AD_____

Award Number: W81XWH-11-1-0585

TITLE: ROLE OF NATURAL KILLER T CELLS IN IMMUNOGENIC CHEMOTHERAPY FOR BREAST CANCER

PRINCIPAL INVESTIGATOR: Dr Christophe Paget

CONTRACTING ORGANIZATION: University of Melbourne
Parkville, VIC, 3052 Australia

REPORT DATE: September 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual Summary	1 September 2012 – 31 August 2013

4. TITLE AND SUBTITLE
Role of Natural Killer T Cells in Immunogenic Chemotherapy for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0585

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr Christophe Paget, Helene Duret, Prof. Mark J. Smyth

E-Mail: christophe.paget@petermac.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Melbourne
Grattan Street
Parkville, VIC, 3052
Australia

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During this second year of investigation, we have confirmed the important role of γδ T cells in the efficacy of immunogenic chemotherapy using anthracyclines. In the meantime, the NKT cells seem to be dispensable for this effect at least in this particular situation. Moreover, we have also identified a population of γδ T cells with a unique phenotype never described before ($CD3^{bright}$). Interestingly, this population has a clear intrinsic ability to produce IL-17 compared to any other γδ T cells and are represented at a high frequency in breast cancer tumor environment. We have highlighted several key homeostatic factors on this population and identified their precise phenotype. Of interest a human γδ T cell population display a similar phenotype ($CD3^{bright}$) with γδT17-like cell hallmarks. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer.

15. SUBJECT TERMS
Breast cancer, Immunogenic chemotherapy, NKT cells, gamma/delta T cells, IL-17, mouse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	28	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction:

The role of immunity in controlling and regulating development of breast cancer is largely accepted. Recent studies have also demonstrated that some common chemotherapy used for breast cancer treatment relies on a competent immune system of the host to achieve an optimal response[1-3]. Interestingly seminal findings emphasized on the importance of various components of the immune system in this phenomenon[1,4-6]. Recently, we and others have demonstrated that IL-17-producing γδ T cells are well positioned and equipped to participate in this effect.

Body:

As assigned in the previous report (Year 1), we have identified an unpreviously described subset of γδ T cells displaying a high level of CD3 in comparison to the remaining population and pre-programmed to secrete high amounts of IL-17. In addition to the phenotype presented in the last report, we have formerly highlighted several key factors involved in the homeostasis of the $CD3^{bright}$ γδ T cell population. In this context, $CD3^{bright}$ γδ T cells are largely influenced by the microbiota and are fully dependent on the expression of RORγt, a mandatory transcription factor for IL-17 production (**Figure 1**).

Using an approach of single cell PCR, we have demonstrated that $CD3^{bright}$ γδ T cells express a fully germline encoded invariant Vγ6Vδ1 TCR (**Figure 2**).

Of upmost interest, similar to mice, we observed a subset of γδ T cells displaying a bright expression of CD3 in the bone marrow, but not in the peripheral blood lymphocytes of healthy donors (**Figure 3A**). Of interest, these cells displayed hallmarks of human IL-17-producing cells, uniformly expressing CD161[7] (**Figure 3B**). Similar to the observations made in mice, human $CD3^{bright}$ γδ T cells expressed high levels of IL-7R and IL-18R compared to the conventional population and harboured a phenotype of memory cells (**Figure 3B**). Finally, culture of purified γδ T cell subsets with a pre-coated anti-CD3 induced γδ T cell expansion. Interestingly, association

with a recombinant IL-7, a selective growth factor for γδ T17 cells[8] favoured CD3^{bright} γδ T cell expansion but had a minimal effect on conventional γδ T cells (**Figure 3C**). This observation is of high importance considering that identification of human IL-17-producing γδ T cells in healthy donors remains challenging[9], despite the fact that these cells have been associated with various immune responses[10].

A manuscript has been submitted to *PNAS* and is currently under review.

Having this paper submitted and as mentioned in the previous report, we will focus our efforts in investigating the contribution of this population in development of breast cancer or immunogenic chemotherapy for breast cancer considering their specialized function (IL-17 production) and their abundance in breast cancer tumors. Of note, this population is also found at a high frequency in various solid tumors (transplantable and spontaneous) in mice from different background (C57BL/6 and BALB/C) (**Figure 5**).

Moreover, all points from the original proposal, and as discussed in the previous report, will be tested in the coming next months. In February, I have quitted my position at Melbourne University and joined the INSERM in France. Recently I have been offered a tenured position and the transfer of the award is still pending between Melbourne University and INSERM.

Key Research Accomplishment:

- CD3^{bright} γδ T cells express a **unique Vγ6Vδ1 TCR**.

- Discovery of a **similar population of CD3^{bright} γδ T cells in humans also associated with a signature of IL-17-producing cells.**

- Submission of an original manuscript to *PNAS* describing a new way to define mouse and human γδ T cells with high capacity to produce IL-17.

Conclusions:

We have identified a population of γδ T cells both in mouse and humans with a unique phenotype never described before ($CD3^{bright}$). Interestingly, this population has a potent intrinsic ability to produce IL-17 compared to the other γδ T cells and is enriched in breast cancer tumor environment. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer and more generally in the development of breast cancer.

References:

1. Apetoh, L*., et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 13, 1050-1059 (2007).

2. Ghiringhelli, F*., et al.* Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 15, 1170-1178 (2009).

3. Park, S*., et al.* The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell* 18, 160-170 (2010).

4. Mattarollo, S.R*., et al.* Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. *Cancer Res* 71, 4809-4820 (2011).

5. Obeid, M*., et al.* Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 13, 54-61 (2007).

6. Stagg, J*., et al.* Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A* 108, 7142-7147 (2011).

7. Maggi, L*., et al.* CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol* 40, 2174-2181 (2010).

8. Michel, M.L*., et al.* Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. *Proc Natl Acad Sci U S A* 109, 17549-17554 (2012).

9. Caccamo, N.*, et al.* Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118, 129-138 (2011).

10. Nembrini, C., Marsland, B.J. & Kopf, M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* 123, 986-994; quiz 995-986 (2009).

Appendices:

Figures shown in this section are unpublished.

Figure 1

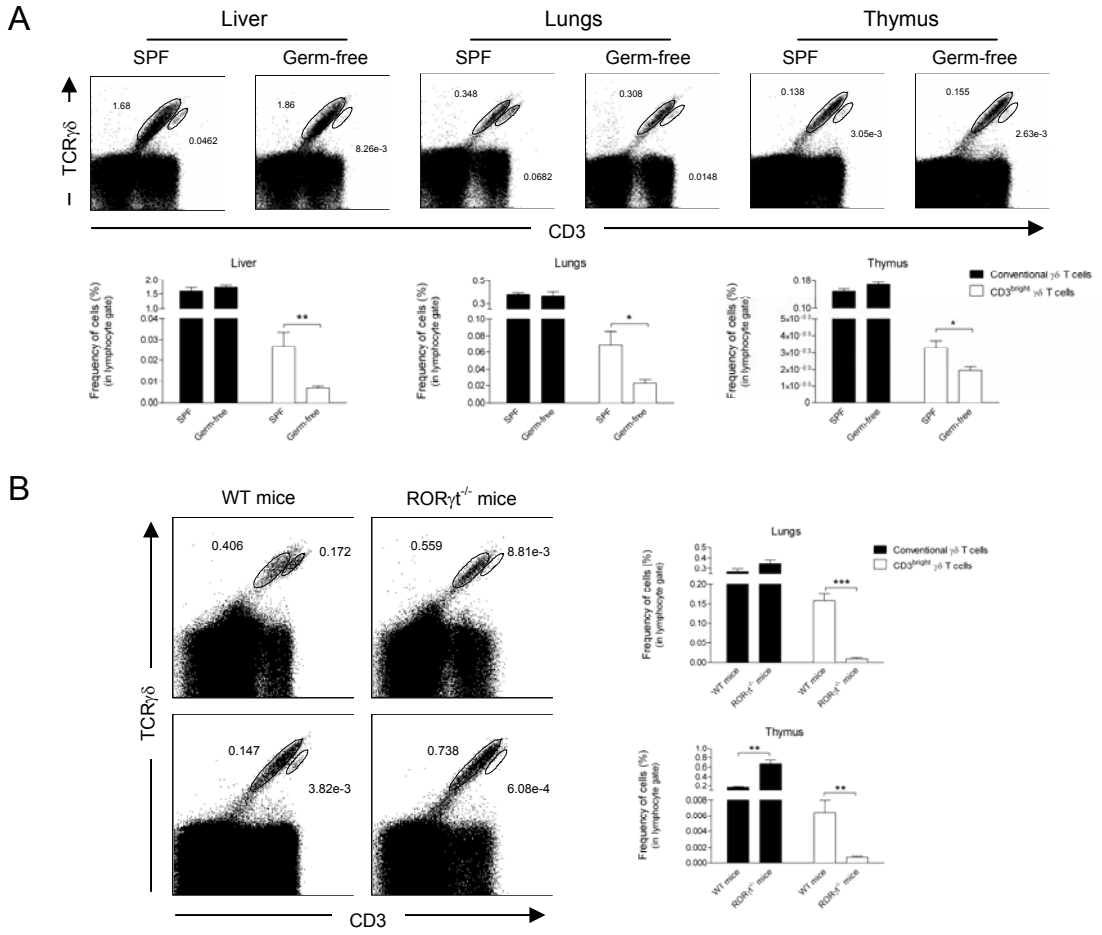
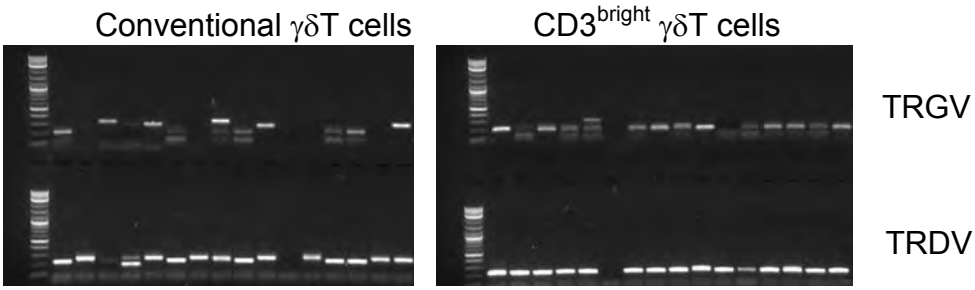


Figure 1: Factors involved in CD3^{bright} γδ T cell homeostasis. *A*, frequency of γδ T cell subsets based on CD3 expression was analyzed by flow cytometry in mice bred either in GF or SPF conditions. The percentage of γδ T cell subsets is shown (*upper panel*). The average ± SEM of γδ T cell subset frequencies pooled from two independent experiments is shown in the *lower panel*. Of note, the total number of cells in each organ was not different between GF and SPF mice. Differences in mean were analyzed using a Mann-Whitney test. *, $p < 0.05$ and **, $p < 0.01$. *B*, frequency of γδ T cell subsets was analyzed by flow cytometry in WT *vs* RORγt$^{-/-}$ mice. The percentage of γδ T cell subsets is shown (*left panel*). The mean ± SEM of γδ T cell subset frequencies pooled from two independent experiments is shown in the *right panel*. Differences in mean were analyzed using a Mann-Whitney test. **, $p < 0.01$ and ***, $p < 0.001$.

Figure 2

A



B

Subsets	#	Vγ	CDR3γ	Jγ	Vδ	CDR3δ	Dδ	Jδ
Conventional γδ T cells	1	**2**	CAVWMRYSSGFHKVF	**2**	**5**	CASGYSWHIGGIRSSSTDKLVF	**2**	**1**
	2		-	**-**	**5**	CASGVYGTLYRRDTDKLVF	**1**	**1**
	3		-	**-**	**4**	CALMERGAFPPYRRDKGTDKLVF	**1**	**1**
	4		-	**-**	**5**	CASGYIGGIRATDKLVF	**2**	**1**
	5	**2**	CAVCRLARKF#KVF	**2**	**2**	CALMLYGLLSEGPTTDKLVF	**1**	**1**
CD3bright γδ 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**

Figure 2: CD3^{bright} γδ T cells have an invariant Vγ6Vδ1 TCR. *A*, PCR analysis of cDNA isolated from single cell-sorted CD3^{bright} and conventional γδ T cells and amplified with a set of primers specific for each TCRγ V-gene segment or the corresponding constant region (Cγ) (upper row) or specific for each TCRδ V-gene segment or the corresponding constant region (Cδ) (lower row). *B*, Sequences for CD3^{bright} and conventional γδ T TCRs including diversity (D) (for δ chains), junction (J) and CDR3 region.

Figure 3

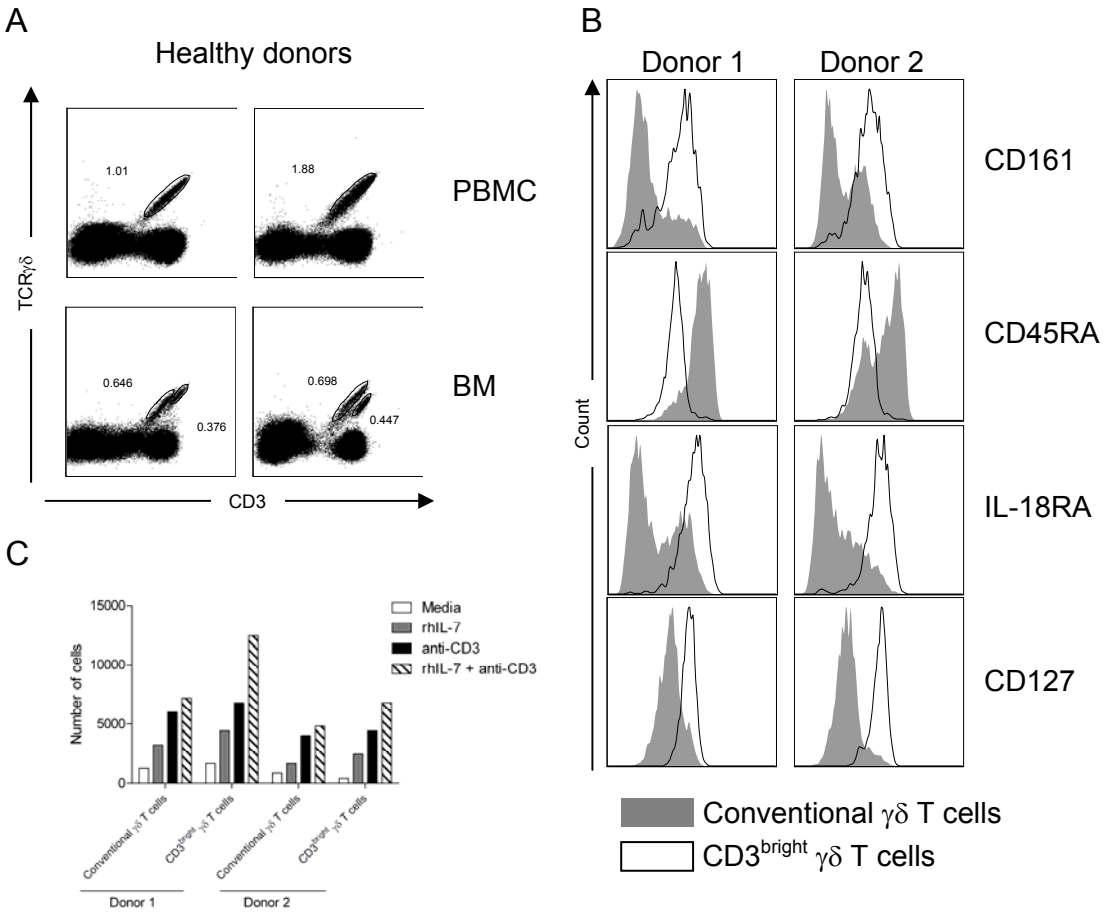


Figure 3: Human CD3^{bright} but not conventional γδ T cells display characteristics of IL-17-producing cells. *A*, Single cell suspensions of peripheral blood mononuclear cells (PBMC) or bone marrow (BM) from healthy donors were analyzed by flow cytometry for CD3 and TCRδ expression. *B*, γδ T subsets (based on CD3 expression) were stained with different combinations of antibodies and were analyzed for CD161, CD45RA, IL-18RA and CD127 differential expression. *C*, purified γδ T subsets (based on CD3 expression) from bone marrow of two donors were cultured for 4 days in presence of recombinant IL-7 (20 ng/ml) or/and pre-coated anti-CD3 (0.5 μg/ml/clone: OKT3). At end point, number of cells was counted under the microscope.

CD3 expression reveals a gamma/delta T cell subset with specialized functions

Christophe Paget[a,b,c,1], Melvyn T. Chow[a,b,d], Nicholas A. Gherardin[a,b,g], Paul A. Beavis[a,b], Adam P. Uldrich[g], Helene Duret[a,b], Maya Hassane[c], Fernando Souza-Fonseca-Guimaraes[d], Geoffrey R. Hill[d,e], Paul Neeson[a,b], David S. Ritchie[a,b], François Trottein[c], Gabrielle T. Belz[h], Dale I. Godfrey[g] and Mark J. Smyth[a,b,d,f,1]

[a]Cancer Immunology Program, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria 3002, Australia.
[b]Sir Peter MacCallum Department of Oncology and Department of Pathology, University of Melbourne, Parkville 3010, Australia.
[c]INSERM U1019, Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, 59000 Lille, France.
[d]QIMR Berghofer Medical Research Institute, Herston, Queensland 4006, Australia.
[e]Department of Bone Marrow Transplantation, Royal Brisbane Hospital, Herston, Australia.
[f]School of Medicine, University of Queensland, Herston, Queensland 4006, Australia.
[g]Department of Microbiology and Immunology, University of Melbourne, Parkville, VIC 3010, Australia.
[h]Division of Molecular Immunology, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, 3052, Australia.
[1]Correspondence: christophe.paget@pasteur-lille.fr or mark.smyth@qimrberghofer.edu.au

Running title: Level of CD3 expression as a marker of γδT17

Classification: Biological sciences; Immunology

Key-words: γδT cells, IL-17, IL-23, IL-1β, invariant TCR, microbiome, RORγt, alpha-galactosylceramide, lipopolysaccharide, innate immunity, neutrophils, mucosal immunity.

Conflict of interest: All authors declare that they have no conflict of interest

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

Summary

Interleukin-17A is a pro-inflammatory cytokine that plays an important role in a wide range of immune responses including infection, allergy and auto-immunity. γδT cells are recognized as secretors of IL-17, but now we define the remarkable ability of a $CD3^{bright}$ Vγ6Vδ1 γδT cell subset with effector memory phenotype to rapidly produce IL-17A, but not IFN-γ. These cells are widely distributed with a preferential representation in the lungs and do not develop properly in absence of RORγt expression or in germ-free mice. Interestingly this population responded promptly to various stresses in a mechanism involving IL-23 and NLRP3-inflammasome-dependent IL-1β. Finally, we demonstrated that pulmonary $CD3^{bright}$ γδ T cells expanded/were recruited and produced an early substantial amount of IL-17 in response to pneumococcal infection. Given the versatile role of IL-17 in pulmonary immunity, the prevalence of this population in lungs suggests a pivotal role of this γδT cell subset in respiratory disorders.

Introduction

γδT 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 cancer (1). In particular, mouse γδT cells are known as major early IL-17 producers (2). In this context, IL-17-producing γδT cells (γδT17) 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, γδT cells have been reported to be a major source of this cytokine (5, 7-9). On the other hand, IL-17 has been proposed to participate in the development of a diversity of inflammation or auto-immune disorders including arthritis, asthma and EAE (6) and similarly, γδT17 cells play an important part in these processes (10). Finally, the role of IL-17 in tumorigenesis or control of tumor progression has recently been studied but remains controversial (11). However, γδT17 cells appear to be important in eliciting a positive outcome during immunogenic anti-cancer chemotherapy (12). In humans, little is known about the real functional relevance of γδT17 cells in IL-17-dependent pathologies (13) despite the fact that IL-17-production by γδT cells has been detected during infections and chronic inflammation (14-17). However, characterization, isolation and activation of these cells in humans remains extremely challenging (14).

Numerous recent studies have also focused on the factors influencing γδT17 cell differentiation, homeostasis and activation (10, 18). γδT17 cells express a fairly restricted TCR repertoire ($V\gamma4^+$ and $V\gamma6^+$ chain predominantly) (9, 19-21) and originate from fetal thymus without signs of TCR-dependent positive selection (19, 22). Within the thymus, γδT17 cells have a pre-programmed IL-17-producing phenotype, although these cells do not require the transcription factors RORγt and STAT3 to develop (23), even though STAT3 remains mandatory for their activation in periphery. Moreover, the strength of engagement with cognate antigen(s) (Ag) by γδTCRs in the thymus seems to be the key factor influencing the commitment towards IFN-γ- or IL-17-producing γδ T cell lineages (19, 22, 24). In this context, absence (or weak) TCR ligation seems to determine a transcriptional program leading to the development of γδT17 cells (19, 22, 24). In addition, Notch/Hes1 signaling pathways are involved in the development and homeostasis of γδT17 cells (23). While IL-6 and IL-23 are dispensable for the development of γδT17 cells (23, 25), TGF-β may be required since *Tgfb*$^{-/-}$ mice show a significant reduction in thymic γδT17 cells (26). Whilst IL-23 is not required for γδT17 cell development, this cytokine along with IL-1β, IL-6, IL-7 and IL-18, potently promotes γδT17 cell functions (expansion and/or cytokine production) (25, 27-29).

Here, we demonstrate in mice that the level of cell surface CD3 expression reveals a population of γδ T cells bearing a clonal Vγ6Vδ1 TCR associated with a great ability to secrete IL-17. We characterize 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 by increasing in absolute number and producing high levels of IL-

17 compared to other acknowledged innate IL-17-producing cells. Given the versatile role of IL-17 in pulmonary immunity, the prevalence of this population in lungs of adult mice suggests a pivotal role of this γδT cell subset in respiratory disorders.

Experimental procedures

Mice

Male C57BL/6J wild-type mice were purchased from the Walter and Eliza Hall Institute for Medical Research (WEHI). C57BL/6 TCRδ-deficient (TCRδ$^{-/-}$) mice, C57BL/6 IL-23p19-deficient (IL-23p19$^{-/-}$) mice, C57BL/6 IL-12p35-deficient (IL-12p35$^{-/-}$) mice, C57BL/6 IL-12p40-deficient (IL-12p40$^{-/-}$) mice, C57BL/6 IL-21-deficient (IL-21$^{-/-}$) mice, C57BL/6 IL-6-deficient (IL-6$^{-/-}$) 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 RORγt$^{-/+}$-GFP knock-in mice, RORγt$^{-/-}$-GFP knock in (RORγt-GFP reporter) mice, Germ-free mice and SPF controls were obtained from WEHI. C57BL/6 MyD88/TRIF-deficient (MyD88$^{-/-}$ TRIF$^{-/-}$) mice (kindly provided by Dr. Geoff Hill, QIMR Berghofer) and C57BL/6 FcεRIγ-deficient (FcεRIγ$^{-/-}$) 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.

Mouse 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: PB-conjugated), NK1.1 (PK136: PECy7- or PE-conjugated), anti-Cδ (GL3: APC- or FITC-conjugated), CD69 (FN50: FITC-conjugated), IFN-γ (XMG1.2: PE-conjugated), IL-17A (TC11-18H10: PE-conjugated), CD27 (LG.7F9: PECy7-conjugated), TCRβ (H57-597: PE-conjugated), CD44 (IM7: FITC-conjugated), CD62L (MEL-14: PE-conjugated), CD127 (A7R34: FITC-conjugated), CD122 (TM-β1: FITC-conjugated), NKG2D (C7: APC-conjugated), IL-18Rα (112614: APC-conjugated), CD121a (35F5: PE-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-1β, 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 elsewhere (30). Mice were anesthetized and administered i.n. with 2×10^6 live bacteria.

Preparation of splenic, pulmonary and liver cells

Splenic, pulmonary and hepatic mononuclear cells from control, LPS- or α-GalCer-treated mice were prepared as previously described (31). 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 µg/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.

Flow cytometry and cytospin

Mice were injected with PBS, α-GalCer (2 μg/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. Golgi Plug/Golgi Stop (BD Biosciences) was added for 2 hrs. Then cell suspensions were blocked in the presence of 2.4G2 prior to staining with appropriate dilutions of APC-conjugated TCRγδ, PB-labeled anti-CD3 and PeCy7-conjugated NK1.1 for 30 min in PBS containing 2% FCS and 0.01% NaN_3. Cells were then fixed and permeabilized using the BD Cytofix/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 γδ T subsets and in vitro assays

Livers, lungs and spleens were harvested from naive mice and cell suspensions were prepared as previously described (31). Red blood cells were lysed with ACK lysis buffer prior to γδT cell enrichment with autoMACS (depletion of TCRβ$^+$, CD19$^+$ and F4/80$^+$ cells). Then, γδT-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 γδT cells and CD3^{bright} γδT 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-1β (1 ng/ml), IL-18 (1 ng/ml), PMA (10 ng/ml) and ionomycin (1 μg/ml). In some case purified cells have been stimulated with various concentrations of anti-CD3ε (clone 145-2C11) (BD Pharmingen) pre-coated on the culture plates.

Single-cell PCR

For single-cell PCR, cDNA from sorted mouse and human γδT cell subsets was generated as previously described (11) and then was amplified by two rounds of semi-nested PCR with sense primers for Cγ, Cδ, Vγ and Vδ regions each used with the Cγ and/or Cδ antisense primers (Table 1). PCR products were separated by electrophoresis through a 1.5% agarose gel and were sequenced as described (32). TCR nomenclature is in accordance with the International ImMunoGeneTics database (33).

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 or a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Results with a p value of less than 0.05 were considered significant.

Results

Detection of a γδT cell subset harboring a high level of CD3

We have recently demonstrated that γδT cells are an important early source of cytokines (IFN-γ and IL-17A) in response to α-GalCer (34). During our investigations, we have consistently observed in our control mice at steady-state, a minor population of hepatic γδT cells displaying an intense expression of CD3 compared to the remainder of the γδT cell population, but with a similar level of TCR expression (Fig. 1A). As shown in Figure 1B, this population is widely distributed, however in each organ tested, their frequency as a proportion of the whole γδ T cell population differs. Thus, although this subset was preferentially represented in lungs, it was also detected in thymus and other peripheral organs, but not in the blood. 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 (Figure S1). Moreover, their morphology was similar to normal lymphocytes (Fig. S1) and this population was absent in TCRδ$^{-/-}$ mice (Fig. S1) reinforcing the idea that these cells are true γδT cells. Together our data define a population of γδ T cells with a unique phenotype characterized by a bright expression of the CD3 complex.

CD3^{bright} γδ T cells display a phenotype of γδT17 cells

Since no studies have yet reported a population of γδT cells with such a phenotype, we investigated the markers expressed by these cells in comparison to the conventional γδT cells. CD27 expression segregates γδT cell population into IFN-γ-producing (CD27^{+}) and IL-17-producing (CD27^{-}) γδT cells (35) and so represents a critical marker to define γδT cell functions. Strikingly, the CD3^{bright} cells uniformly lacked CD27 and NK1.1 expression, a phenotype of

γδT17 cells (Fig. 2A). A more extensive investigation of these cells determined that this population had an activated ($CD69^+$) (Fig. 2B) and effector memory phenotype ($CD62L^-$ $CD44^{high}$) (Fig. 2C) and was well equipped to produce T_H17-related cytokines ($IL\text{-}1R1^+$ $IL\text{-}23R^+$ $IL\text{-}18R^+$) (Fig. 2D). Interestingly, we also detected the expression of receptors for soluble factors involved in leukocyte survival including IL-2 and IL-7, but not IL-15 (Fig. 2E). It is noteworthy that CD127 (IL-7R) and CD25 (IL-2R) expression on $CD3^{bright}$ γδ T cells was higher compared to conventional γδT cells. $CD3^{bright}$ γδT cells also expressed the orphan nuclear receptor RORγt, a key transcription factor for IL-17 production (36, 37) (Fig. 2F) and the common marker of γδT cell-mediated cytotoxicity, NKG2D (Fig. 2G). Collectively, these data suggest that these cells have a phenotype of γδT17 cells.

$CD3^{bright}$ γδ T cells express a canonical germline encoded Vγ6 /Vδ1 TCR

The nature of the TCR expressed by γδT cells has also been shown to correlate with their functions (38, 39). Here we have used the Heilig & Tonegawa nomenclature (40) and Elliott's nomenclature (41) to define the Vγ and Vδ chains respectively. Thus, expression of a $V\gamma1^+$ TCR is associated with a T_H1-profile whereas $V\gamma4^+$ and $V\gamma6^+$ TCRs are mainly associated with a T_H17 phenotype (1). 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 encoding the different γ and δ chains (Fig. 3A). To date, all the $CD3^{bright}$ γδT cells were found to express a unique TCRδ rearrangement TRDV4-TRDD2-TRDJ2 encoding for a Vδ1-Dδ2-Jδ2 TCR (Fig. 3B). Of interest, the PCR products indicated that all $V\delta1^+$ sequences included Jδ2 and had a similar CDR3δ amino acid sequence with no N diversity. Study of the TCRγ repertoire also demonstrated a unique rearrangement TRGV6-TRGJ1

encoding for a Vγ6-Jγ1 TCR (Fig. 3B) with a similar CDR3γ. By contrast, sequencing of the PCR products obtained with the conventional population indicated that this population expressed a more diverse TCR repertoire (Fig. 3B). These results suggest that CD3^{bright} γδT cells uniformly express a canonical fully germline encoded Vγ6Vδ1 TCR.

CD3^{bright} γδT cells are influenced by the endogenous flora and are RORγt-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 γδT cells. In line with our hypothesis, C57BL/6 mice bred in germ-free (GF) conditions had a selective reduction in their CD3^{bright} γδT cell compartment in the lungs, thymus, spleen, and liver compared to mice bred in SPF conditions (Fig. 4A). Moreover, mice chronically exposed to antibiotics (neomycin) also displayed a reduced frequency of CD3^{bright} γδT cells in the periphery (Fig. S2). By contrast, the frequency of conventional γδT 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} γδ T cell compartment (Fig. S2).

Expression of RORγt is mandatory in the development of T_H17 cells (36, 37) but appears dispensable for development and maintenance of γδT17 cells (9). However, using RORγt-deficient mice, we determined that CD3^{bright} γδT cells were fully dependent on the expression of RORγt (Fig. 4B). In addition, various cytokines including IL-6, IL-21, IL-1, and IL-23 have been demonstrated to be important in T_H17 cell differentiation and homeostasis (2). The frequency of

CD3^{bright} γδT cells was not modulated in mice lacking IL-23 (IL-23p19$^{-/-}$ and IL-12p40$^{-/-}$ mice) (Fig. 4C). Similarly, we were not able to detect a role for IL-1, IL-6 and IL-21 in the differentiation/maintenance of the CD3^{bright} γδT cell population (Fig. S2). The level of CD3 expression on these cells suggests an atypical CD3/TCR complex structure (42) with a perturbed stoichiometry, an observation already made for some γδ T cell subsets (43). This could be due to an alternate structure with a biased expression of particular CD3 components such as the substitution of a CD3ζ subunit by an FcεRIγ (44, 45). However, using FcεRIγ-deficient mice, we observed no modulation in the prevalence of CD3^{bright} γδ T cells compared to control mice (Fig. S2).

CD3^{bright} γδT cell subset preferentially produces IL-17A upon activation

Having established that this CD3^{bright} γδT 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 γδT cells from lungs according to their CD3 expression and subjected them to PMA/ionomycin stimulation. As depicted in Figure 5A, this resulted in a large secretion of cytokines by γδT cell subsets including IFN-γ and IL-17A. Interestingly, the two subsets displayed a clear dichotomy in their cytokine profile. The conventional γδT 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} γδT cells (Fig. S3). Recently, CD27 has been demonstrated to be a thymic determinant for γδT cell differentiation where the CD27^{-} subset was associated with a T_H17 profile (35). Therefore we investigated in our *in vitro* assay whether the CD3^{bright} γδT cell subset (CD27^{-} NK1.1^{-}) had a distinct ability to secrete IL-17A compared to the CD27^{-} subset represented in the conventional population of γδT cells (Fig. S3).

In line with the study from Ribot et al. (35) we observed that $CD27^+$ γδT cells produced IFN-γ, but no IL-17A, whereas $CD27^-$ γδT cells secreted IL-17A, but no IFN-γ. Importantly however, comparison of conventional $CD27^-$ γδT cells *vs* $CD3^{bright}$ γδT cells indicated that the latter were more capable of producing IL-17A (Fig. S3). We also did a similar analysis to compare $CD3^{bright}$ γδT cells and $V\gamma4^+$ γδT cells, another TCR chain strongly associated with IL-17 production (39). Again, $CD3^{bright}$ γδT cells produced more IL-17A than $V\gamma4^+$ γδ T cells in this setting (Fig. S3). Furthermore, despite a clear ability to produce IL-17, $V\gamma4^+$ γδT cells were also able to secrete a substantial amount of IFN-γ. In contrast, activation of $CD3^{bright}$ γδT cells by means of anti-CD3 stimulation also resulted in IL-17A production, but not IFN-γ (Fig. 5B). Together, these data demonstrate that the $CD3^{bright}$ γδT cells are intrinsically skewed towards an IL-17A-producing cell profile and have a higher ability to produce this cytokine compared to other γδT cells.

IL-23 and NLRP3 inflammasome-dependent IL-1β are required for IL-17A production by $CD3^{bright}$ γδT cells

We have recently demonstrated that α-GalCer injection resulted in a rapid production of IL-17A by γδT cells (34). According to the hallmarks of $CD3^{bright}$ γδT cells, this population represented an interesting candidate to be a preferential IL-17-producing γδ T cell subset in response to α-GalCer. In line with our hypothesis, we observed that hepatic $CD3^{bright}$ subsets of γδT cells rapidly produced IL-17A after α-GalCer administration (Fig. 6A). In stark contrast, only few conventional γδ T cells (despite a significant proportion of $CD27^-$ γδT cells) produced IL-17A (Fig. 6A). Interestingly, we found that a large proportion of $CD3^{bright}$ γδT 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 in concert with our recent report (34), non-CD3^{bright} γδT cells were capable of secreting a substantial amount of IFN-γ (Fig. 6A). We next investigated whether the CD3^{bright} γδT cells were also capable of producing IL-17A in an immune response to LPS. As expected, γδT cells were an important source of IL-17A (46), but only ~11% of conventional γδT cells produced IL-17A in response to LPS, whereas 83% CD3^{bright} γδT cells secreted this cytokine (Fig. 6B).

Recent studies have suggested that IL-1β and IL-23 drive IL-17A secretion by γδT cells in the absence of TCR ligation (27, 29). To test this hypothesis, 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} γδT cells was significantly impaired in both settings (Fig. 6C). As expected, the employment of gene-targeted mice for NLRP3-inflammasome components (NLRP3 and caspase-1) indicated that IL-1β-dependent IL-17A production was dependent on the NLRP3/caspase-1 activation cascade (Fig. S4). Experiments conducted with LPS indicated a similar mechanism of activation (Fig. 6D). To further confirm these findings, we checked if these cytokines were sufficient to induce IL-17A production by highly purified γδT cells. In agreement with our recent report and others (27, 29, 34), combination of IL-12 and IL-18 led to IFN-γ production by conventional γδT cells whereas IL-23 plus IL-1β resulted in IL-17A secretion (Figure S4). The same combinations incubated with CD3^{bright} γδT cells also resulted in the secretion of IL-17A (IL-23 + IL-1β), but not IFN-γ (IL-12 + IL-18) (Fig. S4). Notably, at the doses tested, individual recombinant cytokines failed to promote cytokine production. Together these data indicate that IL-23 and IL-1β are important for IL-17A production by CD3^{bright} γδT

cells in response to α-GalCer and suggest that these cytokines are sufficient to induce IL-17A production by purified CD3^{bright} γδT cells.

CD3^{bright} γδT cells display a feature of specialized cells against pulmonary pathogens.

With respect to the preferential localization in lungs and the ability of CD3^{bright} γδT cells to rapidly produce large amount of IL-17A, we next investigated their potential biological activity in lungs. Intranasal injection of α-GalCer, the prototypical type 1 NKT cell activator induces airway neutrophilia in an IL-17-dependent mechanism (30, 47, 48) and its activity as a mucosal adjuvant is now widely accepted for induction of protective immunity against pathogens (30, 49, 50). Intranasal instillation of α-GalCer resulted in IL-17A and IFN-γ production by γδT and type I NKT cells (Fig. 7A and Fig. S5). Notably, the CD3^{bright} γδT cells were prominent producers of IL-17A on a per cell basis compared to conventional γδT cells and type I NKT cells (Fig. 7A). As expected, this was accompanied by airway neutrophilia 8 hrs after α-GalCer instillation (Fig. 7B); an observation largely dependent on γδ T cells (Fig. 7B) suggesting that the CD3^{bright} γδT cells might participate in this effect. As observed in the liver after systemic administration of α-GalCer, the pool of CD3^{bright} γδT cells in lungs expanded by ~ 10 fold within 24 hrs of i.n. administration of α-GalCer (Fig. 7C). Using a relevant model of respiratory bacterial infection, we observed that CD3^{bright} γδ T cells were early and major sources of IL-17 following intranasal *Streptococcus pneumoniae* infection (Fig. 7D). As expected, unlike the conventional subset, CD3^{bright} γδ T cells failed to secrete IFN-γ (Fig. S5). Recently expression of CCR6 has been correlated with γδT17 cells (51). Although IL-17 production was restricted to the CCR6^{+} subset in conventional γδ T cells, CD3^{bright} γδ T cells could produce IL-17 independently from CCR6

expression (Fig. S6). Moreover, similar to the α-GalCer model, the frequency and number of these cells selectively increased during the early course of infection (Fig. 7E). Altogether these data indicate that $CD3^{bright}$ γδ T cells may play a critical role in 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 distinct subset of γδT cells with a heightened ability to produce IL-17A based on their high expression of CD3. $CD3^{bright}$ γδT cells are a homogenous population predominantly represented in lungs that display a phenotype of effector memory IL-17-producing cells and in different mouse backgrounds (C57BL/6 and BALB/c). These cells use a canonical germline encoded Vγ6Vδ1 TCR. All previous reports on Vγ6Vδ1 γδT cells have used PCR-based strategies or a monoclonal Ab recognizing all $Vδ1^{+}$ γδT cells (including Vγ5/Vδ1 γδT cells) (42, 52). Here we describe a simple gating strategy to specifically analyze Vγ6Vδ1 γδT cells using CD3. This new strategy allowed us to determine new features about their phenotype and factors regulating their development/homeostasis.

Of interest, breeding mice in a germ-free environment profoundly affected the $CD3^{bright}$ γδT 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 $IL\text{-}1R1^{+}$ γδT cells through a signaling pathway involving the proto-oncogene Vav1 (53). Given the potential role of this population in antimicrobial immunity (9, 21, 52), this observation is in line with the consensus that chronic exposure to antibiotics results in transient immunodeficiency and increased susceptibility to (super)-infections (54).

How microbiota impacts on CD3^{bright} γδT cells remains unclear, although these organisms appear to promote IL-7 production under homeostatic conditions (55), a factor important to maintain RORγt expression in innate lymphocytes (56). Interestingly, we have demonstrated that CD3^{bright} γδT cells are completely dependent on RORγt expression for their development and maintenance in the periphery. Moreover, these cells have a bright expression of IL-7R compared to other leukocytes and, in line with a recent report (28) and our unpublished data, this population promptly responds to IL-7, which may be a selective growth factor for γδT17 cells (28). Since Notch1 signaling triggers CD127 expression and IL-7 signaling (57, 58), the intense expression of this receptor may result from the involvement of Notch1 during the development of γδT17 cells (9) and therefore, based on our findings, CD3^{bright} γδT cells. However in the same study, the authors demonstrated that RORγt expression was dispensable for the development and maintenance of γδT17 cells. The reason for this discrepancy is likely to be due to the fact that we focused on the CD3^{bright} γδT subset while the previous report analyzed the entire pool of γδT17 cells that may contain both RORγt-dependent (innate Vγ6^{+} cells) and -independent (acquired Vγ4^{+} cells) γδT cells. Consistent with this idea, γδT17 cells (CD27^{-}) from the conventional population were only minimally affected by the absence of RORγt expression (not shown).

The level of CD3 expression is also intriguing and suggests that these cells may have a unique CD3/TCR complex. Consistent with this observation, a previous report had demonstrated that the 17D1 mAb, which primarily detects Vγ5Vδ1 TCR, can cross-react with Vγ6Vδ1 TCR if the structure is first stabilized with an anti-Cδ mAb suggesting an alternate structure for this CD3/TCR complex (42). Thus, the differential CD3 expression between the Vγ6Vδ1 TCR and the other γδTCRs 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 FcεRIγ (44, 45) cannot be considered as CD3^{bright} γδ T cells from mice deficient for this signaling chain have a normal distribution pattern. Furthermore, the canonical germline encoded Vγ6Vδ1 TCR expressed with the high level of CD3 expressed suggests that this TCR does not engage any recombination processes or positive selection during ontogeny. This observation is important regarding the current controversial hypotheses concerning thymic development of γδT17 cells. While studies suggest that γδT17 arise from thymus during embryonic life and persist in adults through self-renewal process (9, 19, 35), the requirement of TCR ligation in their development is still debated (22, 24, 59). A recent study elegantly demonstrated that γδT17 can produce IL-17 in the embryonic thymus prior to TCR rearrangement suggesting that these cells may be selected in a TCR-independent manner (19). By contrast, a previous report suggested that γδT17 selection might require a weak TCR engagement (24). Overall, our data mainly support the first hypothesis and emphasize the innate imprint of the CD3^{bright} γδT cells.

There remains a critical question about the real function of the TCRs expressed on γδT17 cells, especially on Vγ6Vδ1 T cells (60). It seems clear that TCR engagement by anti-CD3 antibody resulted in cytokine release (IL-17A and IL-22), indicating that this TCR is functional. While soluble factors like IL-1β and IL-23 appear sufficient to induce IL-17A production by highly purified Vγ6Vδ1 T cells, we cannot exclude the possibility that the anti-TCRδ 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} γδT cells

were more sensitive to CD3 triggering compared to conventional γδ and αβ T cells (Figure 5 and not shown). While the higher level of CD3 expressed by this subset is the simplest explanation for this hyperreactivity, it may also reflect their memory phenotype which may render them more sensitive to TCR signaling pathway (61). However, further studies will be required to understand if 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 Vγ6Vδ1 could only produce T_H17-related cytokines, indicating a fully differentiated subset 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 our best 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 $CD3^{bright}$ γδT 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 cell signature. However further studies are required to understand if these populations can be somehow related. Such a classification across

species would represent a significant step in understanding the functional dichotomy of γδT cell subsets.

The presence of Vγ6Vδ1 T cells in various organs has been demonstrated during early life (42). Thus it is possible that Vγ6Vδ1 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 $CD3^{bright}$ γδ 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 $CD3^{bright}$ γδT cells after microbial insults is astounding and indicates that this population is likely to be involved in protection against pathogens especially at mucosal sites. Given that $CD3^{bright}$ γδ T cells represent the major sources of T_H17 cytokines during the early course of *S. pneumoniae* infection, it is now important to investigate the functions of these cells in this context and more broadly during various pulmonary disorders. Accordingly, we have identified a subset of γδ T cells with a potent ability to produce IL-17 and with putative key functions in a broad range of IL-17-driven pathologies.

Acknowledgments

We thank Shin-Foong Ngiow, Josette Fontaine, Deborah Roseingrave 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 1013667). 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 (454569). 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 (454569). DIG was supported by an NHMRC Senior Research Fellowship (1020770). NAG was supported by a Leukaemia Foundation of Australia postgraduate scholarship.

The authors have no financial conflicts of interest.

References

1. Bonneville M, O'Brien RL, & Born WK (2010) 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 (2010) Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 10(7):479-489.
3. Kolls JK & Khader SA (2010) The role of Th17 cytokines in primary mucosal immunity. *Cytokine Growth Factor Rev* 21(6):443-448.
4. Kolls JK & Linden A (2004) Interleukin-17 family members and inflammation. *Immunity* 21(4):467-476.
5. Cho JS, *et al.* (2010) 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 (2009) IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* 123(5):986-994; quiz 995-986.
7. Dejima T, *et al.* (2011) 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 (2006) 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 (2007) 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 (2012) IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur J Immunol* 42(9):2221-2231.
11. Murugaiyan G & Saha B (2009) Protumor vs antitumor functions of IL-17. *J Immunol* 183(7):4169-4175.
12. Ma Y, *et al.* (2011) 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 (2009) 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, *et al.* (2011) Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118(1):129-138.
15. Fenoglio D, *et al.* (2009) 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, *et al.* (2012) 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, *et al.* (2008) 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 (2012) Development and function of interleukin 17-producing gammadelta T cells. *Ann N Y Acad Sci* 1247:34-45.
19. Haas JD, *et al.* (2012) 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 (2009) Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31(2):321-330.

21. Simonian PL, *et al.* (2009) 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 (2009) Cutting edge: Gammadelta intraepithelial lymphocytes of the small intestine are not biased toward thymic antigens. *J Immunol* 182(12):7348-7351.
23. Shibata K, *et al.* (2011) 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 (2011) 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. Petermann F, *et al.* (2010) gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism. *Immunity* 33(3):351-363.
26. Do JS, *et al.* (2010) Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism. *J Immunol* 184(4):1675-1679.
27. Lalor SJ, *et al.* (2011) 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.
28. Michel ML, *et al.* (2012) 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.
29. Sutton CE, *et al.* (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31(2):331-341.
30. Ivanov S, *et al.* (2012) 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.
31. Paget C, *et al.* (2007) 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.
32. Kedzierska K, Turner SJ, & Doherty PC (2004) 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.
33. Lefranc MP, *et al.* (1998) IMGT, the International ImMunoGeneTics database. *Nucleic Acids Res* 26(1):297-303.
34. Paget C, Chow MT, Duret H, Mattarollo SR, & Smyth MJ (2012) Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *J Immunol* 188(8):3928-3939.
35. Ribot JC, *et al.* (2009) 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.
36. Ivanov, II, *et al.* (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126(6):1121-1133.
37. Manel N, Unutmaz D, & Littman DR (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9(6):641-649.
38. Born WK, Yin Z, Hahn YS, Sun D, & O'Brien RL (2010) Analysis of gamma delta T cell functions in the mouse. *J Immunol* 184(8):4055-4061.
39. O'Brien RL & Born WK (2010) gammadelta T cell subsets: a link between TCR and function? *Semin Immunol* 22(4):193-198.
40. Heilig JS & Tonegawa S (1986) Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* 322(6082):836-840.
41. Elliott JF, Rock EP, Patten PA, Davis MM, & Chien YH (1988) The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature* 331(6157):627-631.
42. Roark CL, *et al.* (2004) 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.

43. Thibault G & Bardos P (1995) 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.
44. Koyasu S, *et al.* (1992) 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.
45. Liu CP, *et al.* (1993) 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.
46. Andrews DM, *et al.* (2011) Homeostatic defects in interleukin 18-deficient mice contribute to protection against the lethal effects of endotoxin. *Immunol Cell Biol* 89(6):739-746.
47. Lee KA, *et al.* (2008) 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.
48. Michel ML, *et al.* (2007) Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* 204(5):995-1001.
49. Lindqvist M, Persson J, Thorn K, & Harandi AM (2009) The mucosal adjuvant effect of alpha-galactosylceramide for induction of protective immunity to sexually transmitted viral infection. *J Immunol* 182(10):6435-6443.
50. Noda K, *et al.* (2010) 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.
51. Haas JD, *et al.* (2009) CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *Eur J Immunol* 39(12):3488-3497.
52. Hamada S, *et al.* (2008) Importance of murine Vdelta1gammadelta T cells expressing interferon-gamma and interleukin-17A in innate protection against Listeria monocytogenes infection. *Immunology* 125(2):170-177.
53. Duan J, Chung H, Troy E, & Kasper DL (2010) 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 (2012) Antibiotics, microbiota, and immune defense. *Trends Immunol* 33(9):459-466.
55. Shalapour S, *et al.* (2010) Commensal microflora and interferon-gamma promote steady-state interleukin-7 production in vivo. *Eur J Immunol* 40(9):2391-2400.
56. Vonarbourg C, *et al.* (2010) Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. *Immunity* 33(5):736-751.
57. Balciunaite G, Ceredig R, Fehling HJ, Zuniga-Pflucker JC, & Rolink AG (2005) The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. *Eur J Immunol* 35(4):1292-1300.
58. Magri M, *et al.* (2009) Notch ligands potentiate IL-7-driven proliferation and survival of human thymocyte precursors. *Eur J Immunol* 39(5):1231-1240.
59. Itohara S, *et al.* (1990) Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature* 343(6260):754-757.
60. Kapsenberg ML (2009) Gammadelta T cell receptors without a job. *Immunity* 31(2):181-183.
61. Farber DL (2009) Biochemical signaling pathways for memory T cell recall. *Semin Immunol* 21(2):84-91.

Figure legends

Figure 1: Distribution of $CD3^{bright}$ γδ T cells. *A*, hepatic cells from naïve C57BL/6 WT mice were analyzed by flow cytometry based on CD3 and TCRγδ expression. *B*, cells from lungs, thymus, spleen, lymph nodes, bone marrow and peripheral blood were analyzed as in panel *A* (*upper panel*). The mean percentage ± SEM of $CD3^{bright}$ γδ T cells in total γδ T cells from various organs of three independent experiments (3-5 mice/experiment) is shown in the *lower panel* ND, not detected.

Figure 2: Phenotype of pulmonary $CD3^{bright}$ γδ T cells *vs* conventional γδ T cells. Pulmonary γδ T cell subsets from naive 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), RORγt (F) and NKG2D (G) expression. Of note, expression of IL-23R (D) and RORγt (F) were assessed by the use of reporter mice using IL-$23R^{+/-}$-GFP knock-in and ROR$\gamma t^{+/-}$-GFP knock-in mice, respectively. The histogram in panel *C* (*right panel*) represents the differential CD44 expression between $CD3^{bright}$ and conventional γδ 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} γδ T cells have an invariant TCR. *A,* PCR analysis of cDNA isolated from single cell-sorted CD3^{bright} and conventional γδ T cells and amplified with a set of primers specific for each TCRγ V-gene segment or the corresponding constant region (Cγ) (upper row) or specific for each TCRδ V-gene segment or the corresponding constant region (Cδ) (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 γδ TCRs including diversity (D) (for δ chains), junction (J) and CDR3 region.

Figure 4: Factors involved in CD3^{bright} γδ T cell homeostasis. *A,* frequency of γδ T cell subsets based on CD3 expression was analyzed by flow cytometry in mice bred either in GF or SPF conditions. The percentage of γδ T cell subsets is shown (*upper panel*). The average ± SEM of γδ 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 γδ T cell subsets was analyzed by flow cytometry in WT *vs* RORγt$^{-/-}$ mice. The percentage of γδ T cell subsets is shown (*left panel*). The mean ± SEM of γδ T cell subset frequencies pooled from two independent experiments is shown in the *right panel* (4 mice/group/experiment) **, $p < 0.01$ and ***, $p < 0.001$. *C,* frequency of γδ T cell subsets was analyzed by flow cytometry in WT *vs* IL-23p19$^{-/-}$ mice. The percentage of γδ T cell subsets is shown (*left panel*). The mean ± SEM of γδ T cell subset frequencies of one representative experiment out of two is shown in the *right panel* (5 mice/group/experiment).

Figure 5: Pulmonary $CD3^{bright}$ γδ T cells produce IL-17A *in vitro*. FACS-sorted pulmonary γδ T cell subsets and NK cells (positive control) were incubated with PMA (10 ng/ml) and ionomycin (1 μg/ml) (A) or pre-coated anti-CD3 (10, 5 and 1 μg/ml) (B) for 20 hours. Cytokine production in the supernatants was measured by CBA. Data represent the mean ± SEM of two pooled independent experiments performed in duplicate.

Figure 6: Hepatic $CD3^{bright}$ γδ T cells preferentially produce IL-17A *in vivo* after α-GalCer or LPS administration. *A*, WT mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and were killed at indicated time points. Gated hepatic γδ 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 ± SEM of three independent experiments for IFN-γ- and IL-17A-positive γδ T cell subsets is shown in the *lower panel* (3-4 mice/group/experiment). ***, $p < 0.001$. Total number of hepatic γδ T cell subsets was assessed. ***, $p < 0.001$. *B*, 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 A. One representative experiment out of two is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean ± SEM of two independent experiments for IL-17A-positive γδ T cell subsets is shown in the *lower panel* (3 mice/group/experiment). *C*, WT mice were pre-treated with an anti-IL23p19 or isotype control (agp3) (500 μg/mouse) at day -1 and 0 and then injected i.p. with vehicle or α-GalCer (2 μg/mouse) (*left panels*). WT or $IL\text{-}1R1^{-/-}$ mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) (*right panels*). Mice were sacrificed 2 hours after α-GalCer administration. Liver cells were treated as in panel A. The mean ± SEM of two pooled independent experiments for IL-17A-positive $CD3^{bright}$ γδ T cells is shown (4 mice/group/ experiment) ***, $p < 0.001$. *D*,

IL-23-depleted or controls WT mice were injected with LPS as in panel B. The mean ± SEM of two independent experiments for IL-17A-positive γδ T cell subsets is shown (3 mice/group/experiment). *, $p < 0.05$ and **, $p < 0.01$.

Figure 7: Pulmonary γδ T cells produce IL-17A *in vivo* after i.n. α-GalCer instillation 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 γδ 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 ± SEM for IFN-γ- and IL-17A-positive γδ T cell subsets is shown in the *lower panel* (6 mice/group/experiment). ***, $p < 0.001$. *B,* WT and TCR$\delta^{-/-}$ 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 γδ T cell populations in the lungs was determined by flow cytometry. One experiment out of two is shown (n = 7). **, $p < 0.01$. *D and E,* WT mice were infected i.n. with *Streptococcus pneumoniae* serotype 1 (2×10^6 bacteria) and were sacrificed at indicated time points. *D,* Gated pulmonary γδ 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 ± SEM for IL-17A-positive γδ T cell subsets is shown in the *lower panel* (>3 mice/group/experiment) *, $p < 0.05$ and **, $p < 0.01$. *E,* Percentage of γδ T cell subsets is shown

in the upper panel. Absolute number of IL-17A-positive γδ T cell subsets is shown in the lower panel. *, $p < 0.05$.

Table 1: Primers for mouse TCRγ and TCRδ chains

IMGT	Vγ	External	Internal
TRGV1	1	CGGTCACCAGAGMGACAGATG	ATGTCTCAACAAACTACAATCAACG
TRGV2	2	CGGTCACCAGASAGACAGATG	ATGTCGCAACAAACTACAATCAACG
TRGV3	3	CGGCAAAAACCAAATCAACAGT	CTACAATCAACRACCCTTAGGAG
TRGV4	4	GCAACCCCTACCCATRTTTTCT	GGAGTACAAGAAAATGGARGCAA
TRGV5	5	CCCGAAGACCAAACAAGACG	CCCTGAGACGAATCTTCTATGG
TRGV6	6	AAGTGTTCAGAAGCCCGATG	GACGAAAGATATGAGGCAAGGA
TRGV7	7	AATGTCAATCACCAAGCTAGAGG	TGCTTCGGAATGTGGAGGAG
TRGC Rev1	-	TGACAATACATCTGTGCTCTTTCC	GCAGCAGAAGGAAGGAAAATAGTA
TRGC Rev2	-	TGACAATGCAACTATGTTCTTTAGC	GCAACAGAAGGAAGRAAAATAGTG
	Vδ	External	Internal
TRDV4	1	CTTCTACTGCACTGTAACAGGAGG	CTCGACATTCAGAAGGCAACA
TRDV1	2	GAGCTGCAATTTATCGACATCTT	GGTATAGGCAGGGGGATGAT
TRDV2-2	4	GGACTGCACCTATGACACAAGTGA	TCKCCAAGAAGCATACAAGC

TRDV5	**5**	GTACAATGCGGATTCTCCAAAC	TATCGCAAAAGGCCAGACAG
TRDC Rev	**-**	GCACTGTACTTCCCGCTGG	ATCTTTCACCAGACAAGCAACA

Figure 1

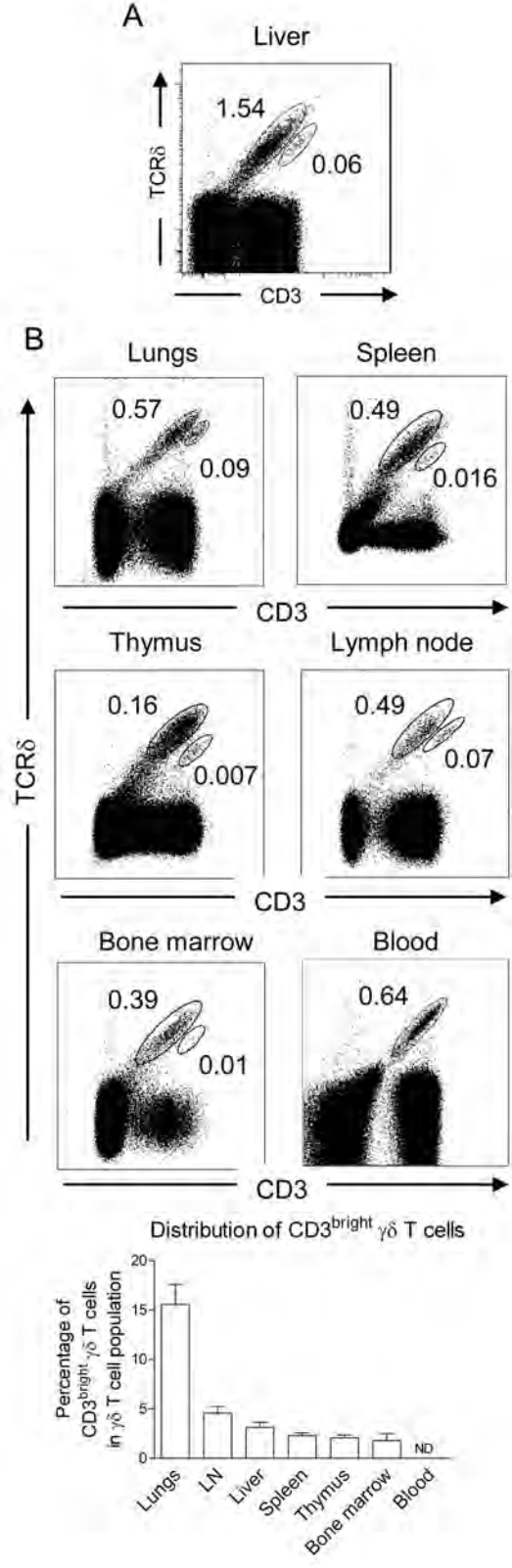

A
Liver
1.54
0.06
TCRδ
CD3
B
Lungs
0.57
0.09
Spleen
0.49
0.016
CD3
Thymus
0.16
0.007
Lymph node
0.49
0.07
TCRδ
CD3
Bone marrow
0.39
0.01
Blood
0.64
CD3
Distribution of CD3bright γδ T cells
Percentage of CD3bright γδ T cells in γδ T cell population
20
15
10
5
0
ND
Lungs
LN
Liver
Spleen
Thymus
Bone marrow
Blood

Figure 2

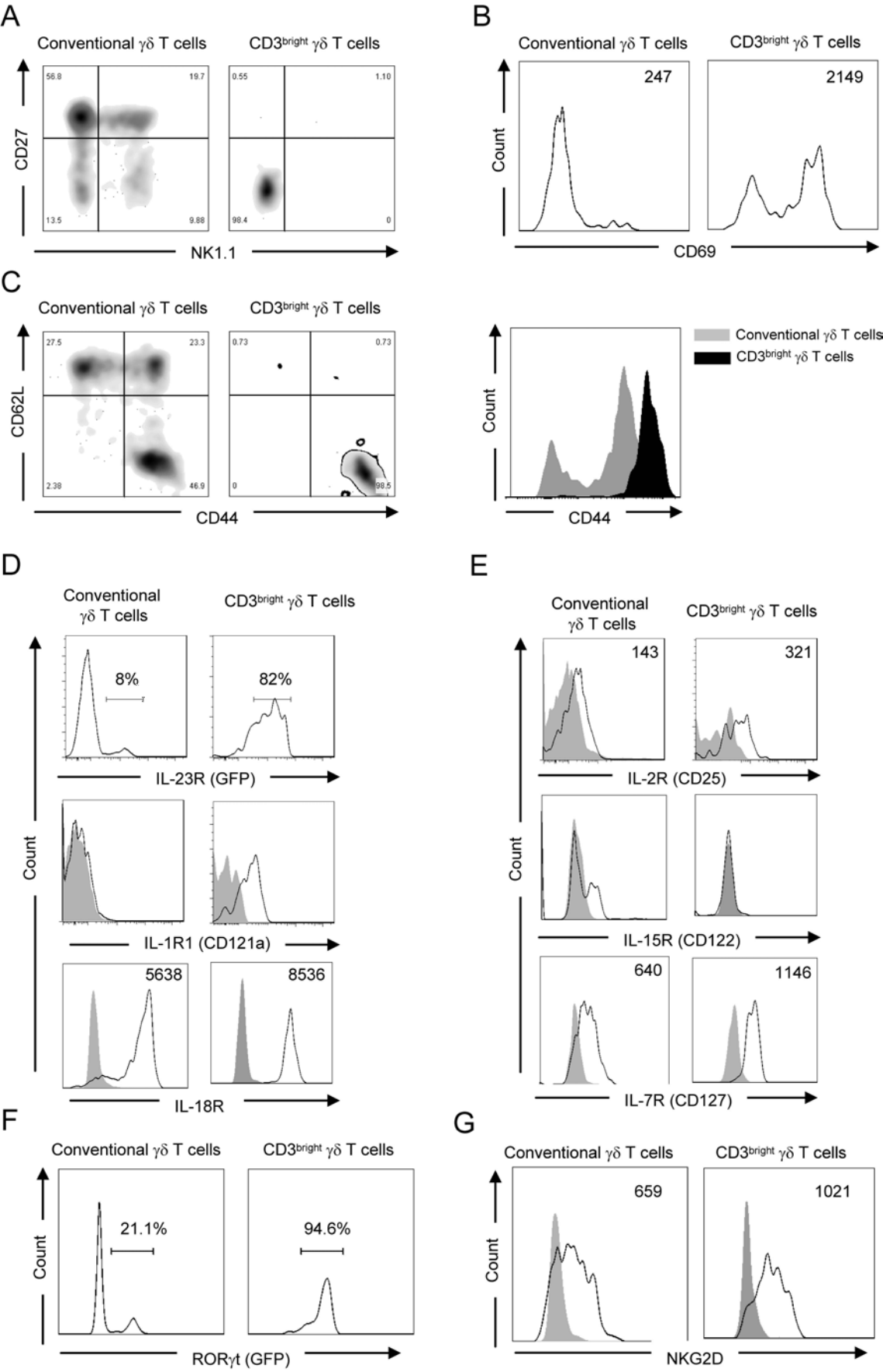
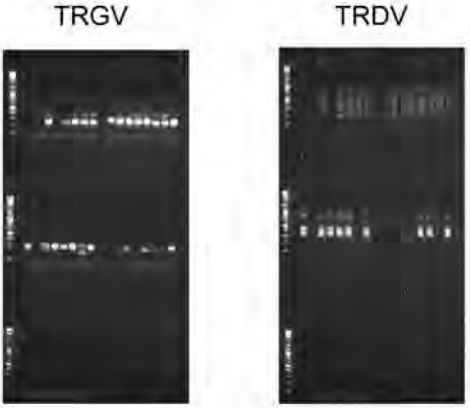

A
Conventional γδ T cells
CD3bright γδ T cells
CD27
NK1.1
56.8
19.7
13.5
9.88
0.55
1.10
98.4
0
B
Conventional γδ T cells
CD3bright γδ T cells
Count
CD69
247
2149
C
Conventional γδ T cells
CD3bright γδ T cells
CD62L
CD44
27.5
23.3
2.38
46.9
0.73
0.73
0
99.5
Count
CD44
Conventional γδ T cells
CD3bright γδ T cells
D
Conventional γδ T cells
CD3bright γδ T cells
8%
82%
IL-23R (GFP)
Count
IL-1R1 (CD121a)
5638
8536
IL-18R
E
Conventional γδ T cells
CD3bright γδ T cells
143
321
IL-2R (CD25)
Count
IL-15R (CD122)
640
1146
IL-7R (CD127)
F
Conventional γδ T cells
CD3bright γδ T cells
Count
21.1%
94.6%
RORγt (GFP)
G
Conventional γδ T cells
CD3bright γδ T cells
659
1021
Count
NKG2D

Figure 3

A



B

Subsets	Clones	Vγ	CDR3γ	Jγ	Vδ	CDR3δ	Dδ	Jδ
Conventional γδT cells	1	2	CAVWMRYSSGFHKVF	2	5	CASGYSWHIGGIRSSSTDKLVF	2	1
	2	1/4	-	-	5	CASGVYGTLYRRDTDKLVF	1	1
	3	1/4	-	-	4	CALMERGAFPPYRRDKGTDKLVF	1	1
	4	1/4	-	-	5	CASGYIGGIRATDKLVF	2	1
	5	2	CAVCRLARKF#KVF	2	2	CALMLYGLLSEGPTTDKLVF	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
CD3bright γδT 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

Figure 4

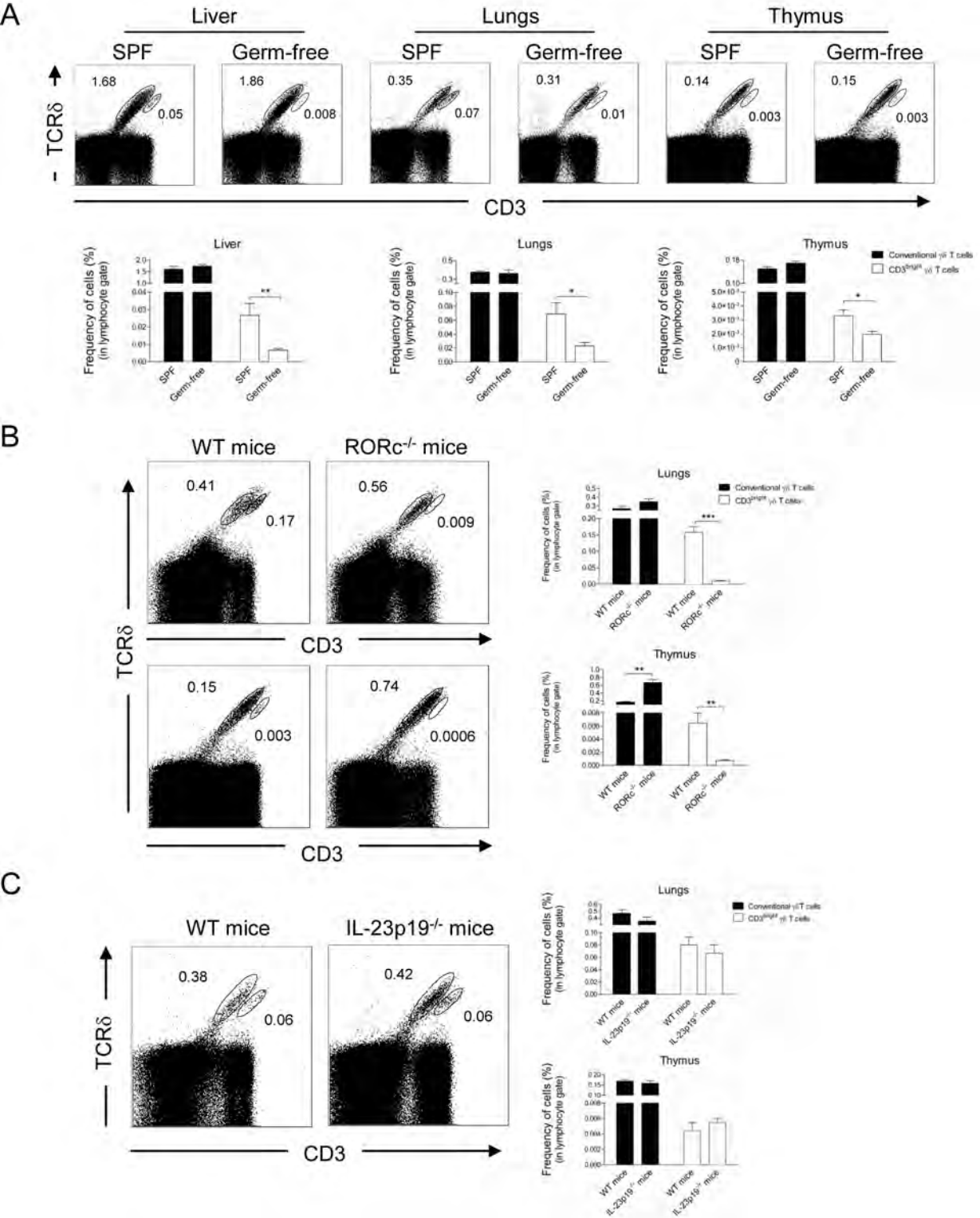

A
Liver
Lungs
Thymus
SPF
Germ-free
1.68
0.05
1.86
0.008
0.35
0.07
0.31
0.01
0.14
0.003
0.15
0.003
TCRδ
CD3
Frequency of cells (%) (in lymphocyte gate)
Conventional γδ T cells
CD3high γδ T cells
B
WT mice
RORc-/- mice
0.41
0.17
0.56
0.009
0.15
0.003
0.74
0.0006
C
IL-23p19-/- mice
0.38
0.06
0.42
0.06

Figure 5

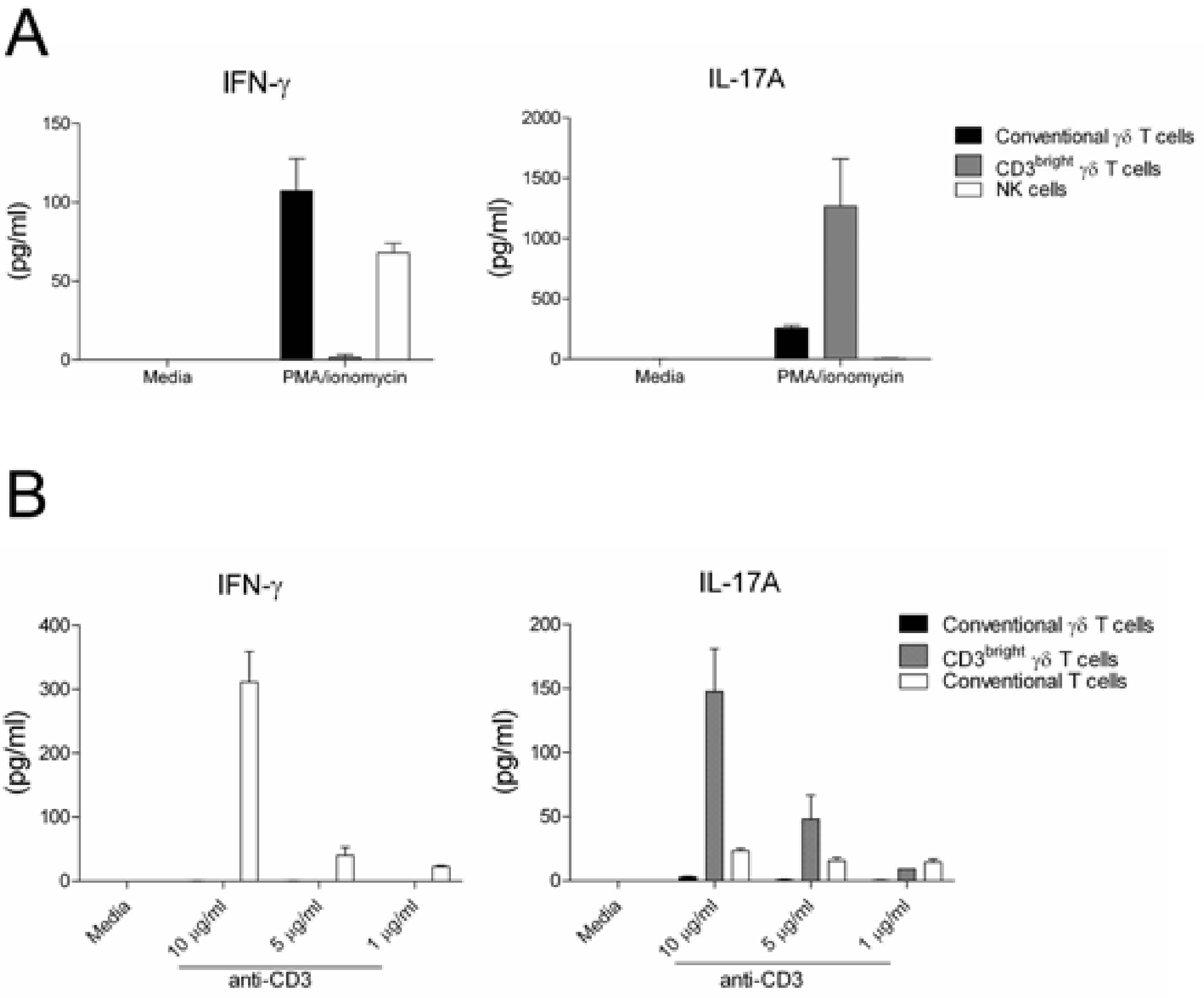

A
IFN-γ
(pg/ml)
150
100
50
0
Media
PMA/ionomycin
IL-17A
2000
1500
1000
500
0
Media
PMA/ionomycin
Conventional γδ T cells
CD3bright γδ T cells
NK cells
B
IFN-γ
(pg/ml)
400
300
200
100
0
Media
10 μg/ml
5 μg/ml
1 μg/ml
anti-CD3
IL-17A
200
150
100
50
0
Media
10 μg/ml
5 μg/ml
1 μg/ml
anti-CD3
Conventional γδ T cells
CD3bright γδ T cells
Conventional T cells

Figure 6

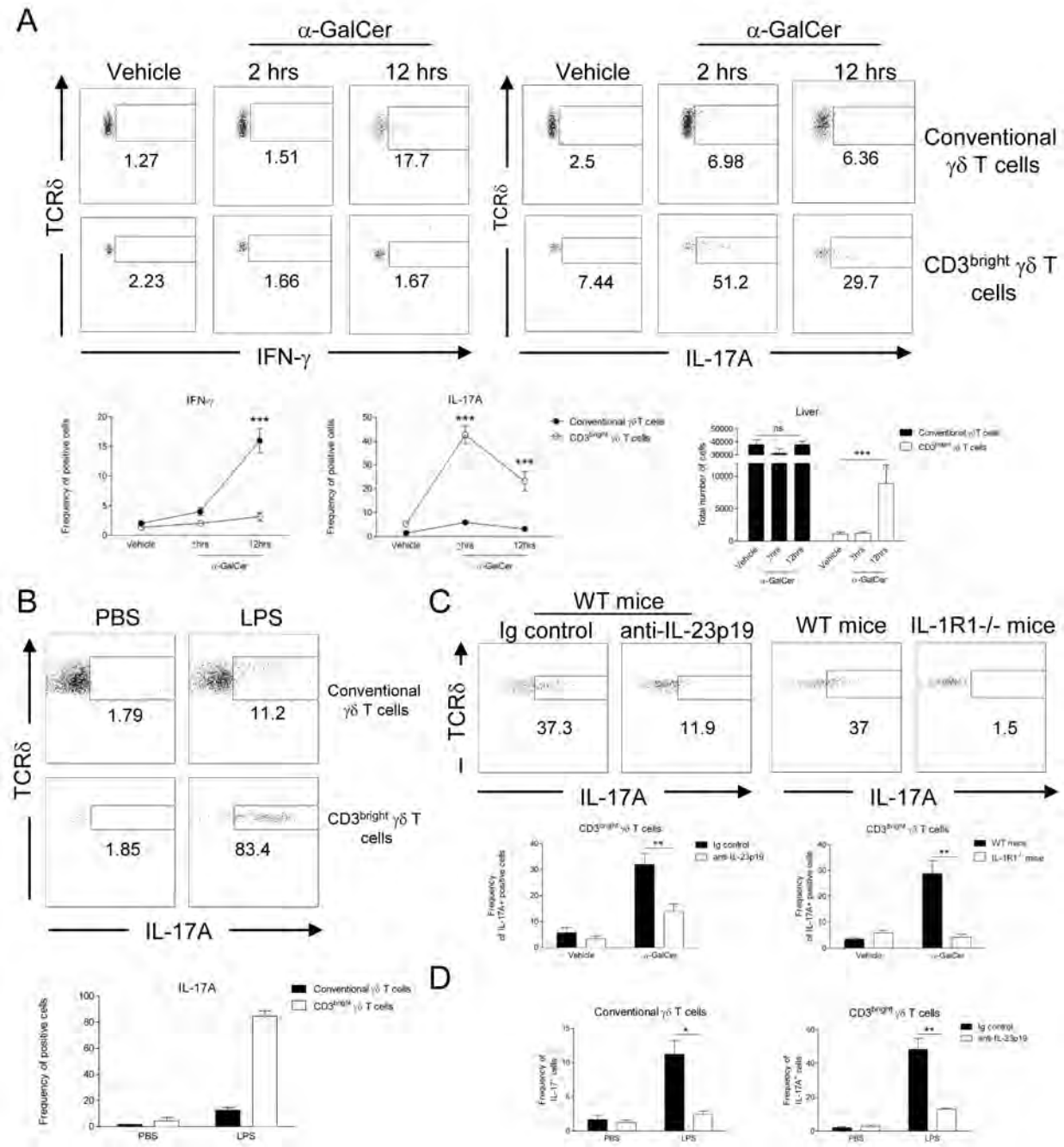
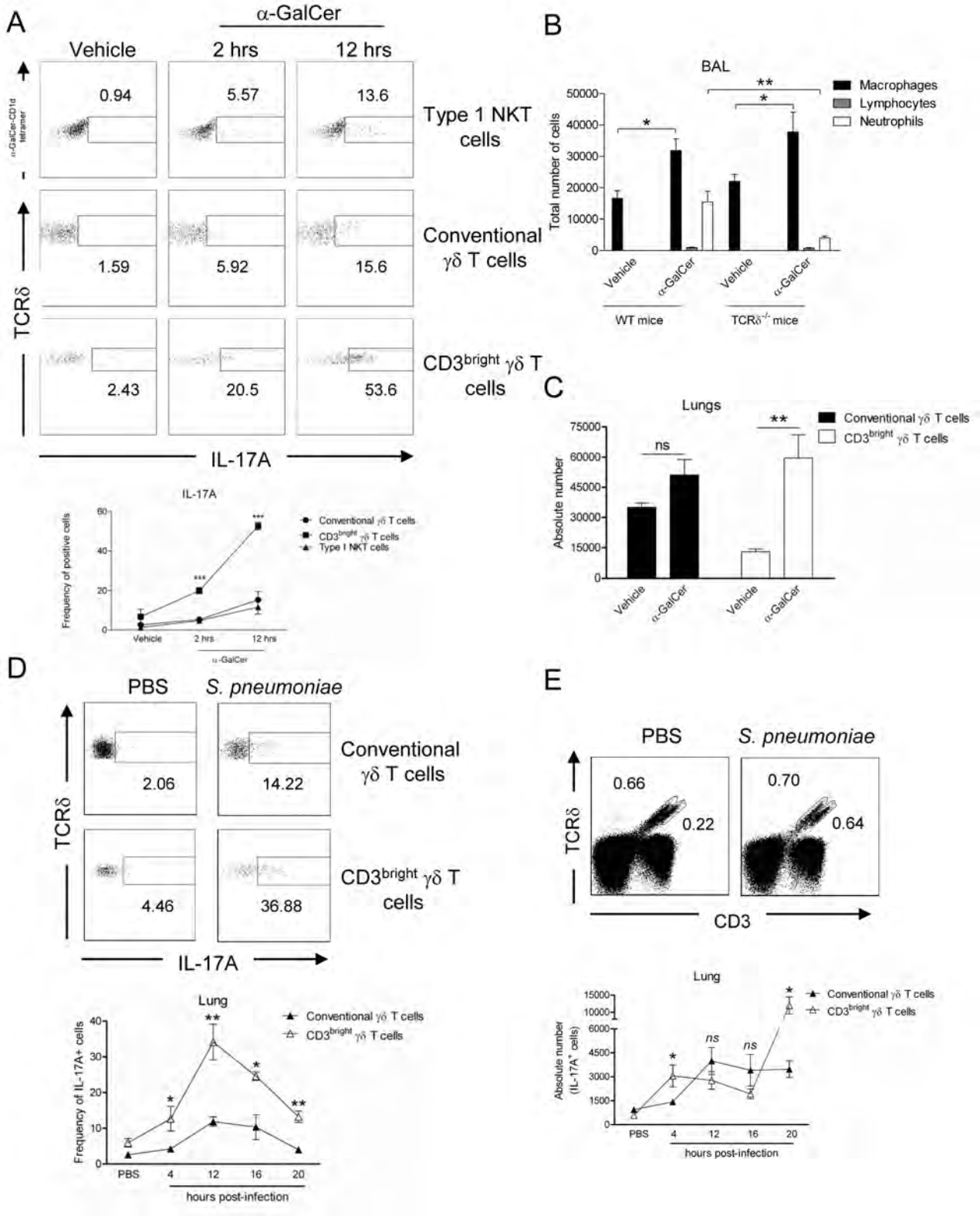

A
α-GalCer
Vehicle
2 hrs
12 hrs
TCRδ
1.27
1.51
17.7
2.23
1.66
1.67
IFN-γ
Conventional γδ T cells
CD3bright γδ T cells
2.5
6.98
6.36
7.44
51.2
29.7
IL-17A
IFN-γ
IL-17A
Liver
Conventional γδ T cells
CD3bright γδ T cells
Frequency of positive cells
Total number of cells
ns
***
B
PBS
LPS
1.79
11.2
1.85
83.4
C
WT mice
Ig control
anti-IL-23p19
IL-1R1-/- mice
37.3
11.9
37
1.5
**
Ig control
anti-IL-23p19
WT mice
IL-1R1-/- mice
D
Conventional γδ T cells
CD3bright γδ T cells
*
**

Figure 7


A
α-GalCer
Vehicle
2 hrs
12 hrs
0.94
5.57
13.6
Type 1 NKT cells
1.59
5.92
15.6
Conventional γδ T cells
2.43
20.5
53.6
CD3bright γδ T cells
α-GalCer-CD1d tetramer
TCRδ
IL-17A
IL-17A
Frequency of positive cells
Conventional γδ T cells
CD3bright γδ T cells
Type I NKT cells
B
BAL
Total number of cells
Macrophages
Lymphocytes
Neutrophils
Vehicle
α-GalCer
WT mice
TCRδ-/- mice
C
Lungs
Absolute number
ns
Conventional γδ T cells
CD3bright γδ T cells
D
PBS
S. pneumoniae
2.06
14.22
Conventional γδ T cells
4.46
36.88
CD3bright γδ T cells
TCRδ
IL-17A
Lung
Frequency of IL-17A+ cells
hours post-infection
E
PBS
S. pneumoniae
0.66
0.22
0.70
0.64
TCRδ
CD3
Lung
Absolute number (IL-17A+ cells)
hours post-infection

Supplemental information:

CD3 expression reveals a gamma/delta T cell subset with specialized functions

Christophe Paget, Melvyn T. Chow, Nicholas A. Gherardin, Paul A. Beavis, Adam P. Uldrich, Helene Duret, Maya Hassane, Fernando S.F. Guimares, Goeffrey R. Hill, Paul Neeson, David S. Ritchie, François Trottein, Gabrielle T. Belz, Dale I. Godfrey and Mark J. Smyth

Supplemental Inventory:

Supplemental Figures:

Figure S1

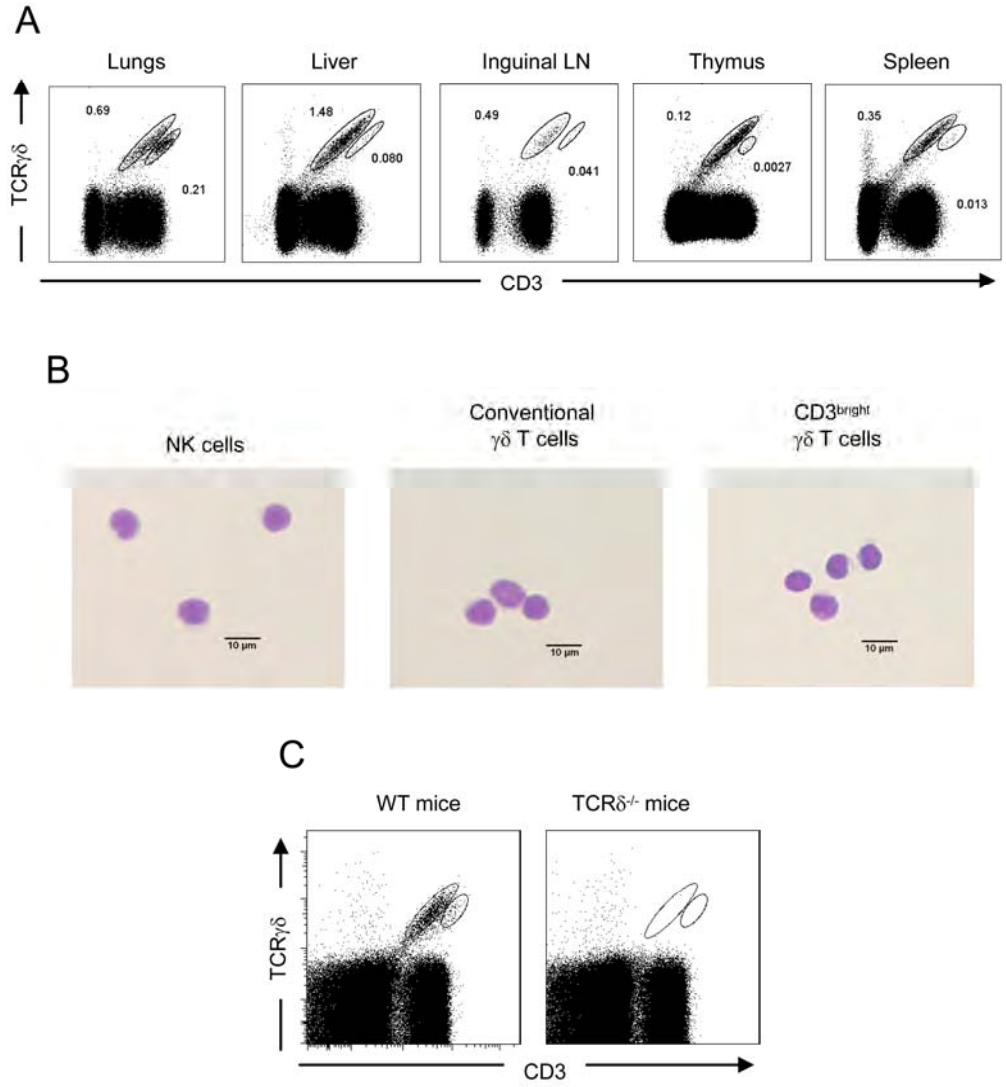


Figure S1: CD3^{bright} γδ T cells are authentic γδ T cells and conserved in different mouse backgrounds.

A, Cells from naïve Balb/c WT mice were analyzed by flow cytometry based on CD3 and TCRγδ expression.

B, Morphology and Giemsa staining of cytospun FACS-sorted C57BL/6 WT lymphocyte populations.

C, Cells from naïve C57BL/6 WT and TCRδ$^{-/-}$ mice were analyzed by flow cytometry based on CD3 and TCRγδ expression.

Figure S2

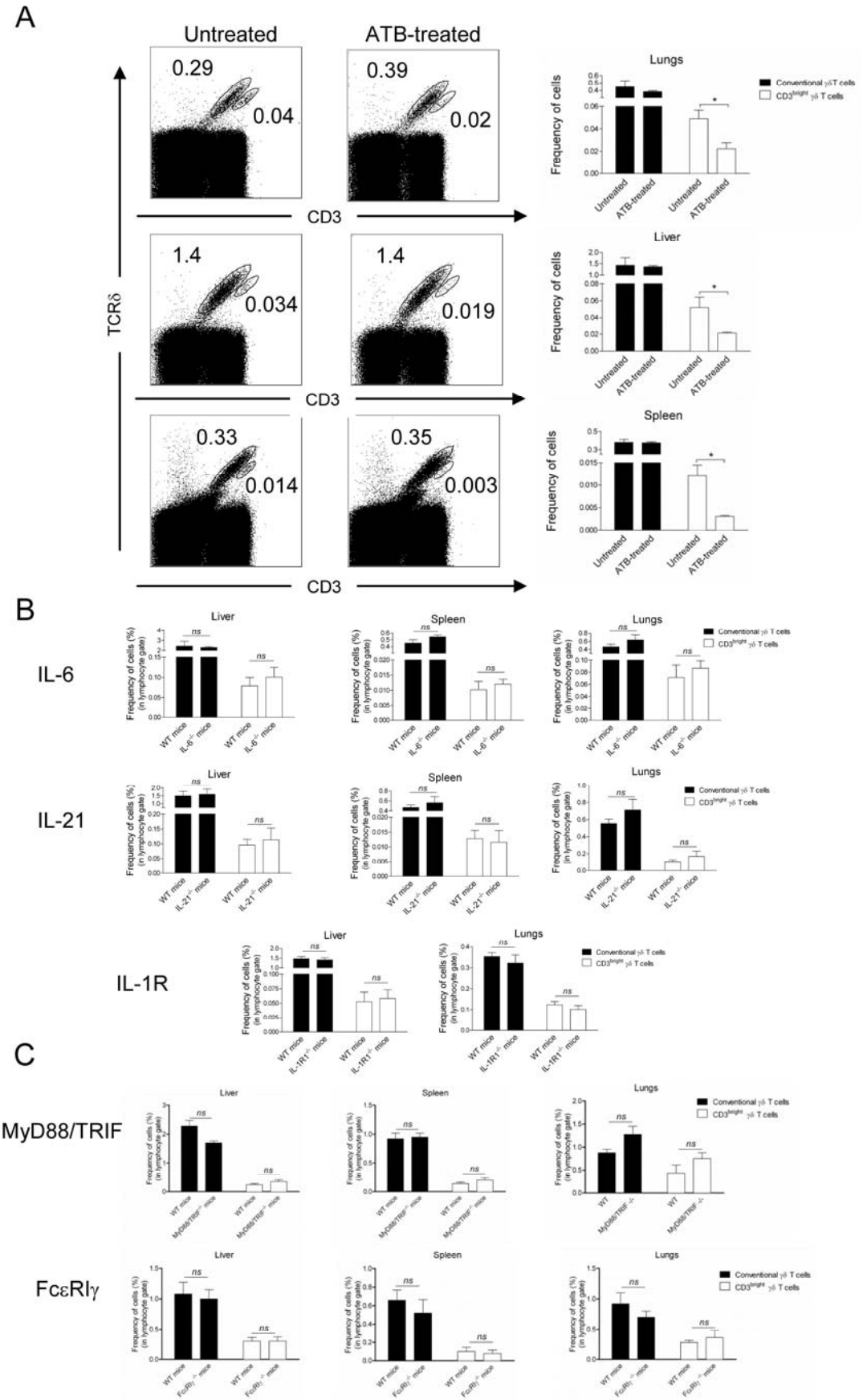

A
Untreated
ATB-treated
0.29
0.04
0.39
0.02
1.4
0.034
1.4
0.019
0.33
0.014
0.35
0.003
TCRδ
CD3
Lungs
Liver
Spleen
Frequency of cells
Untreated
ATB-treated
Conventional γδ T cells
CD3bright γδ T cells
B
IL-6
IL-21
IL-1R
C
MyD88/TRIF
FcεRIγ
WT mice
ns

Figure S2: Factors influencing CD3^{bright} γδ T cells homeostasis

A, **Effect of chronic exposure to antibiotics on CD3^{bright} γδ T cells**. 5-week-old C57BL/6 mice were exposed three weeks with or without neomycin in the drinking water. Frequency of CD3^{bright} and conventional γδ T cells in each group was evaluated by flow cytometry in different organs. Of note, antibiotic exposure did not impact on the total number of cells in each organ.

B, **IL-6, IL21 and IL-1 do not impact on CD3^{bright} γδ T cell frequency**. Frequency of γδ T subsets was analyzed by flow cytometry in WT *vs* IL-6$^{-/-}$, IL-21$^{-/-}$ and IL-1R1$^{-/-}$ mice. The percentage of γδ T cell subsets is shown. The mean ± SEM of γδ T cell subset frequencies of one representative experiment out of two is shown (*n* = 5-6).

C, **TLR-signalling and FcεRIγ are not involved in CD3^{bright} γδ T cell homeostasis**. Frequency of γδ T subsets was analyzed by flow cytometry in WT *vs* MyD88/TRIF$^{-/-}$ mice. The percentage of γδ T cell subsets is shown. The mean ± SEM of γδ T cell subset frequencies of one representative experiment out of two is shown (*n* = 5-6).

Figure S3

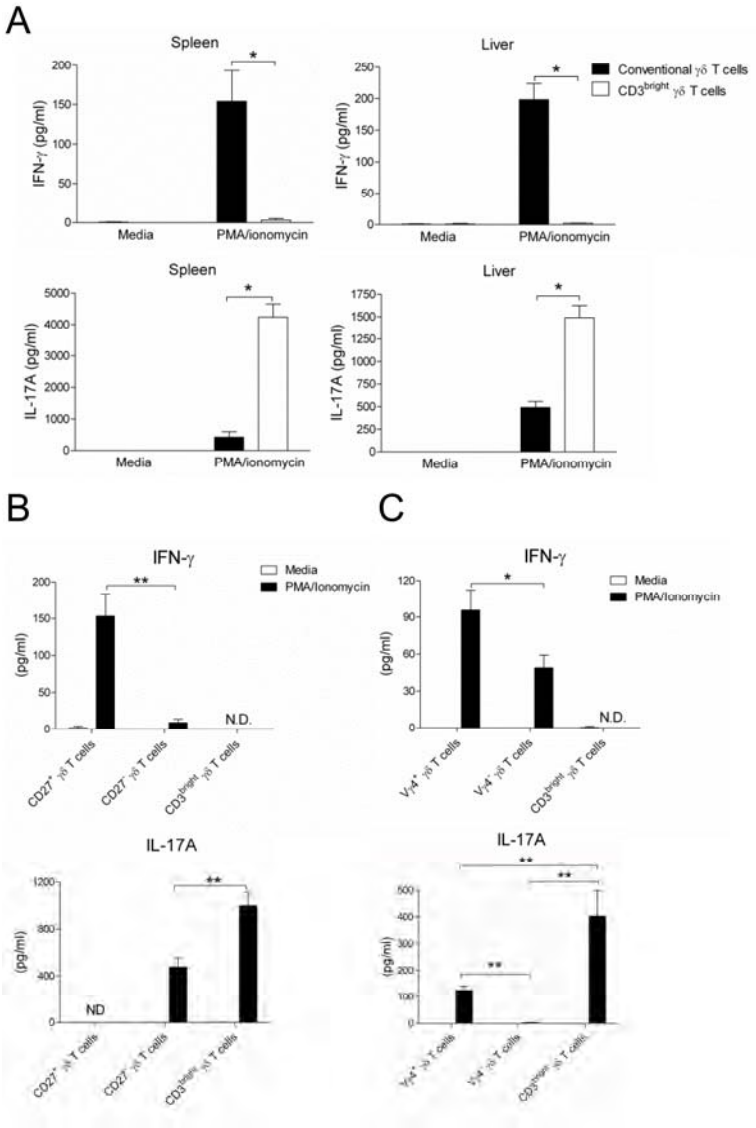


Figure S3: Capacity of CD3^{bright} γδ T cells *vs* other γδT17 populations to produce IL-17A.

A, FACS-sorted splenic or hepatic γδ T cell subsets were incubated with PMA (10 ng/ml) and ionomycin (1 μg/ml). Data represent the mean ± SEM of two independent experiments performed at least in duplicate. *, $p < 0.05$.

B and C, FACS-sorted pulmonary γδ T cell subsets were incubated as in panel *A*. Cytokine production in the supernatants was measured by CBA. Data represent the mean ± SEM of two independent experiments performed at least in duplicate. *, $p < 0.05$ and **, $p < 0.01$.

Figure S4

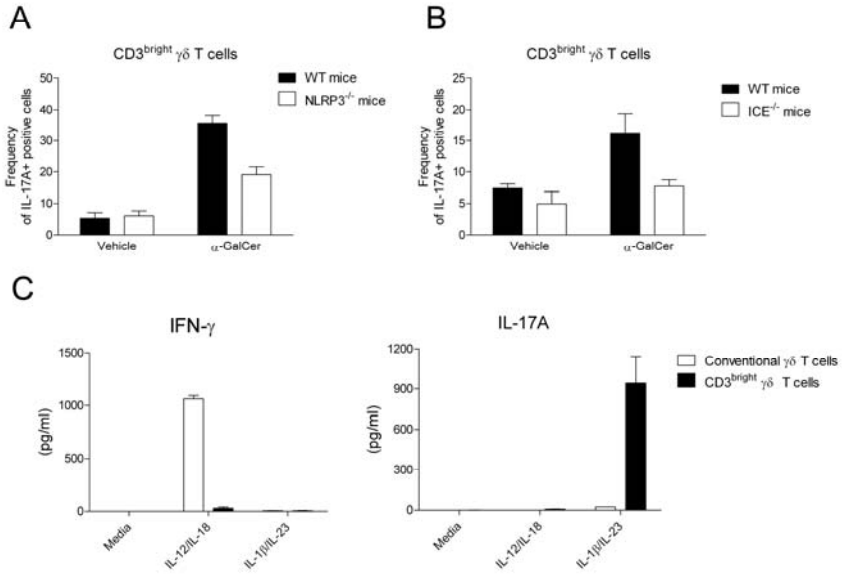


Figure S4: IL-17 production by CD3^{bright} γδ T cells requires NLRP3 inflammasome components *in vivo*.

A-B, WT, NLRP3$^{-/-}$ (A) or ICE$^{-/-}$ (B) mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and were sacrificed after 2 hrs. Liver cells were treated with GolgiStop for another 2 hrs. Gated CD3^{bright} γδ T cells were screened for IL-17A production. The pooled mean ± SEM of two independent experiments for IL-17A-positive cells subsets is shown (n = 5-6).

C, FACS-sorted γδ T cell subsets were incubated or not with IL-12/IL-18 (50 pg/ml and 1 ng/ml respectively) or IL-1β/IL-23 (1 ng/ml for both) for 20 hrs. Cytokine production in the supernatants was measured by CBA. Data represent the mean ± SEM of two experiments performed in duplicate.

Figure S5

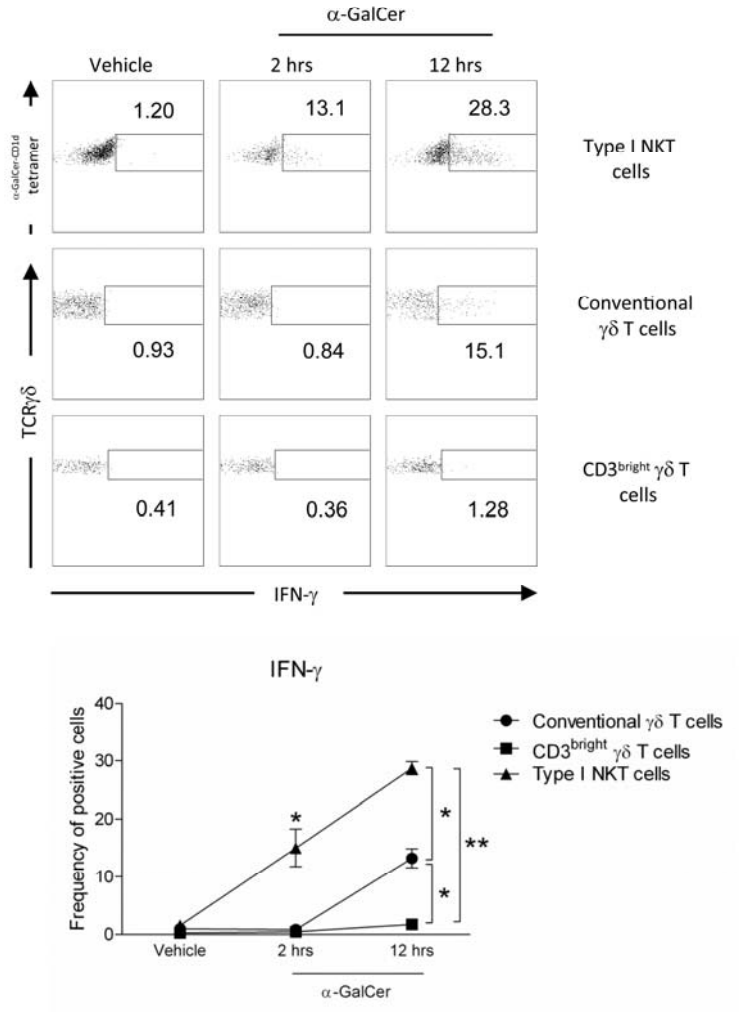


Figure S5: CD3^{bright} γδ T cells do not produce IFN-γ following intranasal instillation of α-GalCer

WT mice were injected i.n. with vehicle or α-GalCer (500 ng/mouse) and were sacrificed at indicated times. Pulmonary cells were treated with GolgiPlug for another 2 hrs. Gated innate-like T cells were screened for IFN-γ production. One representative experiment out of three is shown (*upper panel*). The pooled mean ± SEM of three independent experiments for IFN-γ-positive cells subsets is shown (6 mice/group/experiment) in the lower panel. *, $p < 0.05$ and **, $p < 0.01$.

Figure S6

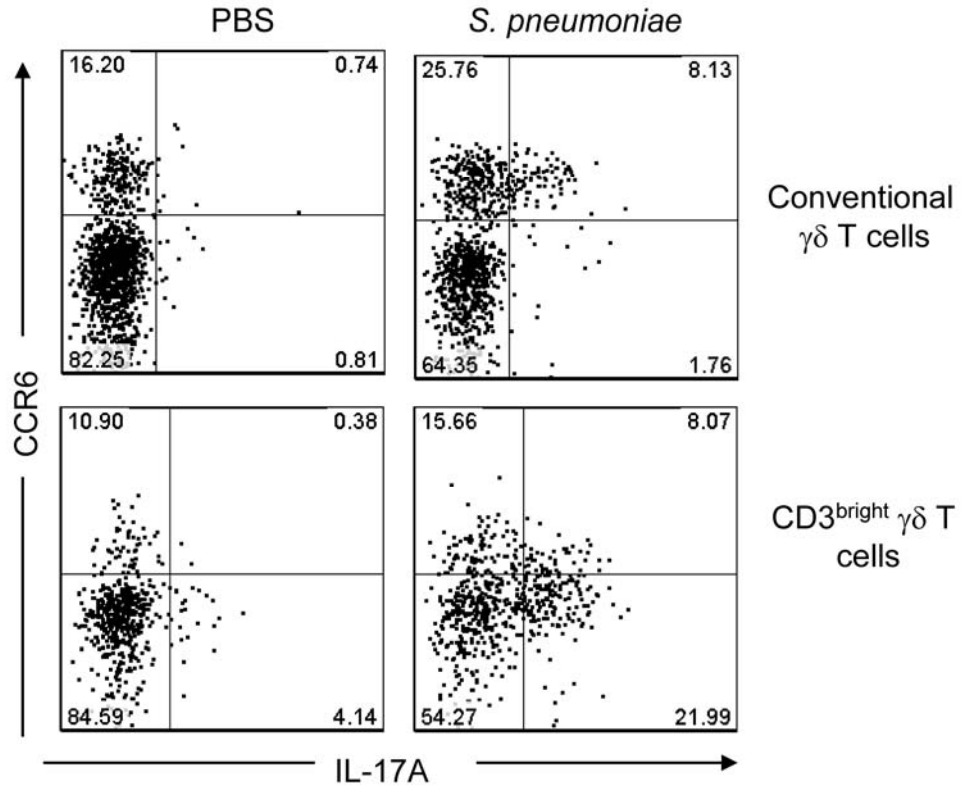


Figure S6: CCR6 expression on conventional, but not on CD3^{bright} γδ T cells correlated with their ability to produce IL-17.

WT mice were infected i.n. with *S. pneumoniae* serotype 1 (2 x 10^6 bacteria) and were sacrificed at indicated time points. Gated pulmonary γδ T cell subsets were screened for IL-17A production based on their CCR6 expression. One representative experiment out of two is shown. Gates were set based on the staining with isotype control.