were harvested at different time points and mononuclear cells (MNCs) were prepared as described above. Then, Golgi Plug/Golgi Stop (BD Biosciences) was added for 2 hrs. For psoriasis-like model, cells were isolated as described above and were stimulated with PMA (100 ng/ml) and ionomycin (1 μ g/ml) for 4 h in the presence of Golgi Plug/Golgi Stop (BD Biosciences). Then cell suspensions were blocked in the presence of 2.4G2 prior to staining with appropriate dilutions of APC-conjugated TCR δ , PB-labeled anti-CD3 and PeCy7-conjugated NK1.1 for 30 min in PBS containing 2% FCS and 0.01% NaN₃. Cells were then fixed and permeabilized using the BD Cytotfix/Cytoperm™. Fixation/Permeabilization Kit and incubated with PE-conjugated mAb against IFN- γ , IL-17A or matching isotype control mAbs in permeabilization buffer. Cells were acquired and analyzed on either Canto2 or LSR-II cytometer (BD Biosciences CA). FACS analysis was performed with FlowJo (Treestar, OR USA). A morphology-based differential cell count was conducted on cytospin preparations from the bronchoalveolar lavage (BAL) fluid samples and stained with Giemsa solution.

Detection of cytokines

Cytokines were detected using the BD Cytometric Bead Array (CBA) system (BD Bioscience CA). Acquisition was performed on an LSR-II (BD Bioscience). A total of 300 bead events for each cytokine were collected. Analysis was performed using FCAP array software (BD).

Isolation of $\gamma\delta$ T subsets and in vitro assays

Livers, lungs and spleens were harvested from naive mice and cell suspensions were prepared as previously described⁶³. Red blood cells were lysed with ACK lysis buffer prior to $\gamma\delta$ T cell enrichment with autoMACS (depletion of TCR β^+ , CD19 $^+$ and F4/80 $^+$ cells). Then, $\gamma\delta$ T-enriched cells were sorted using an ARIA2 and purity was always greater than 97%. Of note, the pre-enrichment step does not modulate the ratio between conventional $\gamma\delta$ T cells and CD3^{bright} $\gamma\delta$ T cells. For the stimulation assay, purified cells were cultured for the indicated amount of time in complete media containing a combination of recombinant mouse IL-12p70 (50 pg/ml), IL-23 (1 ng/ml), IL-1 β (1 ng/ml), IL-18 (1 ng/ml), PMA (10 ng/ml) and ionomycin (1 μ g/ml). In some case purified cells have been stimulated with various concentrations of anti-CD3 ϵ (clone 145-2C11) (BD Pharmingen) pre-coated on the culture plates.

Single-cell PCR

For single-cell PCR, cDNA from sorted $\gamma\delta$ T cell subsets was generated as previously described¹¹ and then was amplified by two rounds of semi-nested PCR with sense primers for C γ , C δ , V γ and V δ regions each used with the C γ and/or C δ antisense primers (Table 1). PCR products were separated by electrophoresis through a 1.5% agarose gel and were sequenced as described⁶⁵. TCR nomenclature is in accordance with the International ImMunoGeneTics database⁶⁶.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software (Graphpad Software Inc., San Diego, USA). The statistical significance between experimental groups was calculated using

a Mann-Whitney test for comparison of two groups or, for comparison of three or more groups, a Kruskal-Wallis test followed by a Dunn's post-test. Results with a p value of less than 0.05 were considered significant. Correlation calculation between two parameters has been performed using the Spearman's rho test.

Acknowledgments

We thank Josette Fontaine, Shin-Foong Ngiow, Deborah Knight, Sébastien Fleury, Julien Wartelle and Kim Steegh for technical assistance. We thank Qerime Mundrea, Ben Venville, Jessica May, Joanne Sutton and Liam Town for maintaining and caring for the mice. We also thank the Peter MacCallum flow cytometry core facility for technical assistance. This work was supported by an NHMRC Program Grant (454569 and 1013367). CP was supported by a postdoctoral fellowship from the US Department of Defense (W81XWH-11-1-0585) and INSERM. MJS and FSFG were supported by a National Health and Medical Research Council of Australia (NHMRC) Australia Fellowship and Program Grant (1013367). MTC was supported by a Cancer Research Institute PhD scholarship. GTB was supported by an ARC Future Fellowship. FT was supported by CNRS. PN was supported by a National Health and Medical Research Council of Australia (NHMRC) Program Grant (1013367). DIG was supported by an NHMRC Senior Principal Research Fellowship (1020770). NAG was supported by a Leukaemia Foundation of Australia postgraduate scholarship. TM is supported by research grants from the Canadian Institutes of Health Research and the Crohn and Colitis Foundation of Canada, as well as a Tier 2 Canada Research Chair. NE is a recipient of a Vanier Canada Graduate Scholarship. DAM, DS and DD were supported by the Agence Nationale de la Recherche (ANR-10-LABX-46) and the “Fondation de France”.

Supplementary information is available at Immunology and Cell Biology’s website

References

1. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* , **10**(7): 467-478.
2. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* , **10**(7): 479-489.
3. Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. *Cytokine Growth Factor Rev*, **21**(6): 443-448.
4. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*, **21**(4): 467-476.
5. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, *et al.* IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest*, **120**(5): 1762-1773.
6. Nembrini C, Marsland BJ, Kopf M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol*, **123**(5): 986-994; quiz 995-986.
7. Dejima T, Shibata K, Yamada H, Hara H, Iwakura Y, Naito S, *et al.* Protective role of naturally occurring interleukin-17A-producing gammadelta T cells in the lung at the early stage of systemic candidiasis in mice. *Infect Immun*, **79**(11): 4503-4510.
8. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J Immunol*, **177**(7): 4662-4669.
9. 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. *J Immunol*, **178**(7): 4466-4472.

10. Sutton CE, Mielke LA, Mills KH. IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur J Immunol*, **42**(9): 2221-2231.
11. Murugaiyan G, Saha B. Protumor vs antitumor functions of IL-17. *J Immunol*, **183**(7): 4169-4175.
12. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, *et al.* Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J Exp Med*, **208**(3): 491-503.
13. Deknuydt F, Scotet E, Bonneville M. Modulation of inflammation through IL-17 production by gammadelta T cells: mandatory in the mouse, dispensable in humans? *Immunol Lett*, **127**(1): 8-12.
14. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, *et al.* Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood*, **118**(1): 129-138.
15. Fenoglio D, Poggi A, Catellani S, Battaglia F, Ferrera A, Setti M, *et al.* Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to *Candida albicans*. *Blood*, **113**(26): 6611-6618.
16. Kenna TJ, Davidson SI, Duan R, Bradbury LA, McFarlane J, Smith M, *et al.* Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive gamma/delta T cells in patients with active ankylosing spondylitis. *Arthritis Rheum*, **64**(5): 1420-1429.
17. Peng MY, Wang ZH, Yao CY, Jiang LN, Jin QL, Wang J, *et al.* Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol*, **5**(3): 203-208.
18. Korn T, Petermann F. Development and function of interleukin 17-producing gammadelta T cells. *Ann N Y Acad Sci*, **1247**: 34-45.

19. 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*, **37**(1): 48-59.
20. 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*, **31**(2): 321-330.
21. Simonian PL, Roark CL, Wehrmann F, Lanham AM, Born WK, O'Brien RL, *et al.* IL-17A-expressing T cells are essential for bacterial clearance in a murine model of hypersensitivity pneumonitis. *J Immunol*, **182**(10): 6540-6549.
22. Jensen KD, Shin S, Chien YH. Cutting edge: Gammadelta intraepithelial lymphocytes of the small intestine are not biased toward thymic antigens. *J Immunol*, **182**(12): 7348-7351.
23. 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*, **118**(3): 586-593.
24. Turchinovich G, Hayday AC. Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells. *Immunity*, **35**(1): 59-68.
25. Paget C, Chow MT, Duret H, Mattarollo SR, Smyth MJ. Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *J Immunol*, **188**(8): 3928-3939.
26. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, *et al.* CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol*, **10**(4): 427-436.

27. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, *et al.* The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell*, **126**(6): 1121-1133.
28. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor ROR γ . *Nat Immunol*, **9**(6): 641-649.
29. Born WK, Yin Z, Hahn YS, Sun D, O'Brien RL. Analysis of gamma delta T cell functions in the mouse. *J Immunol*, **184**(8): 4055-4061.
30. O'Brien RL, Born WK. gammadelta T cell subsets: a link between TCR and function? *Semin Immunol*, **22**(4): 193-198.
31. Heilig JS, Tonegawa S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature*, **322**(6082): 836-840.
32. Elliott JF, Rock EP, Patten PA, Davis MM, Chien YH. The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature*, **331**(6157): 627-631.
33. 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*, **343**(6260): 754-757.
34. Roark CL, Aydintug MK, Lewis J, Yin X, Lahn M, Hahn YS, *et al.* Subset-specific, uniform activation among V gamma 6/V delta 1⁺ gamma delta T cells elicited by inflammation. *J Leukoc Biol*, **75**(1): 68-75.
35. Thibault G, Bardos P. Compared TCR and CD3 epsilon expression on alpha beta and gamma delta T cells. Evidence for the association of two TCR heterodimers with three CD3 epsilon chains in the TCR/CD3 complex. *J Immunol*, **154**(8): 3814-3820.

36. Koyasu S, D'Adamio L, Arulanandam AR, Abraham S, Clayton LK, Reinherz EL. T cell receptor complexes containing Fc epsilon RI gamma homodimers in lieu of CD3 zeta and CD3 eta components: a novel isoform expressed on large granular lymphocytes. *J Exp Med*, **175**(1): 203-209.
37. Liu CP, Ueda R, She J, Sancho J, Wang B, Weddell G, *et al.* Abnormal T cell development in CD3-zeta^{-/-} mutant mice and identification of a novel T cell population in the intestine. *The EMBO journal*, **12**(12): 4863-4875.
38. Andrews DM, Chow MT, Ma Y, Cotterell CL, Watt SV, Anthony DA, *et al.* Homeostatic defects in interleukin 18-deficient mice contribute to protection against the lethal effects of endotoxin. *Immunol Cell Biol*, **89**(6): 739-746.
39. Lalor SJ, Dungan LS, Sutton CE, Basdeo SA, Fletcher JM, Mills KH. Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J Immunol*, **186**(10): 5738-5748.
40. 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.
41. Cai Y, Shen X, Ding C, Qi C, Li K, Li X, *et al.* Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity*, **35**(4): 596-610.
42. van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, *et al.* Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol*, **182**(9): 5836-5845.
43. MacLeod AS, Hemmers S, Garijo O, Chabod M, Mowen K, Witherden DA, *et al.* Dendritic epidermal T cells regulate skin antimicrobial barrier function. *J Clin Invest*, **123**(10): 4364-4374.

44. Nielsen MM, Lovato P, Macleod AS, Witherden DA, Skov L, Dyring-Andersen B, *et al.* IL-1beta-Dependent Activation of Dendritic Epidermal T Cells in Contact Hypersensitivity. *J Immunol*.
45. Ivanov S, Fontaine J, Paget C, Macho Fernandez E, Van Maele L, Renneson J, *et al.* Key role for respiratory CD103(+) dendritic cells, IFN-gamma, and IL-17 in protection against *Streptococcus pneumoniae* infection in response to alpha-galactosylceramide. *J Infect Dis*, **206**(5): 723-734.
46. Lee KA, Kang MH, Lee YS, Kim YJ, Kim DH, Ko HJ, *et al.* A distinct subset of natural killer T cells produces IL-17, contributing to airway infiltration of neutrophils but not to airway hyperreactivity. *Cell Immunol*, **251**(1): 50-55.
47. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, *et al.* Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med*, **204**(5): 995-1001.
48. Lindqvist M, Persson J, Thorn K, Harandi AM. The mucosal adjuvant effect of alpha-galactosylceramide for induction of protective immunity to sexually transmitted viral infection. *J Immunol*, **182**(10): 6435-6443.
49. Noda K, Kodama S, Umemoto S, Abe N, Hirano T, Suzuki M. Nasal vaccination with P6 outer membrane protein and alpha-galactosylceramide induces nontypeable *Haemophilus influenzae*-specific protective immunity associated with NKT cell activation and dendritic cell expansion in nasopharynx. *Vaccine*, **28**(31): 5068-5074.
50. 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. *Eur J Immunol*, **39**(12): 3488-3497.

51. Hamada S, Umemura M, Shiono T, Hara H, Kishihara K, Tanaka K, *et al.* Importance of murine Vdelta1gammadelta T cells expressing interferon-gamma and interleukin-17A in innate protection against *Listeria monocytogenes* infection. *Immunology*, **125**(2): 170-177.
52. Sheridan BS, Romagnoli PA, Pham QM, Fu HH, Alonzo F, 3rd, Schubert WD, *et al.* gammadelta T cells exhibit multifunctional and protective memory in intestinal tissues. *Immunity*, **39**(1): 184-195.
53. 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*, **7**(2): 140-150.
54. Ubeda C, Pamer EG. Antibiotics, microbiota, and immune defense. *Trends Immunol*, **33**(9): 459-466.
55. Shalapour S, Deiser K, Sercan O, Tuckermann J, Minnich K, Willimsky G, *et al.* Commensal microflora and interferon-gamma promote steady-state interleukin-7 production in vivo. *Eur J Immunol*, **40**(9): 2391-2400.
56. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, *et al.* Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity*, **33**(5): 736-751.
57. 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. *Proc Natl Acad Sci U S A*, **109**(43): 17549-17554.
58. Balciunaite G, Ceredig R, Fehling HJ, Zuniga-Pflucker JC, Rolink AG. The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. *Eur J Immunol*, **35**(4): 1292-1300.

59. Magri M, Yatim A, Benne C, Balbo M, Henry A, Serraf A, *et al.* Notch ligands potentiate IL-7-driven proliferation and survival of human thymocyte precursors. *Eur J Immunol*, **39**(5): 1231-1240.
60. Kapsenberg ML. Gammadelta T cell receptors without a job. *Immunity*, **31**(2): 181-183.
61. Farber DL. Biochemical signaling pathways for memory T cell recall. *Semin Immunol*, **21**(2): 84-91.
62. Yokobori N, Schierloh P, Geffner L, Balboa L, Romero M, Musella R, *et al.* CD3 expression distinguishes two gammadeltaT cell receptor subsets with different phenotype and effector function in tuberculous pleurisy. *Clin Exp Immunol*, **157**(3): 385-394.
63. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, *et al.* Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity*, **27**(4): 597-609.
64. Geddes K, Rubino SJ, Magalhaes JG, Streutker C, Le Bourhis L, Cho JH, *et al.* Identification of an innate T helper type 17 response to intestinal bacterial pathogens. *Nat Med*, **17**(7): 837-844.
65. Kedzierska K, Turner SJ, Doherty PC. Conserved T cell receptor usage in primary and recall responses to an immunodominant influenza virus nucleoprotein epitope. *Proc Natl Acad Sci U S A*, **101**(14): 4942-4947.
66. Lefranc MP, Giudicelli V, Busin C, Bodmer J, Muller W, Bontrop R, *et al.* IMGT, the International ImMunoGeneTics database. *Nucleic Acids Res*, **26**(1): 297-303.

Figure legends

Figure 1: Distribution of CD3^{bright} $\gamma\delta$ T cells. *a*, hepatic cells from naïve C57BL/6 WT mice were analyzed by flow cytometry based on CD3 and TCR δ expression. *b* and *c*, cells from lungs, thymus, spleen, lymph nodes, bone marrow, skin, peripheral blood and various sections of intestine tract were analyzed as in panel *a*. *d*, The pooled mean percentage \pm SEM of CD3^{bright} $\gamma\delta$ T cells in total $\gamma\delta$ T cells from various organs of three independent experiments (3-5 mice/experiment) is shown.

Figure 2: Phenotype of pulmonary CD3^{bright} $\gamma\delta$ T cells vs conventional $\gamma\delta$ T cells. Pulmonary $\gamma\delta$ T cell subsets from naïve C57BL/6 WT mice have been stained with different combinations of antibodies and were analyzed for CD27 and NK1.1 (*a*), CD69 (*b*), CD62L and CD44 (*c*), IL-23R, CD121a and IL-18R (*d*), CD25, CD122 and CD127 (*e*), ROR γ t (*f*) and NKG2D (*g*) expression. Of note, expression of IL-23R (*d*) and ROR γ t (*f*) were assessed by the use of reporter mice using IL-23R^{+/-}-GFP knock-in and ROR γ t^{+/-}-GFP knock-in mice, respectively. The histogram in panel *c* (*right panel*) represents the differential CD44 expression between CD3^{bright} and conventional $\gamma\delta$ T cell subsets. *d*, *e* and *g*, line histograms demonstrate expression of each marker and filled histograms show their respective isotype controls. Numbers indicated in dot plots/histograms represent either mean of fluorescence intensity (MFI) or percentage of positive cells for the marker tested from at least 3 independent experiments (4-5 mice/experiment).

Figure 3: CD3^{bright} $\gamma\delta$ T cells have an invariant TCR. *a*, PCR analysis of cDNA isolated from single cell-sorted pulmonary CD3^{bright} and conventional $\gamma\delta$ T cells and amplified with a set of

primers specific for each TCR γ V-gene segment or the corresponding constant region (C γ) (upper row) or specific for each TCR δ V-gene segment or the corresponding constant region (C δ) (lower row). Clones have been sorted from two independent experiments using a cell suspension of a pool of at least 5 mice. *b*, Sequences for CD3^{bright} and conventional $\gamma\delta$ TCRs including diversity (D) (for δ chains), junction (J) and CDR3 region. *c*, pulmonary $\gamma\delta$ T cell subsets from naïve C57BL/6 WT mice were analyzed by flow cytometry for V γ 1, V γ 4 and V δ 6.3 expression. Representative dot plots from two independent experiments are shown.

Figure 4: Factors involved in CD3^{bright} $\gamma\delta$ T cell homeostasis. *a*, frequency of $\gamma\delta$ T cell subsets based on CD3 expression was analyzed by flow cytometry in mice bred either in GF or SPF conditions. The percentage of $\gamma\delta$ T cell subsets is shown (*upper panel*). The average \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments (3-6 mice/group/experiment) is shown in the *lower panel*. *, $p < 0.05$ and **, $p < 0.01$. Of note, the total number of cells in each organ was not different between GF and SPF mice. *b*, frequency of $\gamma\delta$ T cell subsets was analyzed by flow cytometry in WT vs ROR γ t^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown (*left panel*). The mean \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments is shown in the *right panel* (4 mice/group/experiment) **, $p < 0.01$ and ***, $p < 0.001$.

Figure 5: CD3^{bright} $\gamma\delta$ T cells produce IL-17A *in vitro* and *in vivo* in response to IL-1 β and IL-23. *a* and *b*, FACS-sorted pulmonary $\gamma\delta$ T cell subsets and NK cells (positive control) were incubated with PMA (10 ng/ml) and ionomycin (1 μ g/ml) (*a*) or pre-coated anti-CD3 (10, 5 and 1 μ g/ml) (*b*) for 20 hours. Cytokine production in the supernatants was measured by CBA. Data represent

the mean \pm SEM of two pooled independent experiments performed in duplicate. *c-f*, Hepatic CD3^{bright} $\gamma\delta$ T cells preferentially produce IL-17A *in vivo* after α -GalCer or LPS administration. *c*, WT mice were injected i.p. with vehicle or α -GalCer (2 μ g/mouse) and were killed at indicated time points. Gated hepatic $\gamma\delta$ T cell subsets were screened for IFN- γ and IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The pooled mean \pm SEM of three independent experiments for IFN- γ - and IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (3-4 mice/group/experiment). ***, $p < 0.001$. Total number of hepatic $\gamma\delta$ T cell subsets was assessed. ***, $p < 0.001$. *d*, WT mice were injected i.p. with PBS or LPS (0.5 mg/mouse) and were sacrificed after 8 hrs. Liver cells were treated as in panel *c*. One representative experiment out of two is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean \pm SEM of two independent experiments for IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (3 mice/group/experiment). *e*, WT mice were pre-treated with an anti-IL23p19 or isotype control (agp3) (500 μ g/mouse) at day -1 and 0 and then injected i.p. with vehicle or α -GalCer (2 μ g/mouse) (*left panels*). WT or IL-1R1^{-/-} mice were injected i.p. with vehicle or α -GalCer (2 μ g/mouse) (*right panels*). Mice were sacrificed 2 hours after α -GalCer administration. Liver cells were treated as in panel *c*. The mean \pm SEM of two pooled independent experiments for IL-17A-positive CD3^{bright} $\gamma\delta$ T cells is shown (4 mice/group/ experiment) ***, $p < 0.001$. *f*, IL-23-depleted or controls WT mice were injected with LPS as in panel *b*. The mean \pm SEM of two independent experiments for IL-17A-positive $\gamma\delta$ T cell subsets is shown (3 mice/group/experiment). *, $p < 0.05$ and **, $p < 0.01$.

Figure 6: IMQ increases infiltration of IL-17A producing CD3^{bright} $\gamma\delta$ T cells into the skin in a psoriasis-like mouse model. C57BL/6 WT mice were treated with a daily topical dose of IMQ cream (IMQ) or control “Lanette” cream (Control) for 5 days: presented data corresponds to 5 days after the first cream application. *a*, Flow cytometry analysis of conventional and CD3^{bright} $\gamma\delta$ T cells in abdominal skin. Cells gated on CD45⁺ are shown. Numbers indicate per cent of conventional and CD3^{bright} $\gamma\delta$ T cells. Conventional and CD3^{bright} $\gamma\delta$ T cells in abdominal skin were calculated as per cent of CD45⁺ cells (*right panel*). *, $p < 0.05$. *b*, Spearman's rank-order correlation analysis of conventional and CD3^{bright} $\gamma\delta$ T cells in abdominal skin calculated as per cent of CD45⁺ cells and average interfollicular epidermal thickness in both IMQ cream and control “Lanette” cream-treated mice. *c*, Intracellular IL-17A production assessed by flow cytometry in conventional and CD3^{bright} $\gamma\delta$ T cells in abdominal skin. Cells gated on CD45⁺ CD3⁺ TCR δ ⁺ are shown. Numbers indicate percentage of IL-17A⁺ of the indicated population. Gates were set based on the staining with isotype control. IL-17A⁺ conventional and CD3^{bright} $\gamma\delta$ T cells in abdominal skin were calculated as percentage of CD45⁺ cells (*right panel*). Data are shown as mean \pm SEM, $n = 7$ mice per group. *, $p < 0.05$.

Figure 7: Pulmonary CD3^{bright} $\gamma\delta$ T cells produce IL-17A *in vivo* and contribute to airway neutrophilia. *a-c*, WT mice were administered i.n. with vehicle or α -GalCer (500 ng/mouse) and were sacrificed at indicated time points. *a*, Gated pulmonary $\gamma\delta$ T cell subsets were screened for IFN- γ and IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean \pm SEM for IFN- γ - and IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (6 mice/group/experiment). ***, $p < 0.001$. *b*, WT and TCR δ ^{-/-} mice were injected i.n. with vehicle or α -GalCer and were

sacrificed after 8 hours. The total number of cells in BAL was determined and differential proportion of leukocytes was counted under the microscope. One experiment out of two is shown (n = 7). *, p < 0.05; **, p < 0.01. *c*, WT mice were injected i.n. with vehicle or α -GalCer and were sacrificed after 24 hours. The frequency and total number of $\gamma\delta$ T cell populations in the lungs was determined by flow cytometry. One experiment out of two is shown (n = 7). **, p < 0.01. CD3^{bright} $\gamma\delta$ T cells are major producers of IL-17A during skin inflammation and lung bacterial infection. *d and e*, WT mice were infected i.n. with *Streptococcus pneumoniae* serotype 1 (2 x 10⁶ bacteria) and were sacrificed at indicated time points. *d*, Gated pulmonary $\gamma\delta$ T cell subsets were screened for IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean and absolute number \pm SEM for IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (>3 mice/group/experiment) *, p < 0.05 and **, p < 0.01. *e*, Percentage of $\gamma\delta$ T cell subsets is shown in the upper panel. Absolute number of IL-17A-positive $\gamma\delta$ T cell subsets is shown in the lower panel. *, p < 0.05.

Figure 1

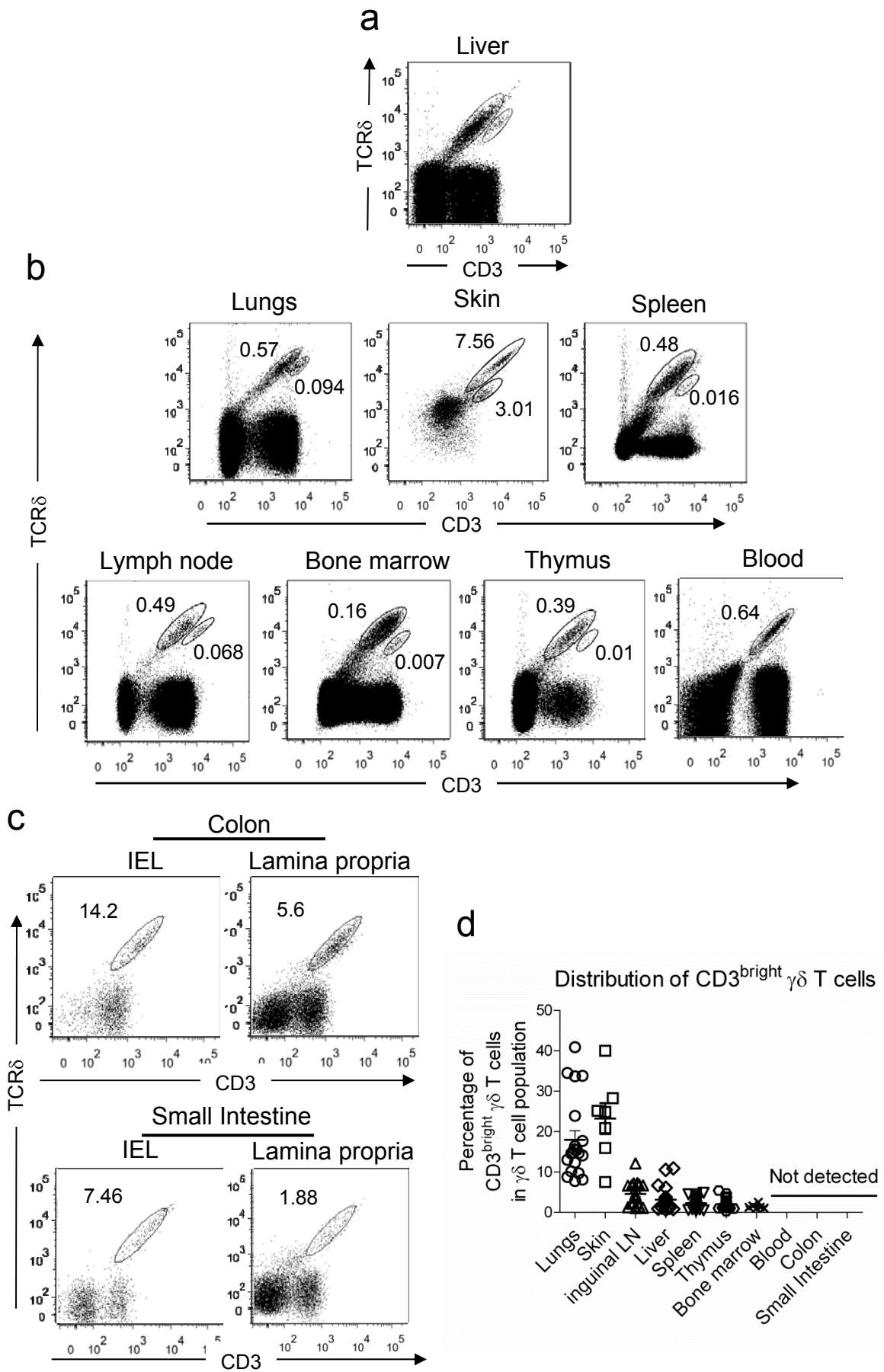


Figure 2

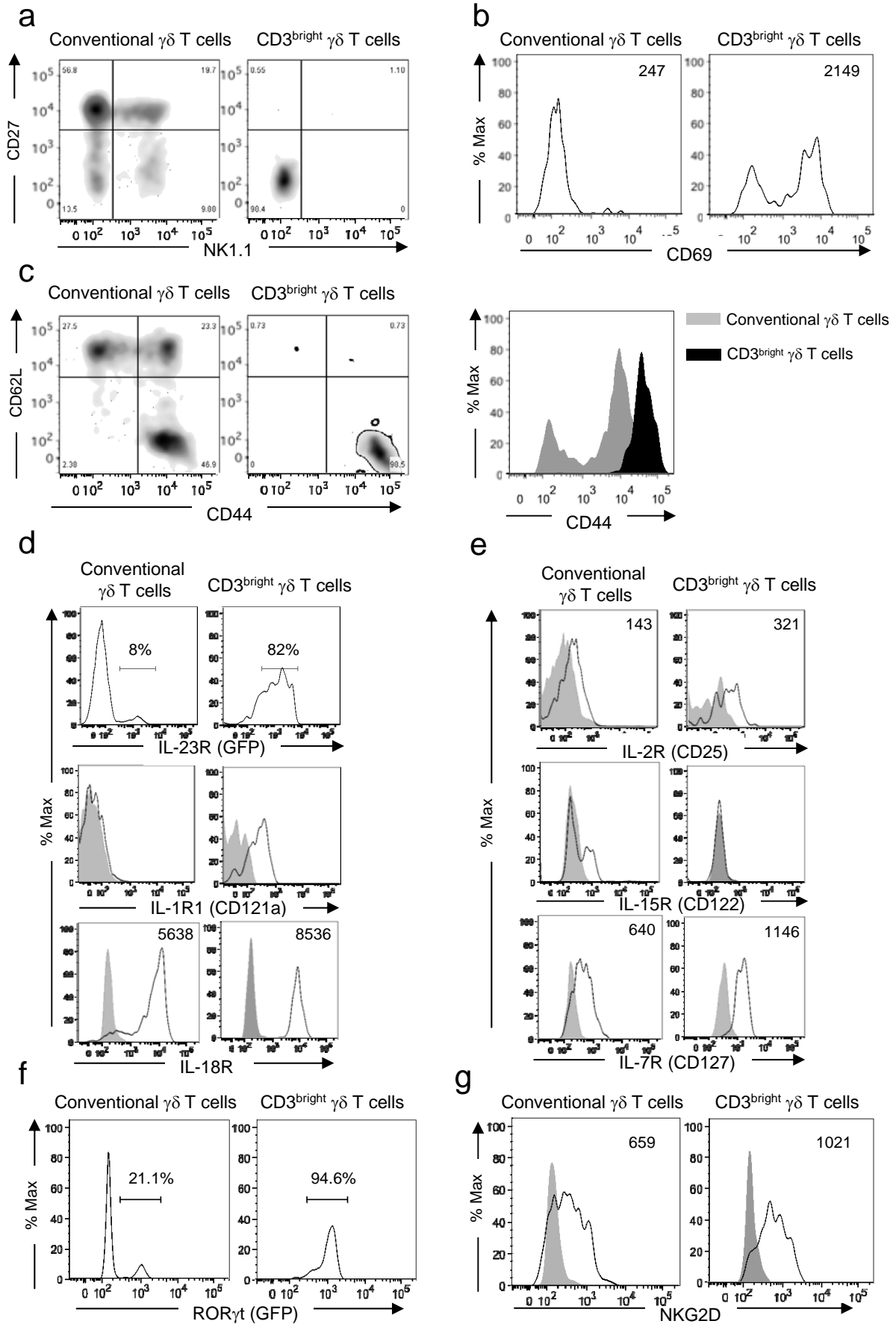
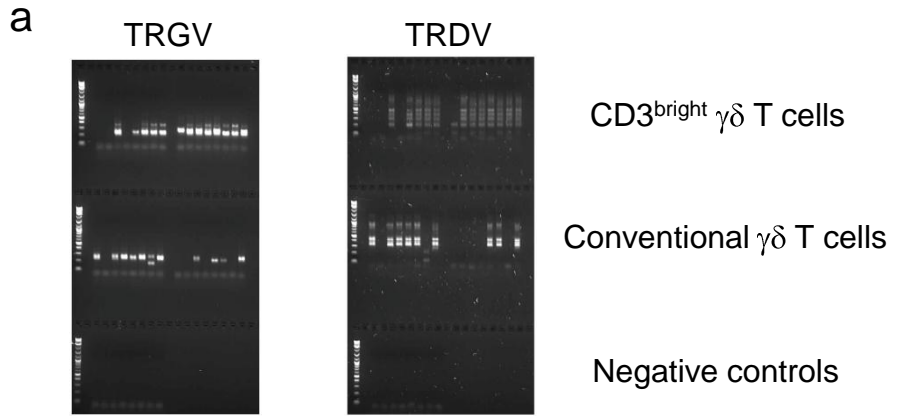


Figure 3



b

Subsets	Clones	V γ	CDR3 γ	J γ	V δ	CDR3 δ	D δ	J δ	
Conventional $\gamma\delta$ T cells	1	2	CAVWMRYSSGFHKVF	2	5	CASGYSWHIGGIRSSSTDKLVF	2	1	
	2	1/4	-	-	5	CASGVYGLTYRRDTKLVF	1	1	
	3	1/4	-	-	4	CALMERGAFPPYRRDKGTDKLVF	1	1	
	4	1/4	-	-	5	CASGYIGGIRATDKLVF	2	1	
	5	2	CAVCRLARKF#KVF	2	2	CALMLYGLLSEGGPTTDKLVF	1	1	
	6	2	CAVRISSGFHKVF	2	5	CASGPIGGIRATDKLVF	1	1	
	7	2	CAVLDSSGFHKVF	2	5	CASGYIGGIRATDKLVF	1	1	
	8	2 sequences - undecipherable				5	CASGYIGGIRATDKLVF	1	1
	9	2 sequences - undecipherable				5	CASGWGYETDKLVF	1	1
CD3 ^{bright} $\gamma\delta$ T cells	1	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	2	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	3	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	4	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	5	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	6	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	7	6	CACWDSSGCHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	8	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	9	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	10	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	11	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	
	12	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2	

c

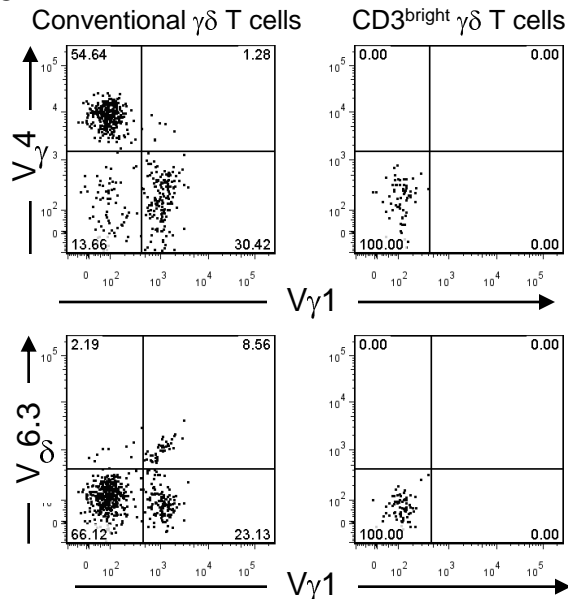


Figure 4

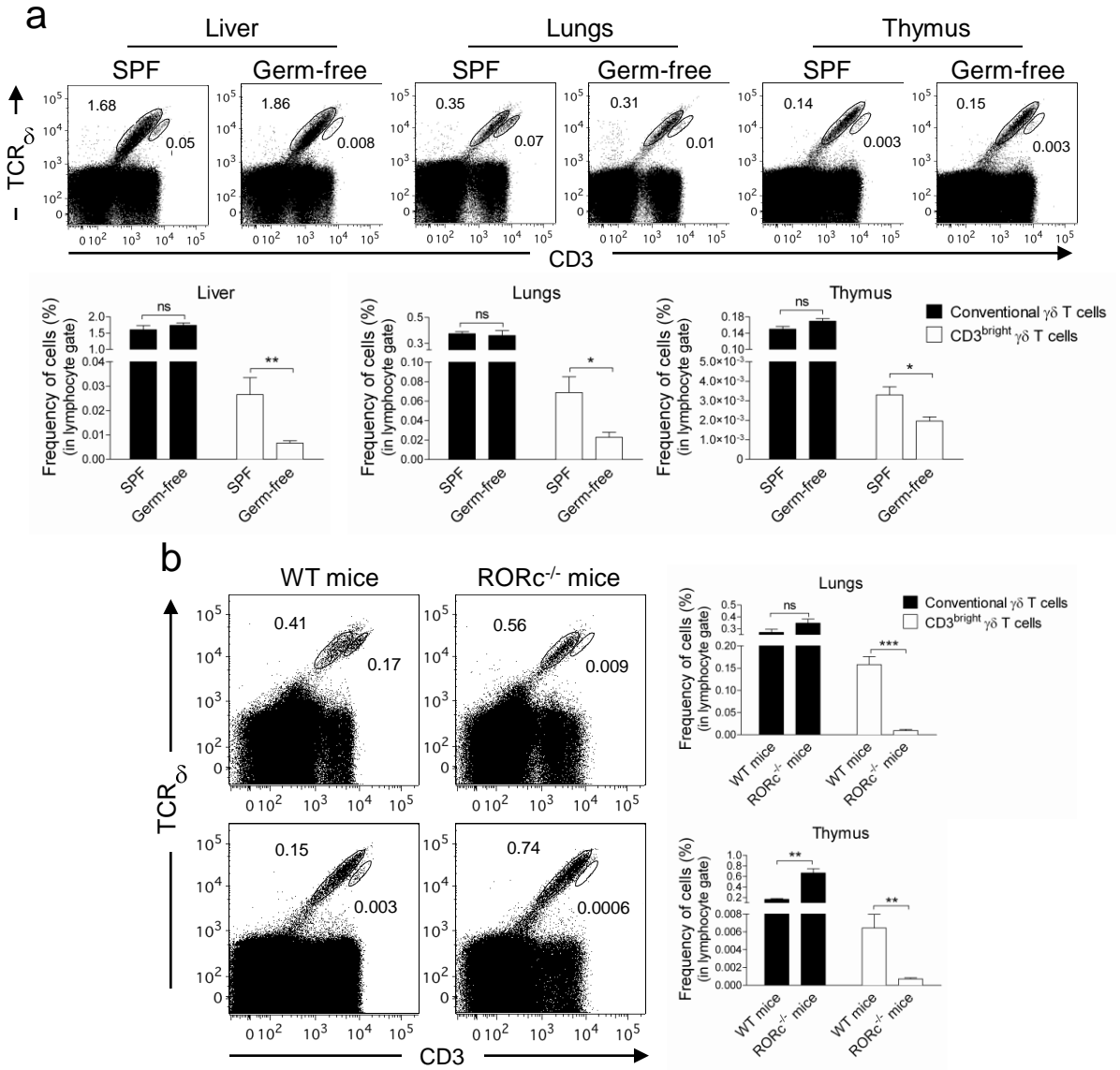


Figure 6

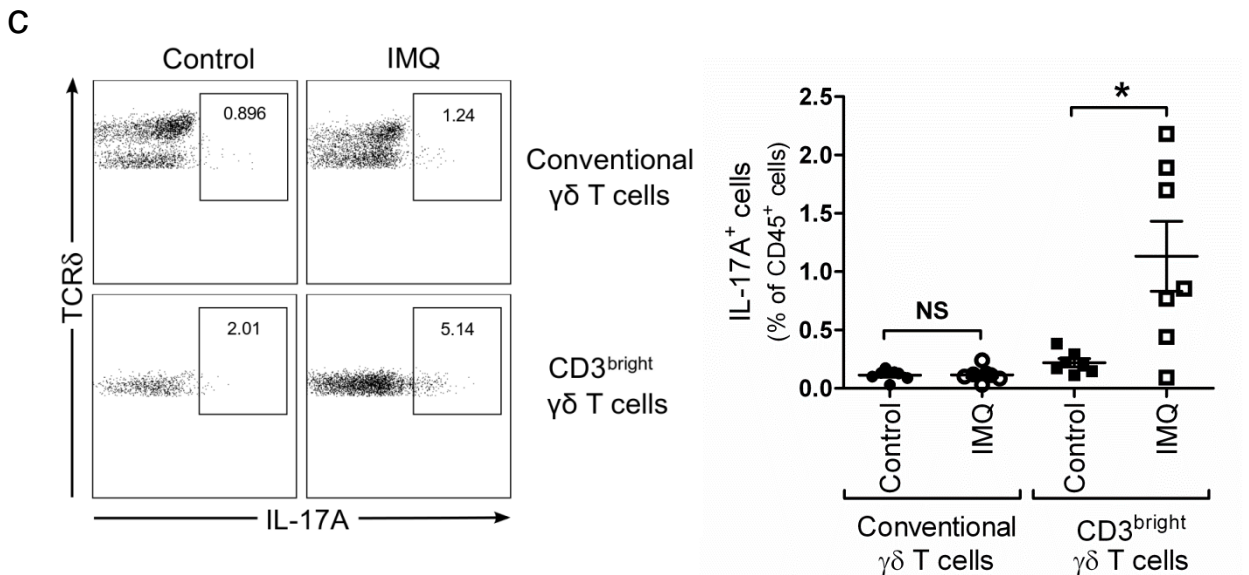
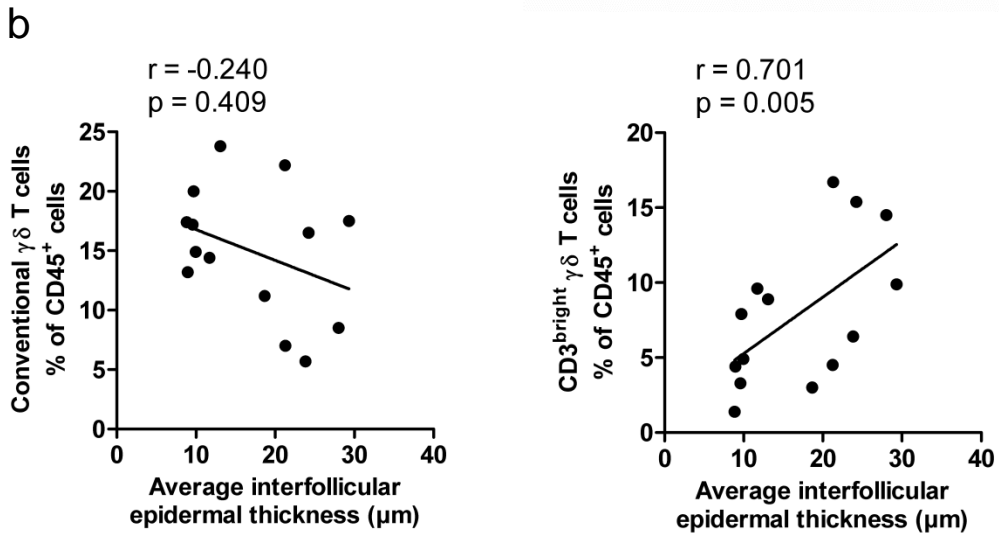
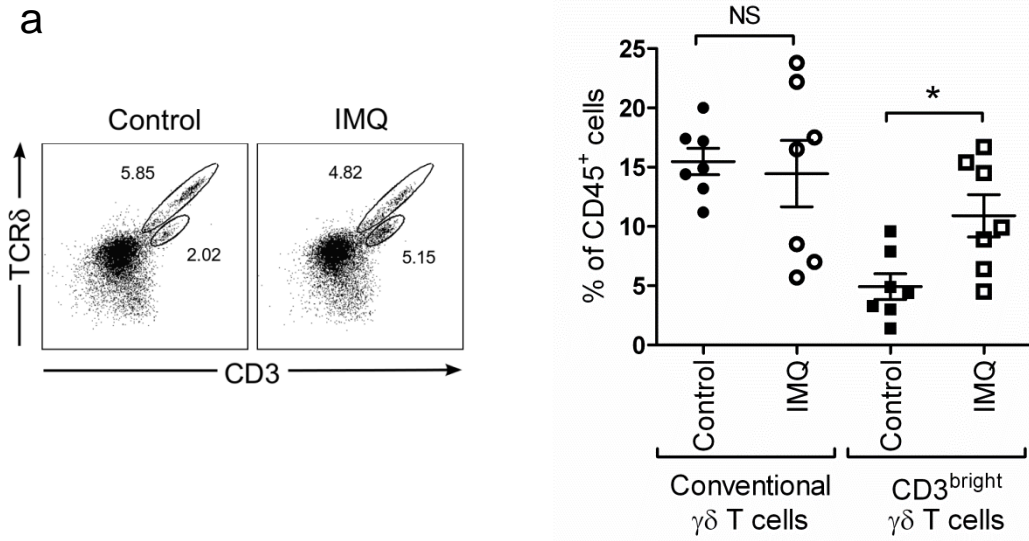


Figure 7

