Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

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Joana Luísa de Barros Martins

Dissertação

Mestrado em Bioquímica

Especialização em Bioquímica Médica

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Tese orientada pela Doutora Karine Serre e pelo Professor Doutor António Ferreira

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I. RESUMO

O contacto contínuo com o exterior e a vasta exposição a patogéneos levou a que, durante a evolução, organismos superiores desenvolvessem mecanismos de protecção especializados. O sistema imunitário, constituído por um vasto número de células e tecidos especializados, pode ser subdividido em dois grupos – sistema imune inato ou adaptativo – de acordo com o tipo de resposta que é desenvolvido. As células do sistema imune inato, maioritariamente localizadas em locais de alto contacto com o exterior (p.e. o epitélio), caracterizam-se pela sua resposta não especializada – e portanto, inata – que permite um rápido controlo de infecções. As células T $\gamma\delta$, natural killer (NK) e macrófagos são exemplos de células pertencentes a este tipo de imunidade. Por outro lado, células do sistema imune adaptativo como as células T $\alpha\beta$ e células B, possuem a capacidade de desenvolver uma resposta mais duradora e especializada, que lhes permite não só combater mais eficazmente uma infecção mas também desenvolver uma memória imunitária.

Uma característica funcional partilhada entre as células do sistema imune inato e adaptativo é a produção de citocinas. A sua produção e consequente secreção permitem uma melhor e mais eficaz resolução de uma infecção, através do recrutamento e activação de células do sistema imune para o local afectado. Os linfócitos T $\gamma\delta$, em conjunto com os linfócitos T $\alpha\beta$ CD4, produzem citocinas pro-inflamatórias como o IFN-γ e a IL-17A. Todavia, as células T $\gamma\delta$ distinguem-se dos últimos pela sua rápida capacidade de resposta a estímulos externos, através da produção e secreção de citocinas na periferia. Esta característica advém da diferenciação dos seus progenitores em células T $\gamma\delta$ especializadas na produção de IFN-γ ($CD27^+$) ou de IL-17A ($CD27^-$) no timo, ao passo que linfócitos T CD4 abandonam o timo sob a forma de linfócitos *naïve*, necessitando de estímulos prolongados para que se diferenciem em linfócitos T efectores. Os mecanismos moleculares que regulam os processos de diferenciação e produção de citocinas encontram-se extensamente estudados em linfócitos T $\alpha\beta$. O factor de transcrição T-bet, por exemplo, regula a diferenciação dos linfócitos T CD4 em linfócitos Th1 CD4 bem como a produção de IFN-γ. Contudo, pouco é conhecido relativamente aos reguladores da produção de citocinas nos linfócitos T $\gamma\delta$. Por esta razão propusemo-nos estudar o papel de alguns dos factores de transcrição na regulação da transcrição de citocinas como o IFN-γ e IL-17A.

O paralelismo entre os linfócitos Th1 CD4 produtores de IFN-γ e os linfócitos T $\gamma\delta27^+$ e entre os linfócitos Th17 CD4 produtores de IL-17A e os linfócitos T $\gamma\delta27^-$ levou-nos a escolher como objecto de estudo vários factores de transcrição associados à produção destas citocinas nos

linfócitos T CD4. Tendo isto, escolhemos o T-bet e o factor de transcrição Eomes, envolvido na produção de IFN-γ em linfócitos T CD8. Associados à diferenciação dos linfócitos T CD4 em Th17, vários factores de transcrição foram ainda associados à regulação da transcrição de IL-17A nestas mesmas células. De entre estes os factores de eleição para o nosso estudo foram o RORγt, BATF e o RORα. De modo a estudar o papel de cada factor de transcrição nos linfócitos T γδ usámos ratinhos geneticamente modificados, nos quais cada um destes factores de transcrição se encontra deletado. Para o estudo do factor de transcrição Eomes, recorremos ao uso de ratinhos nos quais apenas células que expressam o gene RAG1 - células T e B - apresentam a deleção específica para o factor de transcrição Eomes, visto a deleção total ser letal durante a embriogénese.

Através de estudos *in vitro* usando diversas condições de estimulação (citocinas e anticorpos monoclonais), foi-nos possível aferir quais destas permitem induzir a produção de IFN-γ em linfócitos T γδ27^{+} e de IL-17A em linfócitos T γδ27^{-}. De forma a determinar o impacto que a ausência de cada factor de transcrição teria na produção de IFN-γ e IL-17A, optámos pelo o uso dos anticorpos monoclonais anti-CD3 e anti-CD28 no caso dos linfócitos T γδ27^{+} e das citocinas IL-1β mais IL-23 no caso dos linfócitos T γδ27^{-}. É de salientar que na presença de IL-1β e IL-23 os linfócitos T γδ27-, como já anteriormente descrito pelo laboratório, demonstraram não só a capacidade de produzir outras citocinas em simultâneo – como a IL-17F e a IL-22 – mas também a capacidade de co-produzir IFN-γ e IL-17A.

Os nossos resultados *in vitro* demonstraram que, na ausência de T-bet, os linfócitos T γδ27^{+} possuem a mesma capacidade de produção de IFN-γ apesar de o número de linfócitos que produz esta citocina ser menor. Este resultado sugere que os linfócitos T γδ27^{+} possam depender parcialmente do T-bet. Por outro lado, a ausência do factor de transcrição Eomes não revelou diferenças na produção de IFN-γ, demonstrando que os linfócitos T γδ27^{+} não requerem Eomes para a produção de IFN-γ. Já a produção de IFN-g pelos linfócitos T γδ27^{-} não sofreu quaisquer alterações na ausência tanto do factor de transcrição T-bet assim como na do Eomes. Estes resultados sugerem então que estes linfócitos apresentam um diferente mecanismo na regulação da transcrição do IFN-γ. Contudo, as experiências *in vitro* nem sempre replicam a realidade *in vivo*. Por esta razão e com o intuito de compreender os mecanismos de regulação da produção destas citocinas recorremos a dois modelos murinos – *EAE* e *Listeria monocytogenes* – nos quais foi demonstrada a presença de linfócitos T γδ27^{-} produtores de IFN-γ. Contudo, o modelo experimental de esclerose múltipla (EAE), não nos permitiu observar a produção de IFN-γ em linfócitos T γδ. No entanto, através do uso do

modelo de infecção (*Listeria monocytogenes*) foi possível observar que a ausência de T-bet leva a um desaparecimento completo das células co-produtoras de IFN-γ e IL-17A. Estes resultados sugerem assim, que linfócitos T γδ27$^-$ requererem o factor de transcrição T-bet para a produção de IFN-γ.

A análise da produção de IL-17A em linfócitos T γδ27$^-$ demonstrou que estes são inteiramente dependentes do factor de transcrição RORγt. Verificámos ainda que esta dependência não se encontra limitada à produção de IL-17A estendendo-se também ao desenvolvimento de uma população específica de linfócitos T γδ27$^-$ que apenas produz IL-17A, designada CCR6$^+$γδ27$^-$. Foi também possível observar que os linfócitos T γδ27$^-$ não requerem os factores de transcrição BATF e RORα para a produção de IL-17A. Durante esta análise, contrariamente ao esperado, foi ainda detectado um aumento no número de linfócitos T γδ27$^-$ que produziam IL-17A na ausência do RORα. Estes resultados levaram-nos a prosseguir um estudo mais detalhado sobre o papel do RORα em linfócitos T γδ27$^-$.

Para estudar o papel do factor de transcrição RORα em células T γδ foram usados ratinhos que expressam uma forma truncada, não funcional, do factor RORα. Ao investigar o papel do RORα nos linfócitos T γδ, deparámo-nos que o aumento no número de células produtoras de IL-17A não se devia a um impacto na diferenciação dos linfócitos T CCR6$^+$γδ27$^-$. No entanto, observámos um aumento da proporção de linfócitos T γδ expressando a cadeia Vγ4 (maioritariamente produtores de IL-17A) e uma diminuição na proporção de linfócitos T γδ expressando a cadeia Vγ1 (maioritariamente produtores de IFN-γ) no seu TCR. Este aumento foi não só detectado na periferia como também no timo, em linfócitos T γδ imaturos (CD24$^+$), sugerindo a existência de um papel do RORα no rearranjo das cadeias gamma do TCR dos linfócitos T γδ. Mais ainda, podemos observar que o aumento no número de linfócitos produtores de IL-17A, após estimulo com IL-1β mais IL-23 durante a noite, não se devia apenas a um aumento do número de linfócitos Vγ4, mas também a um aumento do número de células produtoras de IL-17A de entre a população Vγ1$^-$Vγ4$^-$, que acreditamos ser constituída por linfócitos T Vγ6$^+$ γδ. Estes resultados sugerem assim que o factor de transcrição RORα possui dois papéis distintos em linfócitos T γδ, através do controlo do rearranjo das cadeias gamma dos linfócitos T γδ e da regulação da produção de IL-17 em linfócitos T Vγ1$^-$Vγ4$^-$ γδ.

Em suma, os nossos resultados sugerem a existência de algumas semelhanças nos mecanismos de regulação da produção de IL-17A e IFN-γ entre os linfócitos Th1 CD4 e os linfócitos T γδ27$^+$ e

γδ27$^-$. Especificamente, estes resultados parecem sugerir um papel do factor de transcrição RORa no desenvolvimento e produção de IL-17A em linfócitos T γδ.

Palavras-Chave: linófictos T gamma-delta, citocinas, factores de transcrição, IL-17A, IFN-γ

II. SUMARY

γδ T cells are a major source of the proinflammatory cytokines IFN-γ and IL-17, together with αβ CD4 T helper cells. However in stark contrast with the latter, one of the main features of γδ T cells is their briskness at cytokine production in the periphery. This is mostly attributable to their programmed phenotype(s) that distinguishes IFN-γ-producing $CD27^+$ ($\gamma\delta27^+$) and IL-17-producing $CD27^-$ ($\gamma\delta27^-$) subsets. Importantly, $\gamma\delta27^+$ cells are stably committed to express *Ifng* but not *Il17*, whereas $\gamma\delta27^-$ cells spontaneously make IL-17 but can be induced to produce IFN-γ (as well as IL-17F, IL-22, GM-CSF) under specific inflammatory conditions. Our understanding of the transcriptional regulators of the production of these cytokine in both γδ T cell subsets lags behind that of CD4 T cells. Here we show that γδ T cells do not rely entirely on the same transcriptional mechanisms for their functional differentiation. Remarkably, IFN-γ production by both $\gamma\delta27^+$ and $IL17^+\gamma\delta27^-$ T cells relies on the Th1 transcription factor (TF) T-bet but is dispensable of Eomes. This suggests a T-bet-dependent module for IFN-γ production shared by conventional CD4 T cells and innate-like γδ T cells. By contrast and to our surprise, regarding the Th17 TFs and IL-17 production, neither BATF nor RORα showed to be required by $\gamma\delta27^-$ T cells, which appear to depend solely on RORγt. Interestingly, mice lacking RORα showed an increased number of IL-17-producing $V\gamma1^-V\gamma4^-$ cells upon stimulation as well as a change in the proportion of $V\gamma4^+$ and $V\gamma1^+$ γδ T cells. Together our findings illustrate a simpler mechanism for regulation of cytokine production by γδ T cells which allows a faster response to stimulus. In addition, our work revealed a role for RORα in the development of certain γδ T cell subsets as well as in the functional differentiation of IL-17-production.

Keywords: Transcription factors, cytokine production, IL-17, IFN-γ, γδ T cells.

The nomenclature for Vγ chain usage used in this thesis was described by Heilig and Tonegawa, 1986[1].

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
Blk	B lymphoid tyrosine kinase
BrdU	5-Bromodeoxyuridine
CCR	CC chemokine Receptor
CD	Cluster of Differentiation
DC	Dendritic Cell
DETC	Dendritic Epidermal T Cell
DN	Double Negative
DP	Double Positive
E15	Embrionic day 15
EAE	Experimental Autoimune Encephalomyelitis
Eomes	Eomesodermin
FACS	Fluorescence-Activated Cell Sorting
γδ27-	gamma-delta CD27- T cells
γδ27+	gamma-delta CD27+ T cells
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HMG	High Mobility Group
I.v.	Intravenous
IFN	Interferon
IL	Interleukin
ILC	Innate Lymphoid Cells
Iono	Ionomycin
KO	Knock-Out mice
LN	Lymph Nodes
mAb	mono-clonal Antibody
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
NKT	Natural Killer T cells
OVN	Over-Night
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate buffered saline
PLO	Primary Lymphoid Organ

PMA	Phorbol 12-myristate 13-acetate
RAG	Recombinant Activating Gene
ROR	Retinoc-acid Orphan Receptor
S.c.	Subcutaneous
sg/sg	Homozygous stagger mice
SLO	Secondary Lymphoid Organ
STAT	Signal Transducer and Activator of Transcription
TCR	T-Cell Receptor
TF	Transcription Factor
TGF	Transforming Growth Factor
Th	T helper
WT	Wild-type

IV. INTRODUCTION

1. GENERAL ASPECTS

As part of evolution, organisms acquired an immune system that gave them the ability to develop competent immune responses and thereby protection against pathogens. It comprises different tissues and cells that regulate two arms of the immune system, innate and adaptive immunity. While innate immunity recognizes wide conserved molecular patterns of pathogens and develops a quick response, adaptive immunity acts slowly but in a highly specific manner by recognition of antigens through generation of a vast range of antigen specific receptors[2]. Originated through a differentiation process called haematopoiesis taking place in primary lymphoid organs (PLOs), immune cells then migrate to the secondary lymphoid organs (SLOs). SLOs function as coordinating platforms where immune cells are exposed and primed to antigens from invading pathogens. Spleen and lymph nodes are some examples of SLOs.[3]

T and B lymphocytes are the main players in the adaptive immune system. Both arise from a common lymphoid progenitor sharing the ability to produce their antigen-receptors by somatic V, D, J gene rearrangements[2]. B cells develop in the bone marrow and migrate to the SLOs where upon the right stimulus they secrete their antigen-receptor in the form of antibodies[4]. T cell development occurs in the thymus starting from a bone marrow common progenitor giving rise to two subsets – $\alpha\beta$ T cells and $\gamma\delta$ T cells. As the largest population, $\alpha\beta$ T cells (conventional T cells) leave the thymus as naïve cells to be activated through their T cell receptor (TCR) by antigen presenting cells (APCs) in the SLOs, leading to their functional differentiation in the periphery[5,6]. On the contrary, it has been showed that innate-like $\gamma\delta$ T cells leave the thymus already differentiated and functionally competent to populate mucosal and epithelial tissues where they will respond rapidly to invasion or stress situations[7].

In order to protect and achieve pathogen clearance T lymphocytes can respond to infection by producing and secreting small proteins called cytokines. They play a critical role in the interface between innate and adaptive immunity. They trigger recruitment and activation of more lymphoid myeloid cells to site of infection[8].

2. CYTOKINES AND IMMUNITY

Innate and adaptive immunity have different weights in immune surveillance and regulation of immune responses but act in similar way. Cells from both systems secrete cytokines as a way of communication and signaling within near or remote lymphoid and non-lymphoid organs[8]. Cytokines can play different roles in protection by like pro- and anti-inflammatory roles and be involved in several others beyond the immune system such as cell growth, tissue repair after infection and consequently inflammation and in the control of tissue homeostasis[9].

Interferon-γ (IFN-γ) and interleukin-17 (IL-17) are two cytokines critical against invading pathogens. However when deregulated they lead to major tissue damage such as chronic inflammation and autoimmune disorders. These cytokines can be produced by several lymphoid cells[10,11],including IFN-γ producing T helper 1 (Th1) or IL-17 producing T helper 17 (Th17) αβ T cells and γδ T cells[12].

2.1 INTERFERON-GAMMA

Originally called macrophage-activating factor, IFN-γ was discovered as an agent which interferes with viral replication together with other IFNs from the type I family (IFN-α and IFN-β), although to a lesser extent [11]. Produced by $CD4^+$ T cells after specific differentiation, $CD8^+$ T cells and Natural Killer (NK) cells, IFN-γ can be also produced by γδ T, B cells, NKT and professional APCs[11,13,14].

The specific effect of IFN-γ on macrophages and cytotoxic cells consists in mounting an effective response towards intracellular pathogens such as viral infections – West Nile virus, Herpes virus and influenza virus[11,14,15], bacterial infections - *Listeria monocytogenes, Bordetella pertussis and Mycobacterium tuberculosis*[14,15] – and parasitic infections - *Toxoplasma gondii* , *Plasmodium chabaudi and Leishmania major*[14,15]. To achieve these functions, IFN-γ triggers macrophages to produce reactive oxygen species (ROS) targeting extracellular pathogens during phagocytosis and reactive nitrogen intermediate (RNI) which targets intracellular pathogens[11,14].

Besides, IFN-γ has also been shown to contribute to tumor development control *in vivo*[16]. This is linked to the up-regulation of cell-surface class I and II major histocompatibility complex (MHC) that leads to increase cytotoxic T cell recognition of tumor peptides and CD4+ T helper cell activation[11,13,14]. IFN-γ can also promote the B cell isotype class switching to immunoglobulin G2a (IgG2a) and suppress IgG1 and IgE production[11,13,14,17]. Controlling

infected cells apoptosis and suppression of proliferation through cell cycle arrest, at the G1/S boundary, are two other main functions of IFN-γ [11].

As an anti-inflammatory role, IFN-γ can inhibit neutrophil recruitment and consequently play a role in autoimmune diseases. In the experimental model of collagen induced arthritis (CIA) the absence of this cytokine associates with an excessive proportion of neutrophils which leads to an aggravation of the joint lesion, compared to the murine *wild-type* controls were there is no manifestation of the disease[13,18].

2.2 INTERLEUKIN-17

Upon its discovery, IL-17A (here on referred as IL-17) was thought to be only produced by conventional αβ T cells[19]. In fact other cell types such as γδ T cells, natural killer T cells (NKT), eosinophils and neutrophils can also secrete IL-17 upon stimulation [20–22]. IL-17 belongs to a family comprising six ligands – IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F - and five receptors – IL-17RA, IL-17RB/IL-25R, IL-17RC, IL-17RD/SEF and IL-17RE [9,21]. IL-17 and IL-17F are two potent inflammatory cytokines that protect against extracelular pathogens, but are also involved in the development of autoimmune diseases and chronic inflammation.

In response to extracellular bacterial infections including *Klebsiella pneumoniae*, *Mycobacterium tuberculosis, Citrobacter rodentium*, *Echerichia coli, Candida albicans* [8,10,21], IL-17 and IL-17F recruit and activate other cells such as neutrophils and macrophages by inducing the production of chemokines by epithelial cells (CXCL1, CXCL2, CXCL8, CXCL10, CCL7, CCL20) other cytokines (IL-1β, IL-6, granulocyte-colony-stimulating factor, tumor necrosis factor-a (TNF-α), RANTES, monocyte chemoattractant protein-1, granulocyte-macrophage colony-stimulating factor (GM-CSF)), metalloproteinases (MMP18, MMP117-184), inflammatory effectors (acute-phase proteins, complement) and antimicrobial proteins (defensins, mucins) [8,10,21,23].

When deregulated, IL-17 leads to chronic inflammation or autoimmune diseases. Inflammatory bowel disease(IBD)[24], psoriasis[25], Rheumatoid arthritis (RA)[26], systemic lupus erythematosus (SLE)[27] and experimental autoimmune encephalomyelitis (EAE)[28] are some of these disorder were IL-17 coordinates tissue inflammation through induction of other pro-inflammatory cytokines, chemokines and metalloproteases cited before, which mediate tissue infiltration and destruction[8].

3. CONVENTIONAL T CELLS

3.1. DEVELOPMENT

Cells of the immune system originate mainly from a pluripotent precursor which arises initially from the fetal liver and subsequently from the bone marrow (BM) during development[29]. Most of the haematopoietic lineages achieve maturation in the BM while T cells need to migrate through blood to a specialized PLO, the thymus, in order to develop[29,30]. T cell progenitors arriving to the thymus lack the expression of the TCR (TCR^-) and T cell co-receptors CD4 and CD8 thus being referred to as double negative (DN). The first steps of T cell development are therefore TCR independent but driven by migration across distinct microenvironments in the thymus, which provide critical cues such as Notch ligands and interleukin-7 (IL-7)[30].

TCR^-DN cells can be subdivided into four successive maturation stages DN1, DN2, DN3, DN4 based on their expression of CD44, CD25 and c-kit (CD117) on the surface. DN1 cells ($CD44^+CD25^-$), also called early T-cell-lineage progenitors (ETPs), are a very heterogeneous population which present an extensive proliferative capacity and the ability to generate both the $\alpha\beta$ and $\gamma\delta$ lineages together with NK and DC lineages[31–33]. The ability to produce also cells of the B and myeloid lineages, although controversial, has also been suggested[34,35]. Activation of Notch signaling pathway in ETPs through binding of the Notch receptor 1 with delta ligands (DL) on thymus epithelial cells, marks the first irreversible step to the T cell lineage commitment at the expense of B lineage differentiation[36,37].

$CD44^+CD117^+CD25^-$ DN1 cells expand in the cortico-medullary junction which leads to the up-regulation of CD25 at the surface and transition to the DN2 stage ($CD44^+CD117^+CD25^+$)[29,30]. After, DN2 cells migrate into the cortex and start experiencing the first rearrangements of the TCRβ, TCRγ and TCRδ loci due to up-regulation of recombination-activating gene 1 (*Rag1*) and *Rag2* expression[29,38]. During migration through the sub-capsular zone, DN3 cells which have down-regulated the CD44 and CD117 becoming only $CD25^+$ start the selection and segregation into a TCR$\gamma\delta^+$ cell ($\gamma\delta$ T cell) or a TCR$\alpha\beta^+$ cell ($\alpha\beta$ T cell) lineage[6,29,30,39,40].

T cell development involves a stringent repertoire selection where only 1–3% of the progenitors prevails and ultimately exits the thymus to the SLOs[41]. By continuing rearrangement of the TCR *loci* during DN3 stage these cells, in order to continue into the next stage, are subjected to an intrinsic checkpoint to assess and ensure that the TCR is properly rearranged and the TCRβ chain is functional[5,42]. Also called as β-selection[43], this checkpoint

occurs after DN3 cells express the pre-TCR (TCRβ paired with an invariant pre-TCRα chain and CD3 signaling molecules)[29,39,42,44,45]. Proper assembly of this complex and downstream signalling leads to switch off RAG proteins[5] and down-regulation of CD25 expression[38].

Progression to the DN4 stage indicates a commitment to the αβ T cell lineage[46]. Cells that do go through these progression either expressed a pre-TCR or a dysfunctional γδTCR during the DN3 stage[47,48]. Promotion of cell survival, proliferation and differentiation through joint signals of the TCR and cytokines grants DN4 cells progression to immature single positive (ISP) cells with acquired small amounts of CD8 (Figure 1). ISP cells then rapidly progress to the double-positive (DP) stage by the acquisition of CD4 expression[5,6,29]. At this stage RAG genes are expressed once again for the rearrangement of the TCRα gene, allowing further progression into the SP stage[5,6].

DP cells displaying TCR αβ heterodimers through different levels of interaction of the TCR with peptide-MHCI or MHCII ligands in the thymus, can further differentiate into single-positive (SP) cells – $CD8^+$ or $CD4^+$ αβ T cells[5,6,49]. In the absence of TCR signalling, DP cells are subjected to apoptosis due to receptor neglect[5]. Engagement with low affinity between TCR and MHC class I/II allows cells to undergo a process called positive selection[50–52]. These cells, which present a low reactivity toward self-antigens are potentially reactive to foreign antigens, receive signals for survival and differentiation. By contrast, DP cells which engage strongly with the antigens presented by MHC class I/II molecules experience negative selection, leading to the deletion of self-reactive T cells through apoptosis, thereby avoiding triggering autoimmunity[53–55].

Commitment to either CD4 or CD8 lineage is accomplished by DP cells during positive selection[5,56]. Recognition by DP cells of MHC class I molecules drives these cells to commit to the CD8 lineage, while recognition of MHC class II molecules drives commitment to the CD4 lineage[5,46,55,57–59].

Selected $TCR\alpha\beta^+$ T cells migrate to the periphery where $CD8^+$ αβ T cells will perform their role as cytotoxic cells against infected/transformed cells[60], and $CD4^+$ αβ T cells will further differentiate in order to act as helpers to other immune cells, thereby orchestrating the immune response[61].

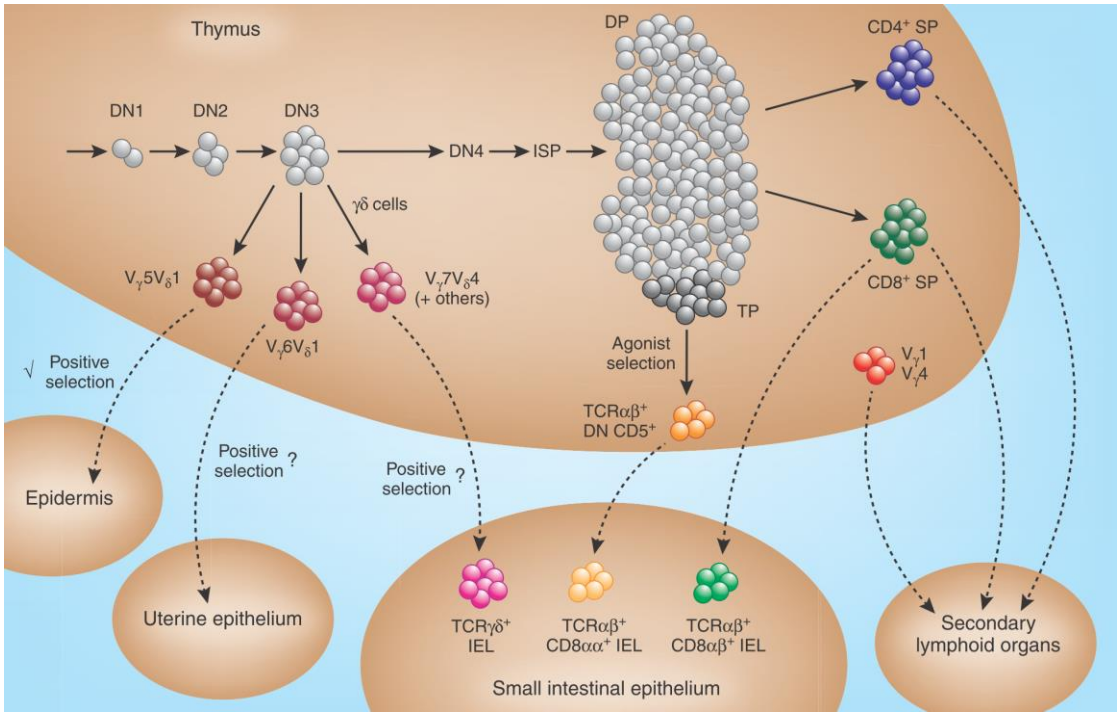


Fig. 1- T cell development in the thymus and homing tissues. Derived from the same progenitors, αβ T cells exhibit a longer and more complex developing pathway through the DN4, ISP, DP and then single positive stages, than γδ T cells. Their differences extend also to their homing tissues, since γδ T cells preferentially home in epithelial tissues and αβ T cells home only in lymphoid organs. (Adapted from Hayday, A. & Pennington, D. Nat. Immunol. 2007[6].)

3.2 CD4$^+$ αβ T CELLS

3.2.1 DIFFERENTIATION

As discussed before, CD4 T cells can produce multiple cytokines including IFN-γ or IL-17. These functions are acquired in the periphery upon antigen (Ag) encounter and segregate CD4 T cells into distinct T-helper (Th) subsets which include Th1, Th2, Th9, Th17, Th22, Tfh, iTreg[61–63]. Of note, strength in TCR-antigen engagement has also been proposed to be a key factor in the differentiation decision[64] and shown to regulate cytokine production[65,66].

CD4 T cell differentiation into T-helper cells relies on the specific expression of transcription factors (TF). Through binding on specific DNA-binding sequences (promoters, enhancers, insulators and silencers), TFs preform repressive or activating transcriptional functions specifically on certain gene targets. Consequently TFs play a pivotal role in regulating the program of differentiation and expression of selective profile of cytokines. To fulfil this regulation, TFs are subdivided according to their function: signal transducers and activators of transcription (STATs) proteins and master regulators (MRs)[62,67]. While STATs are activated by cytokine signaling, they induce MR expression, which will in turn regulate cytokine gene

expression[62]. Another important aspect of TF is to repress differentiation towards opposing Th subsets.

TH1 DEVELOPMENT

One of the first T helper cell subsets to be discovered was Th1, which mainly produce IFN-γ[68]. The differentiation into these cells is induced by IL-12 produced by innate immune cells[69]. Other cytokines such as IL-2, IL-15 and IL-18 can also sustain the signal towards IFN-γ production by these cells. Reliant on STAT1 and STAT4 to mediate cytokine signaling by IL-12 and IFN- γ respectively, Th1 $CD4^+$ T cells also count on *Tbox21* TF (T-bet) as their MR for their differentiation[70–72]. T-bet also mediates the induction of the β2 chain of the IL-12 receptor (IL-12R) expression, through STAT1 and IFN-γ signaling, thus amplifying IL-12 responsiveness[73,74] and the expression of IL-18R1 in synergy with STAT4[67,75]. IFN-γ itself produced by NK and T cells[66] can further trigger his production. Together with others TFs such as Hlx and Runx3, T-bet further promotes IFN-γ production[77,78]. On the other hand, T-bet is not only important for Th1 differentiation, but it has been extensively shown to have a suppressive function in differentiation towards Th lineages through suppression of their MRs [62,63]. By suppressing GATA3 expression, alone or in synergy with RUNX3, T-bet is able to inhibit Th2 polarization[77,79]; and through suppression of interferon regulatory factor 4 (IRF4) and retinoic acid receptor-related orphan receptor γ-t (RORγt)[80,81], T-bet inhibits Th17 polarization.

TH17 DEVELOPMENT

Activated $CD4^+$ T cells differentiated towards IL-17 production, Th17 cells[82–84], are known to require IL-23 for differentiation[85] and expansion[82,86,87]. TGF-β together with other pro-inflammatory cytokines such as IL-6 and IL-1β have also been shown to be important for Th17 differentiation[88–90]. IL-21 has also been pointed to promote Th17 differentiation[91–93]. Signal transducers such as STAT3, with different extents, mediate IL-6, IL-21 and IL-23 activation signal of RORγt and IL-23R[94–96]. As the master regulator of Th17 cells, RORγt is critical for the production of IL-17, IL-17F, IL-21, IL-1R and IL-23R by these cells[63,75,91,97–101]. However, despite the pivotal role of RORγt in Th17, other TF such RUNX1, IRF4 , BATF and RORα are also important for this differentiation since they may regulate RORγt expression and in synergy with it regulate cytokine production[102–105]. On the other hand, signaling through IFN-γ–STAT1, IL-4-STAT6 or IL-2-STAT5 pathways inhibits Th17 differentiation[106–108].

3.2.2 PLASTICITY AND CROSS-REGULATION

Differentiation towards a given T helper cell fate is a not a terminal irreversible step. Indeed, there are *in vitro* studies showing some degree of plasticity between T cell fates[109]. However, Th1 and Th2 cells are two fairly stable populations who lose their plasticity upon proliferation and further commitment to their fate[110]. On the other hand, Th17 cells present some plasticity since they can change phenotype when exposed to different cytokines[63,98,111–113]. While in the presence of TGF-β, IL-6, IL-1β and IL-23, fully differentiated Th17 sustain IL-17 production, when in presence of IL-12 or IL-4 these cells can change and differentiate into Th1 or Th2 cells respectively[63,81,111]. Developing Th17 cells were shown to change into IFN-γ and IL-17 double-producing cells *in vitro* when in the presence of IL-12 or IL-23 and through RUNX1, RUNX3 and T-bet signaling, giving rise to a "Th1-like" Th17 cells ($IL17^+$-IFN-γ^+ cells)[81,111,113,114]. These are also observed *in vivo*, in models like EAE and experimental colitis, where pathogenic IFN-γ-producers were shown to be derived from Th17 still producing IL-17.

4. γδ T CELLS

Firstly described in 1984[115], γδ T cells remain an enigmatic lineage only distinguishable by the expression of a TCR comprising a γ and a δ chain[6,40]. Initially thought to be another T cell subset involved in adaptive immunity, γδ T cells were redefined as "innate-like" lymphotcytes due to their unconventional development, pre-activated phenotype and tropism for epithelial tissues[116]. As with other unconventional T cells (like NKT cells), γδ T cells were also shown to recognize conserved non-proteic antigens that are upregulated by stressed cells[7,116]. As part of the innate system, γδ T cells play an important non-redundant role in the lymphoid immuno-surveillance. Namely, γδ T cells respond in a MHC-independent manner to stress-induced metabolites as well as to pathogen-associated patterns[7,117].

4.1 DEVELOPMENT

γδ T cells arise from ETPs and go through the same early stages of development as αβ T cells. Dendritic epidermal γδ T cells (DETCs) have been shown to require positive selection during development[118], however the need for this step during γδ T development is still highly controversial. DN2-3 cells which have generated low levels of γδ TCR are subjected to γδ-selection. By sustaining the expression of *Sox13*, newly committed γδ T cells, contrary to αβ T cells, continue their development, although with little proliferation and remaining DN[119,120]. Committed cells also start to up-regulate γδ TCR expression, and simultaneously down-regulate CD25 and CD127(IL-7Rα)[39,40,121].

The detailed molecular mechanisms through which γδ T cells commit during development are still unclear. The lineage fate determination does not solely correlate with acquisition of TCR[6,45,46,119,122,123]. Pointed by two different studies, not only cells expressing TCRβ bear the ability to differentiate into both γδ and αβ T cells but also, TCRγδ expressing cells are compatible with both γδ and αβ lineage fates[47,48]. Resorting to TCR transgenic mice, the authors suggested a model in which signal strength is the lineage determination factor, postulating that strong signaling through the TCR promotes adoption of the γδ lineage, whereas weaker signals lead to the adoption of the αβ lineage fate[47,48]. Besides its critical role in survival for both αβ and γδ T cells, in mice and in human, IL-7 signalling was also shown to have a role in controlling accessibility of the TCR-γ locus for recombination[124], through STAT5 activity during embryogenesis[125].

The final step of γδ T cell development is maturation which associates with the down-regulation of CD24 (heat stable antigen–HSA)[126]. Recently it has been suggested that the up-regulation of CD73 defines an intermediate developmental stage after commitment and before acquisition of effector fate of TCR engaged cells[127]. During maturation γδ T cells start to up-regulate the expression CD44[128] and specific surface markers, such as NK1.1, CD122, CCR6 and CD5 which segregates them into effector fates[129–131].

Interestingly γδ T cells are the first T cells to develop during mouse ontogeny. From the day 13 of embryonic development to adulthood γδ T cells develop in waves depending on the rearrangement of their Vγ chain[1]. Murine γδ T cells were postulated to develop through a strict order due to genomic location of the γ- and δ- gene loci giving rise to the known developmental waves[132,133]. γδ T cells with Vγ5 chain are the first to develop from embryonic day 13 (E13) until around E17 followed by Vγ6$^+$ cells which develop from E14 until birth. Finally, Vγ1$^+$ and Vγ4$^+$ cells start to develop on E16-E18 and continue throughout live[134]. Vγ7$^+$ γδ T cells contrary to the other γδ T cells are thought to develop outside the thymus, in the gut during embryonic and perinatal life [135,136](Fig.2). γδ T cell subsets, Vγ5$^+$ and Vγ6$^+$, are essentially oligoclonal, showing no junctional diversity between Vγ and Jγ chains[133,137].

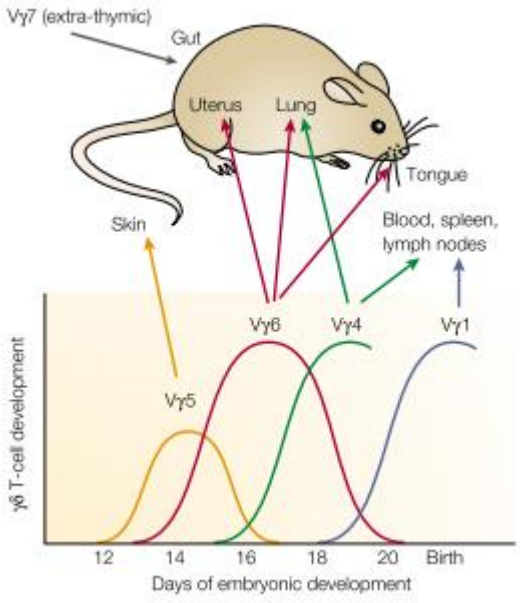


Fig 2- TCR Vγ usage and waves of γδ T cell development and homing tissues. γδ T cells, like Vγ5$^+$ and Vγ6$^+$ subsets develop solely during embryogenesis while Vγ4 and Vγ1 start to arise during late embryonic development and continue to do so during adulthood. (adapted from Carding 2002[134])

γδ T cells with a defined TCR Vγ usage are preferentially distributed to selective epithelial tissues. Vγ5$^+$ T cells migrate to the skin epithelium giving rise to DETCs and Vγ6$^+$ T cells commonly colonize the epithelia of lungs, tongue and reproductive tract. As for Vγ1$^+$ γδ T cells, liver, spleen, intestines and lymph nodes (to a lesser extent) have been pointed to be their

preferential homing tissues. Vγ4$^+$ γδ T cells, on the other hand, recirculate through blood, lymph nodes and spleen and even lung (Fig. 2).

Contrary to αβ T cells, γδ T cells acquire partially their effector functions in the thymus and are able to quickly produce cytokines in response to stimulus. Recently the Vγ-centered view of fetal γδ T cell development was complemented with a functional definition of γδ T cell waves. Indeed, γδ T cells producing IL-17 develop after DETCs and are restricted to the embryonic life, generating long lived, self-renewing cells in the periphery[12]. Development of γδ natural killer T (γδNKT), intraepithelial lymphocyte (IEL) precursors and uncommitted γδ T cells, are generated in the following functional waves from the E16 onwards[137]. Importantly, emergence of each subset in these functional waves does not exactly overlap with the Vγ gene usage waves (Fig 2). Although not exclusive, selective effector function preferentially associates with different Vγ usages[137]. For example, IL- 17 producing γδ T cells preferentially express Vγ4 and Vγ6 chains[138].

4.2 FUNCTIONAL DIFFERENTIATION

γδ T cell functional development occurs in the thymus, which explains their readiness to perform an effector function in the periphery. Differentiation of IFN-γ and IL-17 producers is regulated by selective expression of TFs and exclusive signaling cues, with an additional layer of epigenetic regulation[139]. Furthermore, the divergence between these two subsets occurs early during γδ T cell development, where IL-17$^+$γδ T cells were generated from DN2 (CD117^{high}) while IFN-γ producing γδ T cell were generated from both DN2 (CD117^{high}) and DN3 (CD117$^-$)[140].

Shown by our laboratory, γδ thymocytes develop in successive stages characterised by CD25 and CD27 as follows: CD25$^+$CD27$^+$ cells give rise to CD27$^+$ cells and CD27- cells[130]. γδ T cells which maintain CD27 expression through development promptly secrete specifically IFN-γ after leaving the thymus[141]. It was further shown that CD27 is a decisive determinant that actively regulates acquisition of the IFN-γ fate. However, the specific CD27 signalling, to date, remains elusive. γδ TCR signalling, by specific agonists, was also described to skew γδ T cells towards IFN-γ production[131]. In addition, other intrathymic molecular interactions are also important to regulate γδ T cell differentiation. TCR-independent interactions between early γδ T cell progenitors and DP αβ T cell progenitors, in part through the LTβR (TNF receptor family member), have also been shown to be required for the differentiation and proliferation of IFN-γ producing γδ T cells[142–144]. However, IFN- γ production by γδ T cells was not completely

abrogated in the absence of CD27 or LTβR[130]. These results suggested a crucial role of CD27 together with the TCR signalling in leading to the differentiation of γδ T cells into IFN-γ producers[130].

IFN-γ-producing γδ T cells share similarities with IFN-γ-producing Th1 cells, such as the ability to respond to IL-12 and IL-18 and the high expression of the "master regulator" T-bet[145]. Further, other TFs like ThPOK[128] and Erg3[118] are also important for IFN-γ production and regulation of γδ27$^+$ T cells. These, after development, present maturation markers such as CD27[130], CD122[146] and NK1.1[129] which allow their distinction from IL-17 producers.

On the other hand, the development of IL-17 producing (CD27$^-$) γδ T cells has been linked to the specific expression of Hes1, one of the basic helix-loop-helix proteins involved in Notch signaling [147]**.** IL-17 differentiation pathway of γδ T cells requires also the TF RelB for the expression of RORγt[148]. RORγt in turn acts together with Sox13 and Sox4 to drive differentiation towards IL-17 producing γδ T cells (IL-17$^+$γδ T cells)[149,150]. IRF4, a member of the interferon regulators family, and STAT3, both important in Th17 cell differentiation, were shown to be dispensable for IL-17 production by γδ T cells[147,151]. IL-23, although important for the expansion of γδT17 cells is, contrary to Th17, dispensable for development and maintenance in SLOs[152]. In synergy with IL-18 and IL-1β, IL-23 was shown to promote IL-17 production by γδ T cells[153]. IL-7 was also associated to the expansion IL-17$^+$γδ T cells[154]. Surface expression of CCR6[129], SCART2[155], CD25[156] and IL-23R[152] allows the phenotypic distinction of thymic and peripheral IL-17$^+$γδ T cell subset. Moreover, besides IL-17 this subset of γδ T cells was shown to produce IL-22 (similar to Th17 cells in the periphery[157]) and also IL-17F and IL-21[152].

Although their functional differentiation occurs in the thymus, γδ27$^-$ T cells are also gifted with some functional plasticity. As the host laboratory showed, while *IL-17 locus* is only active in γδ27$^-$ T cells, the *IFN-γ locus* is active in both γδ27$^+$ and γδ27$^-$ T cells[139]. This suggested that γδ27$^-$ T cells can also produce IFN-γ. Through *in vitro* experiments it was shown that, in synergy with IL-1β, IL-23 could induce IFN-γ expression in γδ27$^-$ T cells[139]. These IL-17/IFN-γ double producers were also observed *in vivo* in several different contexts, both in human and in mice[158–161], although the precise physiological role of these cells is still to be established.

5. AIMS OF THIS THESIS

In this thesis we aimed to study the relevance of some of those TFs previously implicated in CD4+ or CD8+ T cell biology in the functional differentiation of γδ T cells.

In order to grasp the role of specific TFs in γδ T cells, we used different mouse models, where the chosen transcription factors (T-bet, Eomes, BATF, RORγt and RORα) were depleted and consequently absent from all the cells of the organism. Using this strategy we assessed the role of these TFs in the functional differentiation of γδ T cells resorting to *in vitro* cultures, flow cytometry, gene expression analysis and experimental disease models. During our experiments we have identify a different role of the TF RORα in γδ T cells. With the aim of determining if this role was specific to RORα, we assessed the relevance of both RORα and RORγt in γδ T cell development by flow cytometry, proliferation and cell death assays and gene expression analysis using the same mouse models.

With this work we aimed to contribute to a better understanding of the molecular mechanisms underlying the γδ T cell development, functional differentiation and cytokine production, which have major implications in infection, cancer and autoimmune diseases

V. MATERIALS AND METHODS

1. MOUSE STRAINS

Tbx21$^{-/-}$ [162] and *BATF*$^{-/-}$ [104] mice *homozygous, Eomes* $^{fl/fl}$ *R26R-RFP* homozygous mice and heterozygous for *Rag1-Cre and Ror*$\alpha^{sg/+}$ [163]and *RORc*^{GFP} [164] mice heterozygous were in a C57BL/6J genetic background. *Tbx21*$^{-/-}$, *BATF*$^{-/-}$, *Ror*$\alpha^{sg/+}$, *RORc*^{GFP} strains were breed and maintained at the IMM rodent/animal facility and *Eomes* $^{fl/fl}$ strain was breed and maintained at the Babraham Institute from which organs were sent for analysis. All procedures and experiments were performed according to national and institutional guidelines.

2. GENOTYPING PCR

All strains kept in the IMM rodent facility where genotyped when arrived. Mice used from heterozygous crossings at the facility where always genotyped before used.

For DNA extraction tissue was harvested from the ears or toes and digested using a NaOH 0,05mM solution for 60 to 90 minutes in a 95°C dry bath. The process of digestion was then stopped with a solution of 1M Tris and 10mM EDTA (pH8).

DNA amplification was performed using T100™ Thermal Cycler (Bio-Rad). The PCR program used was according to the program suggested in the Jackson Laboratory information for each strain. Every program consisted of an initial step of polymerase activation at 94°C for 10 minutes, followed by 35 amplification cycles consisting of 3 steps (DNA denaturation at 94°C for 30 seconds, DNA-primer annealing at between 50-54°C for 60 seconds and polymerase reaction elongation at 72°C for 60 seconds) and a final step of DNA extension at 72°C for 10 minutes. Primer sequences and corresponding PCR DNA-primer annealing TC are detailed in **annex 3**. To each reaction well with 18µL of the PCR Buffer **(annex 2)** 2µL of DNA was added. After the PCR reaction amplified products were resolved in a 2% agarose gel in 1X TAE and containing 3,5µL of green safe (nzytech).

3. MOUSE ANALYSIS

For *in vitro* experiments with $CD4^+$ T cells and/or γδ T cells, mice with ages between 10 days and 20 weeks were dissected and their lymph nodes (Inguinal, Brachial, Axillary, Mesenteric, Popliteal, Caudal Mesenteric, Medial iliac and Accessory Mandibular) and Spleen were harvested. Lymphocytes were obtained by tissue homogenization and filtration through a 70µm cell strainer. The erythrocytes were osmotically lysed using 1X RBC lysis Buffer **(annex 2)** and ressuspended in 1mL after.

In *ex-vivo* analysis the same protocol was applied but LN, Spleen and Thymi were homogenized separately.

For Spinal Cords, mice were perfused before harvesting any organ in order to remove circulating blood out of the spinal cord. When harvested tissue was cut into small pieces, homogenized using glass slides and digested in complete medium with Collagenase IV (0,5mg/mL) and DNAse I (0,01mg/mL) (annex2) for 30min to 1h at 37°C. The digestion was

stopped by adding 10uL of EDTA (1mM). Lymphocytes were then isolated and purified using a one-phase solution of 33% Percoll (GE Healthcare, 17-0891-01) in complete medium (annex2) and by centrifuging the solution for 30min at 2400rpm at RT without brake. Cell pellets from spleen, thymi and spinal cord were then ressuspended in 1mL of complete medium and cell pellets from LN were ressuspended in 500uL of complete medium for cell counting.

4. IN VITRO STIMULATION AND POLARIZATION

CD27+ and CD27- γδ T cells (CD3+TCRδ+) and CD4+ T cells (CD3+CD4+) where sorted by flow cytometry. CD27+ γδ T cells were activated using plate-bound monoclonal antibody (mAb) anti-CD3ε (2 µg/ml; 145.2C11; eBiosciences) and mAb anti-CD28 (2 µg/ml; 37.51; eBiosciences) in the presence or absence of IL-1β (50 ng/ml) or IL-2 (10ng/mL)or IL-12 (50ng/mL) or the cross-reactive human IL-15 (50ng/mL) or IL-23 (50 ng/ml) or IL-1β plus IL-23 for 14h (OVN) or 36h. CD27- γδ T cells were activated using the same conditions mentioned above but without the plate-bound mAb and for 14h (OVN) or 36h.

CD4+ T cells polarization cultures were maintained during 5 days and activated with plate-bound mAb anti-CD3ε and soluble mAb anti-CD28 for all conditions both at 2µg/mL. For Th1 culture conditions, cells were culture in the presence of IL-12 (5 ng/ml) and neutralizing mAb anti-IL-4 (5 µg/ml; 11B11; eBiosciences). For Th17 culture conditions, cells were culture in presence of also TGF-β (2 ng/ml), IL-1β (50 ng/ml), IL-6 (50 ng/ml), IL-21 (100 ng/ml), IL-23(50 ng/ml) and neutralizing anti-IFN-γ (10 µg/ml; eBioscience) and anti-IL-4 were added to the medium.

5. STAINNING AND FLOW CYTOMETRY ANALYSIS

For FACS analysis cells were stained in a 96 well plate in a final volume of 50µL. For surface staining, cells were incubated with 1:200 of FcγR Block (2.4G2; BD Pharmingen), 2% Normal Mouse Serum (serum extracted from blood of WT mice) and the respective Ab mixes. All staining procedures were performed at room temperature (RT) and in the dark. For intracellular cytokine staining, cells where stimulated with PMA (0,05ug/mL) and Ionomycin (0,7ug/mL) for 4h at 37°C and Brefeldin A (10ug/mL) (annex 2), in order to activate cytokine production by the cells and at the same time block their secretion for the last 2 hours. After stimulation and extracellular staining cells where fixed using a Fix solution (annex2) for 20min at 4°C and permeabilized in 1X Perm Buffer (annex 2) for 15 min at RT. Cells where then incubated with the Ab mix in 1X Perm Buffer for the respective cytokines for 30min at RT and wash in 1X Perm Buffer and FACS Buffer (annex 1) before analysis**.** Cells were analyzed on a FACSFortessa or FACSCalibur (BD Biosciences) and data were analyzed with FlowJo software.

For FACS sorting proposes cells were stained in 50mL tube in the dark in final volume between 200µL and 1mL at RT.

Ab used were: anti-γ/δ TCR (eBioGL3), anti-CD3 (17A2), anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-IL17A (eBio17B7), anti-IL22 (Poly5164), anti-IFN-γ (XMG1.2) and anti-CD27 (LG.6F9) from eBioscience, anti-γ/δ TCR (GL3), anti-CD24 (M1/69), anti-CD45 (30F11), anti-IL17A (TC11-18H10.1), anti-GM-SCF (MP1-22E9), anti-CD8a (53-6.7), anti-Vγ1

(2.11), anti-Vγ4 (UC3-10A6) and anti-CCR6 (29-2L17) from BioLegend and anti-CD45 (30-F11), anti-CD4 (RM4-5) from BD Pharmigen.

6. INDUCTION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Mice were immunized with 200µg MOG_{35-35} peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA solution (4mg/mL of mycobacteria (Difco) in incomplete freund adjuvant (Difco)) subcutaneously (100µg in each side flank). On the day of the immunization and 48h after, mice were injected intravenously with 100ng of pertussis toxin (List Bioolgical Laboratories, via Quadratech) in 100µL of 1X PBS. Mice were scored following the score rate in annex 5 daily from day 7 to the day when they were analyzed (highest score day).

7. *LISTERIA MONOCYTOGENES* INFECTION

Listeria monocytogenes bacteria strain EGDe was inoculated from a 25 µl stock in 50 ml brain heart infusion (Sigma; #53286) medium and incubated OVN on a shaker (220 rpm) at 37°C. The *Listeria monocytogenes* bacteria was sub-cultured in a 1:10 dilution into BHI medium and incubated on a shaker (220 rpm) at 37°C for approximately 90-120 min until an OD_{600} of 0.8 was reached. The culture was spin down at 2000 rpm for 20 min at 4°C. An OD_{600} of 0.8 in 20 mL of culture corresponds to approximately $2x10^{10}$ colony forming units (CFU). The pellet was resuspended in PBS so that the concentration was 10^{10} CFU/mL. C57BL/6J and *Tbx21*$^{-/-}$ mice were orally infected with *Listeria monocytogenes*, after OVN deprivation from food and water. Infection was done by feeding a mixture of $2x10^{9}$ CFU (200 µl) *Listeria monocytogenes* with a wet food mash. During feeding, mice were housed separately for approximately 1 h, until most of the food was consumed. Cells from spleen and mesenteric LN were collected at day 8 after infection.

8. GENE EXPRESSION ANALYSIS

RNA was isolated from sorted cells using High Pure RNA Isolation Kit (Roche) following the manufactures instructions. RNAs were converted in cDNA through reverse transcriptase PCR for 1 h at 42 °C using Random oligonucleotides (Invitrogen) and MMLV reverse transcriptase (Promega). Quantification of specific cDNA species was then assessed by real time-PCR using ViiA 7 Real-Time 384-well thermal cycler with SYBR or TaqMan probe relatively to endogenous references (β2-microglobulin, β-actin or HPRT levels). Target gene C_T was subtracted from the C_T for the endogenous references and the relative amount was calculated as $2^{-\Delta CT}$. All sequences used are detailed in annex 3.

9. COUNT OF TOTAL LIVE CELL

Live cells were counted using flow cytometry. For each solution containing the isolated cells a mix containing 10µL of the cell solution, 165µL of FACS Buffer and 20µL of beads (annex 2) was made to which 5uL of propidium Iodide (PI) solution (eBioscience) was added. PI stains for dying cells, allowing the exclusion of the dying cells for each sample and the counting of the number of live cells for a fixed number of beads, using a stopping gate for 500 beads in solution.

10. PROLIFERATION AND APOPTOSIS

To address cell proliferation mice were injected with a fixed concentration of BrdU (BD Pharmigen, FITC BrdU Flow Kit, 51-2354AK), stablished by the manufacturer, and mice were analyzed 16h after injected, being able to access at least two cell divisions. BrdU is a thymidine analog which during replication incorporates the DNA instead of the nucleotide allowing through staining to detect cells in proliferation. Also stablished by the manufacturer staining procedure was performed as indicated.

Apoptotic cells express in their surface phosphatidylserine and phosphatidylethanolamine. Annexin V detects these two proteins in the membrane allowing to access cells going through apoptosis. In order to do so cells were harvested and stained through using the protocol established by the manufacturer (eBioscience, Annexin V - FITC, 11-8005-74).

11. STATISTICS

Statistical analysis was performed using a two-tailed non-parametric Mann-Whitney test. P values of <0.05 were considered significant. Results were scored as * when $p<0.05$, ** when $p<0.01$, and *** when $p<0.001$.

VI. RESULTS

1. γδ27$^+$ AND CCR6$^+$γδ27$^-$ T CELLS ARE PRE-COMMITTED TO PRODUCE DIFFERENT PROFILES OF CYTOKINES

We aim to determine the diversity of inflammatory cytokines produced by distinct lymphoid γδ T cell subsets. To do so we FACS-sorted γδ T cells, derived from SLOs, according to their expression of CD27 and CCR6 as follows: CD27$^+$; CCR6$^-$CD27$^-$ and CCR6$^+$CD27$^-$ γδ T cells (Fig 3A). We then assessed cytokine gene expression by RT-PCR. γδ27$^+$ T cells express high levels of *Ifng*, while very low levels of *Il17*, *Il17f*, *Il22* and *Gmcsf* transcripts (Fig 3B). CCR6$^+$γδ27$^-$T cells, by contrast, express high levels of *Il17* (10.000 fold higher than γδ27$^+$ T cells), *Il17f*, *Il22* (1000 fold higher than γδ27$^+$ cells) and *Csf2* (gene name of GM-CSF) (~50 fold higher than γδ27$^+$ cells) (Fig 3B). CCR6$^-$γδ27$^-$ T cells, on the other hand, showed the intermediate levels of *Il17*, *Il17f* and *Csf2* (compared to CCR6$^+$γδ27$^-$ T cells) and high levels of *Ifng* and *Il22* mRNA (Fig 3B). This suggests that CCR6$^+$γδ27$^-$ cells may represent a more terminal differentiation stage than CCR6$^-$ γδ27$^-$ T cells. Noteworthy, the profile of cytokine transcription of γδ27$^+$ and CCR6$^+$γδ27$^-$ T cell subsets respectively mirrors the one presented by *in vitro* differentiated CD4 Th1 and Th17 cells, respectively (Fig 3B). These data suggest that γδ27$^-$ cells (but not γδ27$^+$ cells) are programmed to produce all five pro-inflammatory cytokines.

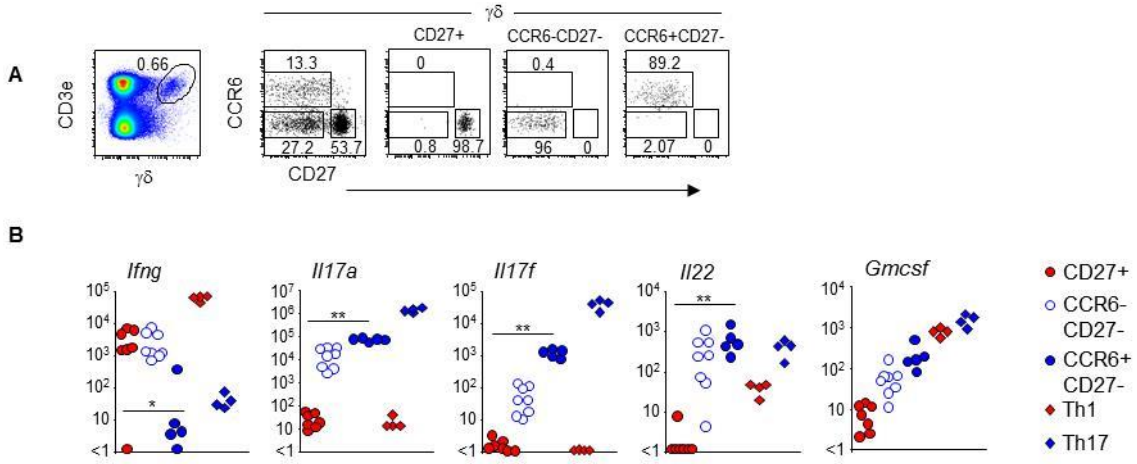


Figure 3 – Cytokine expression profile of CD27$^+$, CCR6$^-$CD27$^-$ and CCR6$^+$CD27$^-$ γδ T cell subsets.
γδ T cells were FACS sorted from pooled spleen and LNs from 8 weeks old C57BL/6J mice. **A** Representative FACS sorting gate strategy for sorted cell subsets. **B** RT-PCR data for *Ifng*, *Il17*, *Il17f* , *Il22 and GM-CSF* expression (relative to *b2m* or *Actb*) on γδ T subsets: CD27$^+$ (CD27$^+$), CD27$^-$ (CCR6$^-$CD27$^-$) and CCR6$^+$ (CCR6$^+$CD27$^-$); and *in vitro*-generated CD4 Th1 and Th17 cells.

2- DIFFERENT SIGNALS CONTROL IFN-γ PRODUCTION BY γδ27$^+$ AND γδ27$^-$ CELLS.

To further understand the mechanisms of cytokine production by γδ T cells, we then questioned the extracellular regulators which lead to IFN-γ production as well as IL-17, IL-17F, IL-22 and GM-CSF production. To do so, γδ T cells were FACS-sorted based on the expression of CD27 (Fig 4A) and stimulated *in vitro* for 16hours (OVN) or 36hours with a broad selection of settings (Fig 4 and Fig 5). The *in vivo* paucity of the CCR6$^+$γδ27$^-$ cells prevented us from studying the functional properties of this subset *in vitro*.

Similarities between γδ27$^+$ T cells and the Th1 cell counterparts led us to question if they respond to identical stimuli. When stimulated with PMA plus Ionomycin (Iono) for 4h, about 20% of γδ27$^+$ cells expressed IFN-γ (Fig 4B-C). This proportion increased upon addition of anti-CD3/28 mAb, which mimics TCR engagement, overnight. γδ27$^+$ T cells respond similarly to IL-12 leading to 50% of γδ27$^+$ T cells to produce IFN-γ. Moreover IL-12 synergized with anti-CD3/CD28 mAb led to induce IFN-γ production by 60-80% of γδ27$^+$ T cells (Fig 4C). Although to a lesser extent, other cytokines, such as IL-2 and IL-15, synergized with anti-CD3/CD28 signalling to induce a two-fold increase in IFN-γ secreting γδ27$^+$ T cells (Fig 4C). The stimulation with IL-2, IL-15 alone led to a minor increase in the frequency of IFN-γ producing γδ27$^+$ T cells. Finally these cells do not respond to stimulation by IL-1β plus IL-23 (Fig 4B-C).

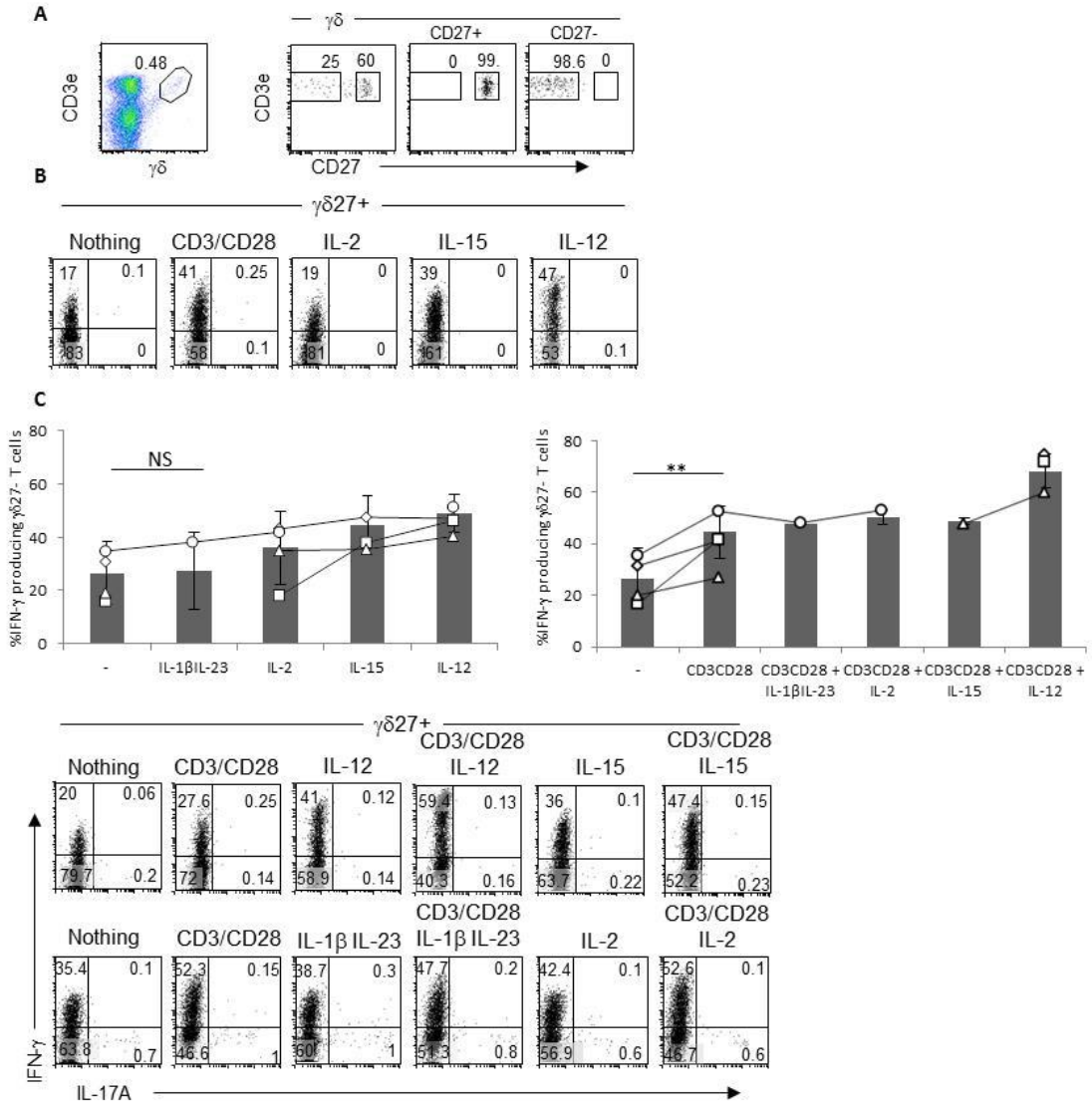


Figure 4 - IFN-γ production by γδ27$^+$ T cells can be activated via TCR and cytokine stimuli.
γδ T cells were FACS sorted from spleen and LNs from 8 weeks old WT mice. **A** Representative FACS sorting gate strategy. **B** Representative FACS plots of **i**ntracellular cytokine staining for IFN-γ and IL-17A from γδ27$^+$ T cells isolated and stimulated over night as indicated in the presence of the following conditions; coated anti-CD3 plus anti-CD28, IL-2, IL-15 and IL-12. **C** On top, statistical data of the frequency of IFN-γ producing γδ27$^+$ T cells after various stimulating conditions (n>9). On the bottom, the representative FACS plots of analyzed cells after stimulation with the following conditions: coated anti-CD3 plus anti-CD28, IL-2, IL-15, IL-12 and IL-1β+IL-23 (alone or in combination); and stained for IFN-γ and IL-17A.

γδ27$^-$ T cells produce IL-17 after PMA plus Iono stimulation. The host laboratory and others have shown that γδ27$^-$ T cells respond to combination of IL-1β and IL-23 cytokines[139,152,165]. We show here that OVN stimulation with IL-1β and IL-23 almost double the proportion of IL-17-producing γδ27$^-$ T cells and increased the mean of IL-17-secretion per cell (Fig 5). Moreover, gene expression suggested that γδ27$^-$ T cells are predisposed to produce IL-17F, IL-22, IFN-γ and GM-CSF, and we hypothesized that IL-1β plus IL-23 could induce their production. . Indeed IL-1β and IL-23 induced strong secretion of IL-17F, IL-22 and GM-CSF from the IL-17-producing

γδ27$^-$ T cell population (Fig 5A and E). Within this effector subset about 10% are also induced to produce IFN-γ. The production of these cytokines, especially of IFN-γ was further increased after 36h of stimulation (data not shown). Interestingly, IL-1β potentiated the effect of IL-23, since IL-23 alone induced IL-17 and a modest production of IL-17F and IL-22 but no IFN-γ production (Fig 5A). Although, it is known that in CD4 T cells IL-17A and IL-17F are often coexpressed[166], our results suggest that in γδ T cells these cytokines are differentially regulated by signaling through PMA plus Iono and IL-1β plus IL-23. In contrast to γδ27$^+$ T cells, γδ27$^-$ T cells were unresponsive to anti-CD3/CD28 mAb and IL-15 stimulation, as there was no increase in the proportion of γδ27$^-$ T cells producing IFN-γ (nor IL-17) (Fig 5B-C). Yet, IL-2 and IL-12 induced around 10% and 18%, respectively, of γδ27$^-$ T cells to produce IFN-γ, but not IL-17 (Fig 5B and D). Therefore, we show here that IL-1β plus IL-23 trigger γδ27- T cells polyfunctionality, through the production of IL-17, IL-17F, IL-22, GM-CSF and IFN-γ.

Taken together, our results show that cytokines such as IL-2, IL-12 and IL-15 are important co-factors in TCR-mediated stimulation of γδ27$^+$ T cells. Additionally, γδ27$^-$ (but not γδ27$^+$) T cells are endowed with a marked polyfunctional capacity which is induced by IL-1β and IL-23.

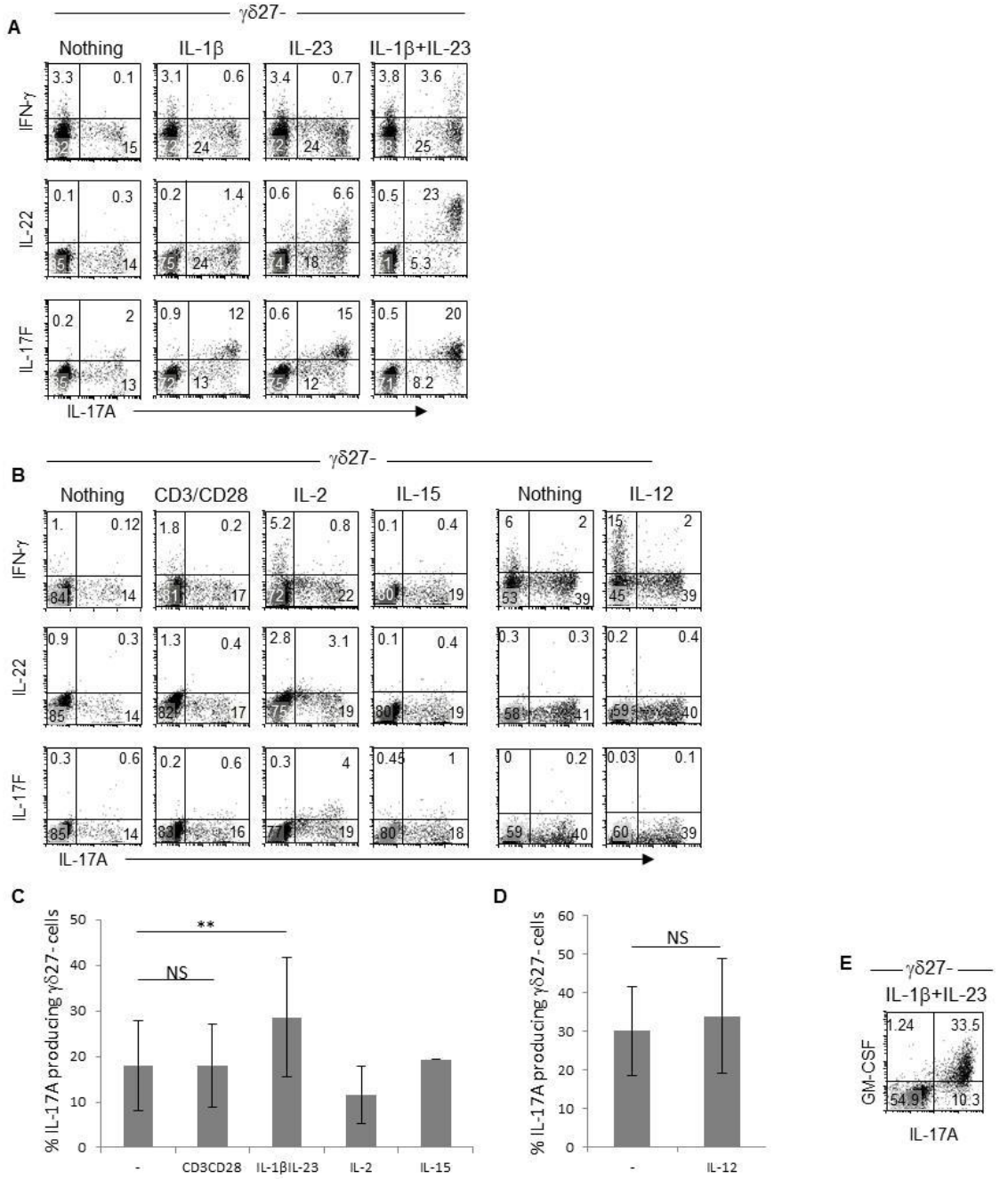


Figure 5 - γδ27$^-$ T cells depend on IL-1β and IL-23 for IL-17 and IFN-γ production.

γδ T cells were FACS sorted from pooled spleen and LNs from 8 weeks old C57BL/6J mice. Sorting strategy was the same as in Figure 4A. Representative FACS plots of intracellular cytokine staining for IFN-γ and IL-17A after stimulation OVN in the presence of the following conditions **A** IL-1β and IL-23 (alone or in combination), **B** coated anti-CD3 plus anti-CD28, IL-2, IL-15, IL-12. **C and D** Statistical data of IL-17A producing γδ27- T cells after submitted to several stimulating conditions used in A and B. **E** Representative FACS plots of intracellular staining for IL-17A and GM-CSF after stimulation OVN with IL-1β and IL-23.

3- γδ27$^+$ and γδ27$^-$ SUBSETS PRESENT DIFFERENT PROFILE OF EXPRESSION OF TH1-AND TH17-RELATED TRANSCRIPTION FACTORS

To get additional insight into the differentiation programs that regulate the selective production of cytokines by γδ27$^+$ versus γδ27$^-$ cells, we assessed the levels of expression of several TFs known to control the IFN-γ and IL-17A in their αβ (Th1 an Th17) counterparts. To do so we performed real-time PCR on γδ27$^+$, CCR6$^-$γδ27$^-$ and CCR6$^+$γδ27$^-$ T cell subsets to assess mRNA levels of type 1 TFs (which control IFN-γ) *Tbx21*, *Eomes*, *Hlx* and *Erg3* and type 17 TFs (which control IL-17) *Rorc, Rorα, Batf and Irf4*. In agreement to what was already published by the hosting laboratory[139], both γδ27$^+$ and CCR6$^-$γδ27$^-$ T cell subsets had similar and high levels of transcripts of the IFN-γ-related TFs (Fig 6,-upper panels). Together, as already suggested by our previous results, CCR6$^+$γδ27$^-$ T cells present a more pronounced phenotype and 10-fold decrease of *Tbx21* transcripts and absence of *Eomes* mRNA when compared to γδ27$^+$ and CCR6$^-$γδ27$^-$ T cell subpopulations (Fig 6-upper panels).

By contrast, both IL-17 related transcription factors, *Rorc* and *Rorα* were highly enriched in both γδ27$^-$ subpopulations, particularly in the CCR6$^+$γδ27$^-$ T cell subset (Fig 6-lower panels). By contrast, *Batf and Irf4* levels of expression were very similar between the three γδ T cell subsets.

Notably, the profile of expression of *Tbx21*, *Eomes*, *Erg3*, *Hlx*, *Rorc*, *Rora*, *Batf* and *Irf4* in γδ27$^+$ and CCR6$^+$γδ27$^-$ T cell subset mirrors that of Th1 and Th17 respectively (Fig 6). Although *Erg3* and *Hlx* are preferentially expressed in Th1 compared to Th17, γδ27$^+$ and CCR6$^-$γδ27$^-$ T cells express similar levels of these TF (Fig 6-upper panels). These results led us to assess the functional role of T-bet and Eomes in IFN-γ-producing γδ27$^+$ T cells and IFN-γ/IL-17-coproducing γδ27$^-$ T cells. Furthermore we choose to assess the function of RORc, RORα and BATF in IL-17-producing γδ27$^-$ T cells *in vivo*. As for IRF4, it was already shown that it does not play a role in the production of IL-17 in γδ T cells[151].

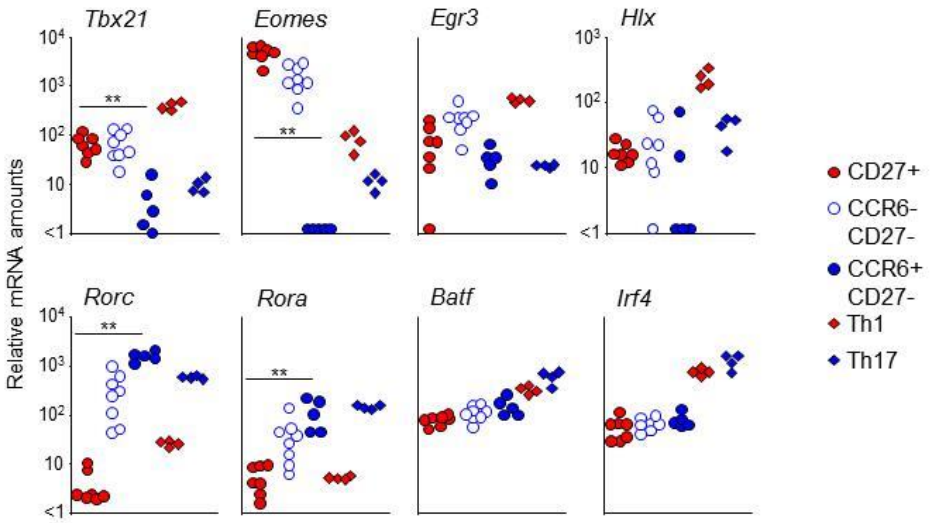


Figure 6 – Transcription factor expression profile of CD27^{+}, CCR6^{-}CD27^{-} and CCR6^{+}CD27^{-} γδ T cell subsets.
γδ T cells were FACS sorted from pooled spleen and LNs from 8 weeks old C57BL/6J mice as shown in Fig 3A. RT-PCR data for *Tbx21, Eomes, Erg3, Hlx, Rorc, Rora, Batf and Irf4* expression (relative to *b2m* or *Actb*) on γδ T subsets and *in vitro*-generated CD4 Th1 and Th17 cells.

4- IN VITRO PRODUCTION OF IFN-γ BY γδ27$^+$ T CELLS PARTIALLY DEPPENDS ON T-BET, BUT NOT EOMES

To address the functional significance of each transcription factor in cytokine production by γδ T cells we employed murine models for single gene deficiency for *Tbx21*, *Eomes*, *Rorc*, *Rora* and *Batf*. As γδ27$^+$ cells present a restricted functional potential (Fig 3B, Fig 4) we focused on their ability to produce IFN-γ. γδ27$^+$ T cells were left untreated or stimulated with anti-CD3/CD28 mAb OVN or for 36h. Within all the conditions tested, to assess IFN-γ production we choose the treatment with anti-CD3/CD28 mAb since it induced about 50% of γδ27$^+$ T cells to produce IFN-γ, in a short-term *in vitro* stimulation. Moreover T-bet expression is known to be triggered upon TCR engagement in γδ T cells[167]. In agreement with some publish data[145,167], we found that in the absence of T-bet (*Tbx21*) there was a significant reduction, but not a total abolishment, of IFN-γ producing γδ27$^+$ T cells (Fig 7A-B). Notably, dependence on T-bet seemed to be diminished with longer time of stimulation (Fig 7B-D). Of note, the IFN-γ MFI between the WT and the Tbx21$^{-/-}$ mice, when stimulated with α-CD3 plus α-CD28 (OVN or 36h), was not changed (Fig 7A and C). These results suggest that there are different layers on the regulation of IFN-γ production by γδ27$^+$ cells and some seem to be through T-bet-independent pathways. Nevertheless, this firmly contrasts with the profound necessity of T-bet by CD4 T cells to produce IFN-γ (Fig 8A-B left panel).

We hypothesized that *Eomes* could account for T-bet-independent IFN-γ production, because of its high level of expression in γδ27$^+$ T cells. Moreover, Eomes plays a major role in IFN-γ production in CD8 T cells[168]. Since *Eomes* deficiency is embryonic lethal, we used a conditional knock-out mice (kindly provided by our collaborator, Marc Veldhoen - Cambridge) in which mice having two *floxed* regions surrounding the *Eomes* gene were crossed to mice expressing Cre recombinase under control of the Rag1 regulatory elements. This induces the deletion of *Eomes* selectively in T and B cells. There was no impairment in the production of IFN-γ by *Eomes*-deficient γδ27$^+$ cells, neither after 16h of stimulation (Fig 7A-B) or after 36h (Fig 7C-D). IFN-γ production by γδ27$^+$ cells was not altered in the absence of *Rorc, Rorα,* or *Batf* (Fig 6A-B) suggesting that none of the Th17-related TF have selective properties to repress IFN-γ.

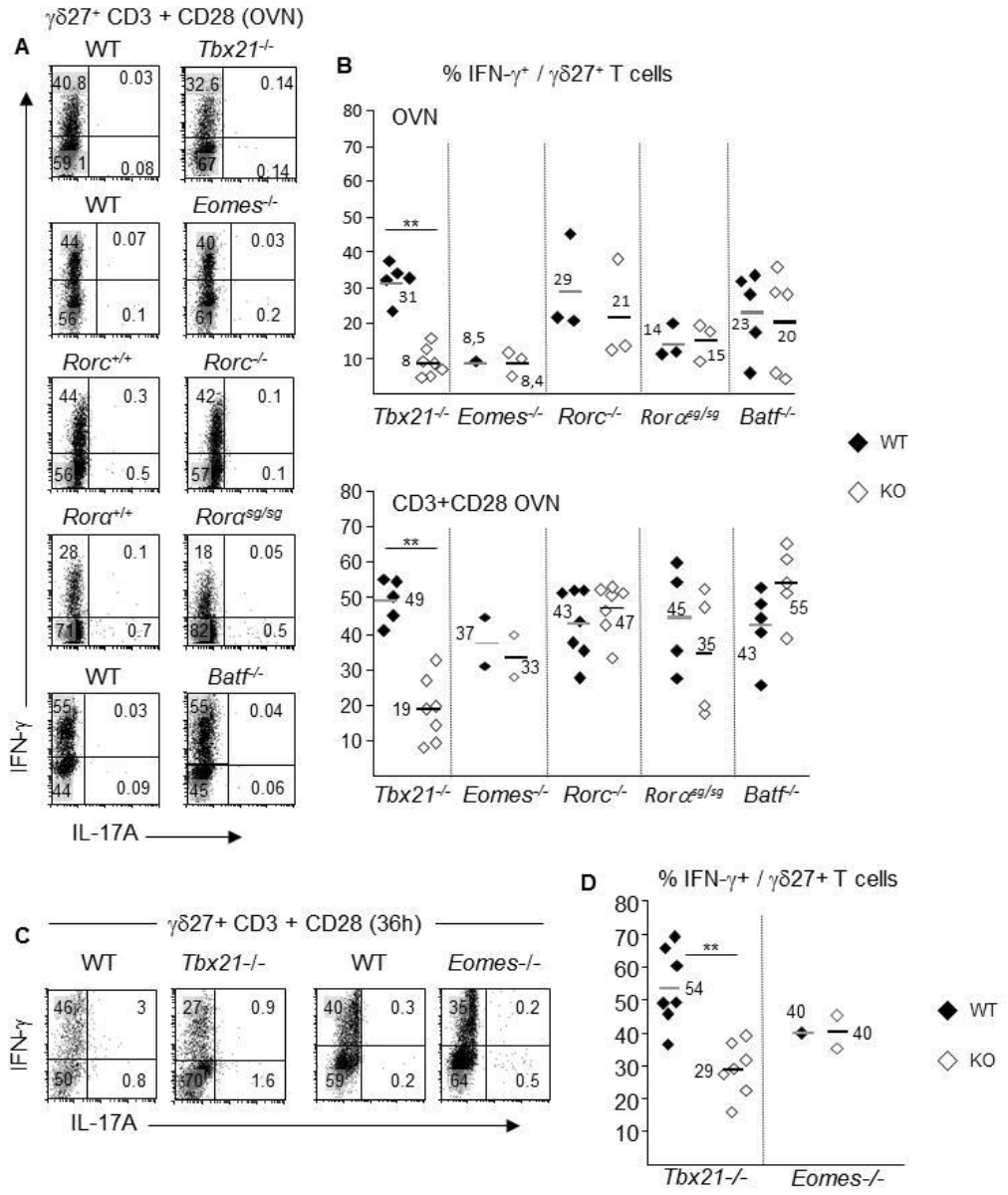


Figure 7 - γδ27^{+} T cells depend partially on T-bet for IFN-γ production.

γδ27^{+} T cells were FACS sorted from pooled spleen and LNs from 8 weeks old *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$, *Rorc*$^{-/-}$, and *Batf*$^{-/-}$ mice and 2 to 3 weeks old *Rorα*$^{sg/sg}$. Wilde type littermates of *Rorα*$^{sg/sg}$ and *Rorc*$^{-/-}$ were used as controls and as for the rest of the mice, C57BL/6J were used as controls. Sorting strategy was the same as in Figure 4A. **A** Representative FACS plots of intracellular cytokine staining for IFN-γ and IL-17A on γδ27^{+} T cells isolated and stimulated OVN with coated α-CD3 plus α-CD28. **B** Percentage of γδ27^{+} T cells producing IFN-γ plated OVN unstimulated (on top) and stimulated (in the bottom). **C** Representative FACS plots of intracellular cytokine staining for IFN-γ and IL-17A after stimulation over 36h with coated anti-CD3 plus anti-CD28 in cells sorted from *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$ and C57BL/6J mice. **D** Percentage of γδ27^{+} T cells producting IFN-γ after 36h of stimulation.

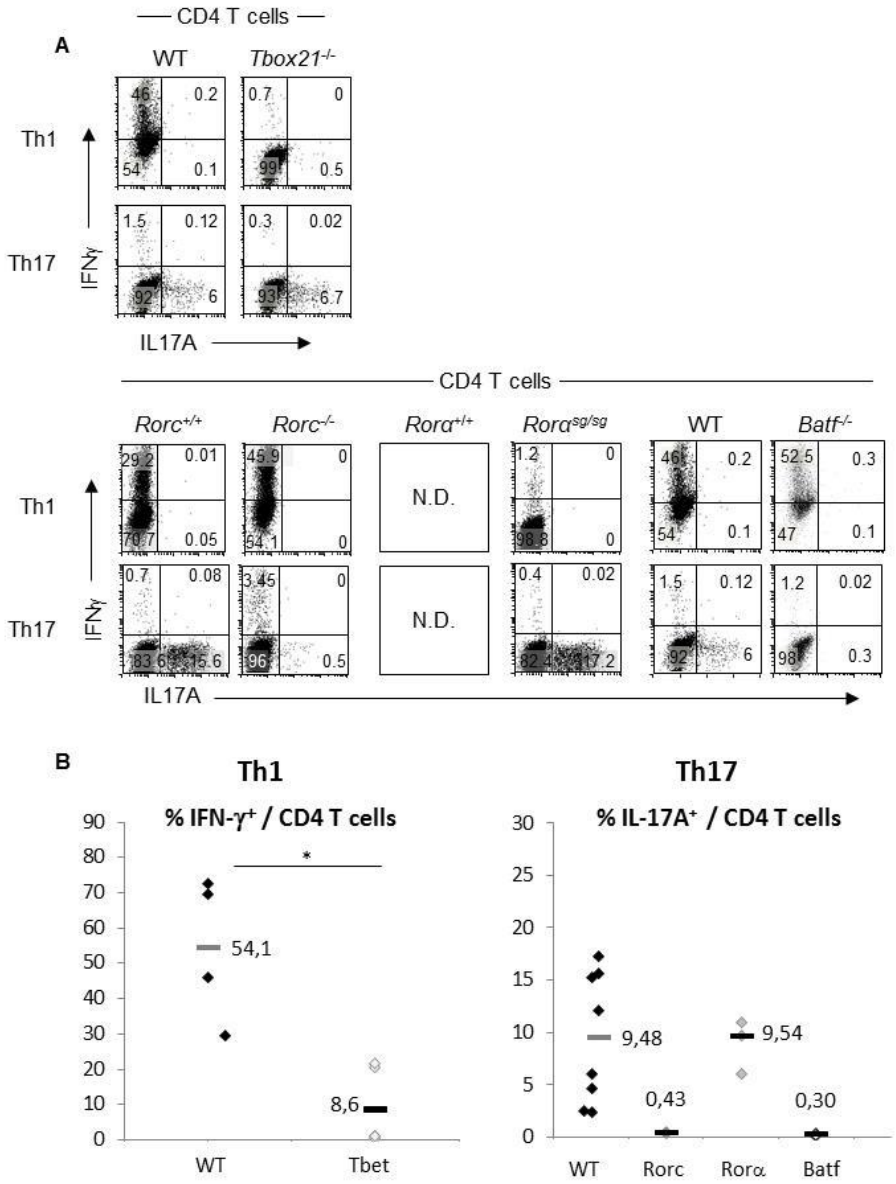


Figure 8- CD4 T cells require T-bet for IFN-γ production and RORc and BATF for IL-17 production
CD4 T cells were FACS sorted from spleen from 8 weeks old *Tbx21*$^{-/-}$, *Rorc*$^{-/-}$, and *Batf*$^{-/-}$ mice and 2 to 3 weeks old *Rorα*$^{sg/sg}$ mice. Wild type littermates of *Rorc*$^{-/-}$ were used as controls and as for the rest of the mice, C57BL/6J were used as controls. **A** Representative FACS plots of intracellular cytokine staining for IFN-γ and IL-17A of CD4 T cells isolated and stimulated over 5 days in polarizing conditions towards Th1 and Th17 CD4 T cell differentiation. **B** Percentage of CD4 T cells polarized towards Th1 producing IFN-γ from WT and *Tbx21* deficient mice on the left (WT n=4, T-bet n=4) and on the right percentage of CD4 T cells polarized towards Th17 producing IL-17 from *Rorc, Rorα* and *Batf* deficient mice (WT n=7, Rorc n=3, Rora n=3, Batf n=2).

In vitro experiments do not always recapitulate the complex *in vivo* signalling pathways. Thus, in order to assess T-bet requirement *in vivo* we choose to use the murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), which has been well characterize to induce Th1, Th17 and Th1-like Th17 cell responses. Moreover γδ T cells play an important role at the first stage of development of the disease[152] and they are important sources of IL-17 at the inflamed SC[114]. Some studies have also reported the emergence of IFN-γ^{+}IL-17^{+} γδ T cell in this model[158].

The site of inflammation in this model is in the central nervous system (CNS) – spinal cord (SC) and brain – where most of the effector CD4 T cells and γδ T cells are found (Fig 9A-B). In the cutaneous inguinal LN, near the site of injection (see the section materials and method), it is also possible to visualize some effector CD4 T cells and γδ T cells that produce IFN-γ and/or IL-17. Knowing that lymphocytes start to invade the CNS from the SC[114], we choose to analyze only the SC. Due to their crucial role in the development of the disease we first analyzed the presence Th1 and Th17 CD4 T cells. In WT mice, CD4 T cells producing IFN-γ or IL-17 where found in both cut LN and SC sites (Fig 9A-B right panels). By contrast, CD4 T cells that co-produce IFN-γ by IL-17 were only detected in the SC. In the absence of T-bet there was a complete disappearance of IFN-γ^+ CD4 T cells and a large inhibition in the IFN-γ^+IL-17^+ CD4 T cells (Fig 9A-B right panels and C).

The frequency of IL-17-effectors was higher within the γδ than the CD4 T cell populations both in the inguinal LN and SC (Fig 9A-B left panels). There were consistently more cytokine-producing effectors in the SC than in the inguinal LN. Notably, over 60% of γδ T cells express IL-17 in the SC and this associated with more γδ27^- than γδ27^+ T cells (Fig 9D). Moreover in the absence of T-bet there was a complete disappearance of IFN-γ producing γδ T cells (Fig 9A-B left panels).

Taken together our *in vitro* results suggest that T-bet signalling pathway is partially required for IFN-γ production by γδ27^+ T cells upon strong TCR signalling. The *in vivo* EAE model was not adequate to look at IFN-γ production by γδ T cells since against our expectation it induces very few of these effectors. Therefore, to specifically question the contribution the role of TF it will be required to use a mouse model inducing largest amount of in IFN-γ-producing γδ27^+ T cells. This could be in response to Malaria infection as shown that it induces great frequency of IFN-γ-effectors γδ27^+ T cells (~45% of IFN-γ^+ γδ27^+).

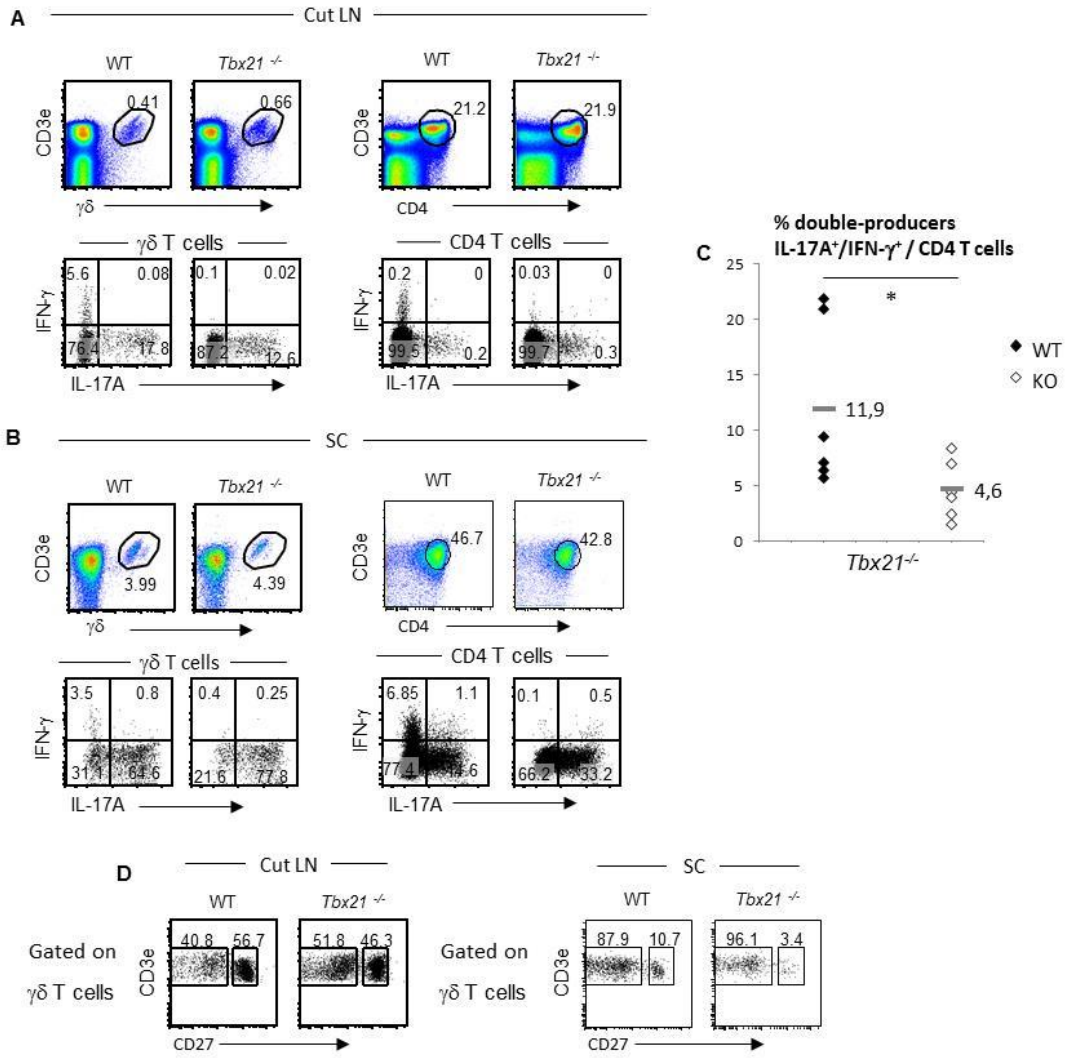


Figure 9- T-bet is partially required for IFN-γ production by IL-17 producing Th17 cells.
Cut LN cells and SC infiltrating cells were isolated from injected 8 weeks old *Tbx21*$^{-/-}$ and C57BL/6J (WT), used as controls. Mice were analyzed at the highest score (between 15 to 19 days after immunization). Representative FACS plots for total γδ and CD4 T cells (identified by CD45^{+} and CD11b^{-}) and intracellular cytokine staining for IFN-γ and IL-17A **A** from the Cut LN and **B** SC. **C** Percentages of IFN-γ producing cells out of IL-17 producing CD4 T cells from SC (WT n=6, *Tbx21*$^{-/-}$ n=5). **D** Representative FACS plots for CD27$^{+/-}$ γδ T cell proportions in cut LN and SC for both WT controls and *Tbx21*$^{-/-}$.

5- IL-17 PRODUCTION BY γδ27$^-$ T CELLS DOES NOT RELY ON THE Th17 TRANSCRIPTIONAL NETWORK

We next went on to determine the transcriptional requirement for IL-17 production by γδ27$^-$ T cells, using similar approach to determine if they share the same transcriptional regulators than CD4 Th17 cells. Along with previous studies[99,147,169], Rorγt showed to be crucial for the production of IL-17 by γδ27$^-$ T cells, since in his absence there was no production of IL-17 (Fig 9A-B). Additionally, accordingly to what was already published by the hosting laboratory[139], our data further support a key role of Rorγt in the development of CCR6$^+$γδ27$^-$ T cells (Fig 13C). On the other hand, and unexpectedly, our results pointed to a dispensable role of both Rorα and Batf for IL-17 production by γδ27$^-$ T cells (Fig 10A-B). Noteworthy, in the absence of Rorα we noticed an increase in the percentage of IL-17 producing cells. This particular point will be further analyzed in the next sections of this report. Consistent with a dispensable role of IRF4 in IL-17 production in γδ T cells[151], our results further support that, although dependent on RORγt, IL-17 production by γδ27$^-$ T cells does not depend on the same transcriptional partners shown to be crucial for Th17 cells.

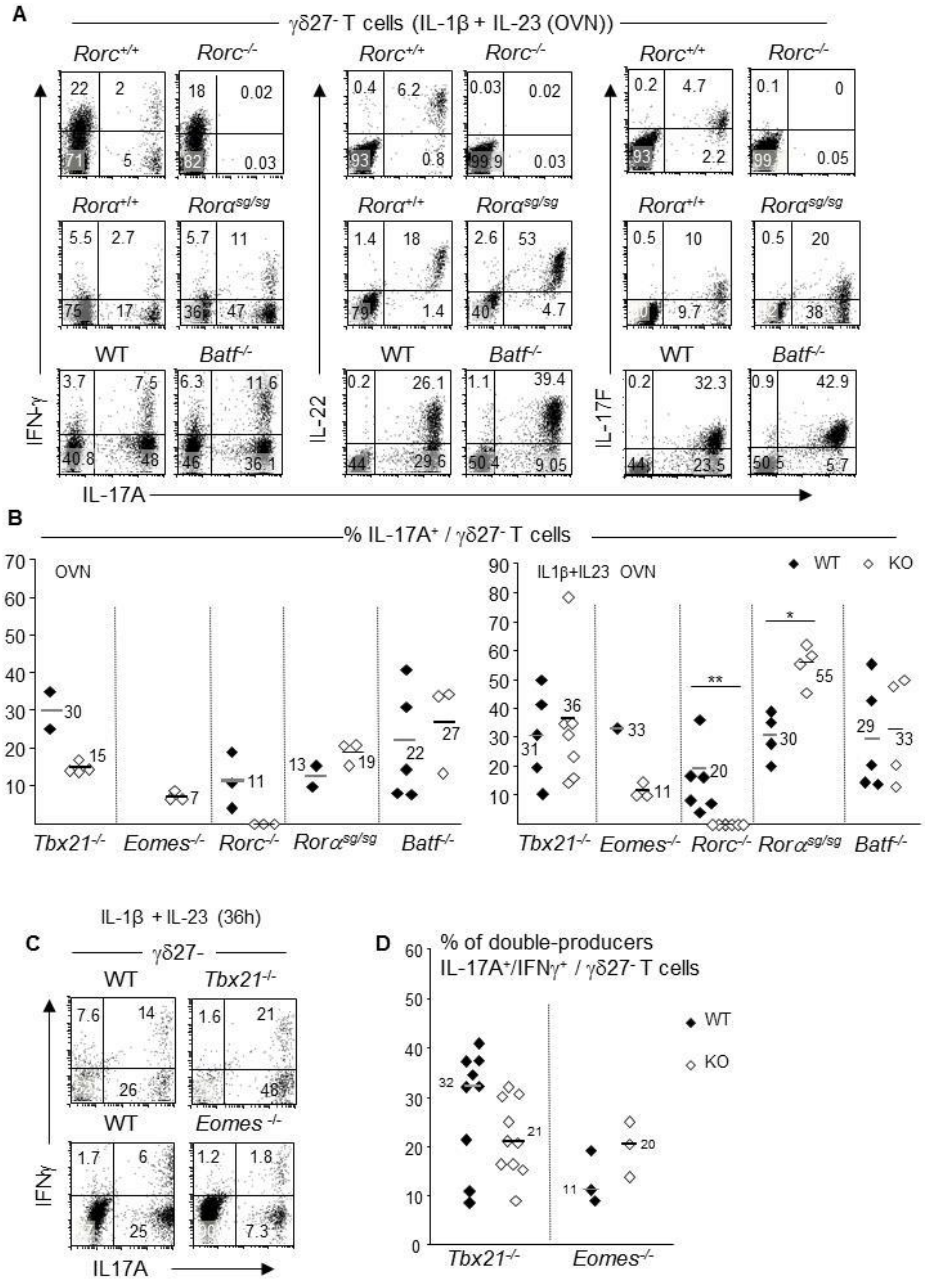


Figure 10- γδ27$^-$ T cells do not depend on Batf nor in Rorα for IL-17 production.

γδ27$^-$ T cells were FACS sorted from pooled spleen and LNs from 8 weeks old *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$, *Rorc*$^{-/-}$, and *Batf*$^{-/-}$ mice and 2 to 3 weeks old *Rorα*$^{sg/sg}$. Wilde type littermates of *Rorα*$^{sg/sg}$ and *Rorc*$^{-/-}$ were used as controls and as for the rest of the mice, C57BL/6J were used as controls. Sorting stategy was the same as in Figure 4A. **A** Representative FACS plots of intracellular cytokine staining for IFN-γ and IL-17A from γδ27$^-$ T cells isolated and stimulated OVN with soluble IL-1β+IL-23. **B** Percentage of γδ27$^-$ T cells producing IL-17 plated OVN unstimulated (on the left) and stimulated (on the right) (WT_{Tbx21} n=5 and *Tbx21*$^{-/-}$ n=7; WT_{Eomes} n=1 and *Eomes*$^{-/-}$ n=3; *RORc*$^{+/+}$ n=6 and *RORc*$^{-/-}$ n=7; *Rorα*$^{+/+}$ n=4 and *Rorα*$^{sg/sg}$ n=4; WT_{BATF} n= 5 and *Batf*$^{-/-}$ n=4). **C** Representative FACS plots of intracellular cytokine staining after stimulation over 36h with with soluble IL-1β plus IL-23 and stained for IFN-γ and IL-17A in cells sorted from *Tbx21*$^{-/-}$, *Eomes*$^{-/-}$ and C57BL/6J mice. **D** Percentage of γδ27$^-$ T cells co-producing IFN-γ and IL-17 within total IL-17$^+$γδ27$^-$ T cells after 36h of stimulation (WT_{Tbx21} n=9 and *Tbx21*$^{-/-}$ n=10; WT_{Eomes} n=3 and *Eomes*$^{-/-}$ n=3).

6- IFN-γ PRODUCTION BY γδ27$^-$ T CELLS IS EOMES INDEPENDENT AND T-BET DEPENDENT.

In response to IL-1β plus IL-23 γδ27$^-$ T cells were induced to coproduce IL-17 and IFN-γ (Fig 5A). CD4 Th17 cells were shown to co-produce IFN-γ and IL-17 upon stimulation with IL-23 or IL-12, giving rise to "Th1-like" Th17 cells[111,114]. However, the transcriptional regulation for IFN-γ production by the IL-17 producing cells is under active debate. To better understand the regulation of IFN-γ production by IL-17$^+$ γδ27$^-$ T cells we assessed possible roles for T-bet and Eomes. Thus, γδ27$^-$ T cells derived from T-bet or Eomes-deficient mice were stimulated with IL-1β plus IL-23 for 36h. Strikingly, in this condition both T-bet and Eomes were dispensable for the production of IFN-γ (Fig 10C-D).

During EAE, IFN-γ$^+$IL-17$^+$ coproducing γδ T cells have been observed in the LN draining the site of injection or the SC[114,158]. However in our hand γδ T cells coproducing IFN-γ and IL-17 were present neither in the LN nor in the SC (Fig 9A-B left panels). Therefore we looked for another mouse model system to study IFN-γ$^+$IL-17$^+$ γδ T cells. These particular cells, have been shown to develop during *Listeria monocytogenes* infection[159]. So, to assess the possible role of T-bet in IFN-γ production by IL-17$^+$γδ27$^-$ T cells, we applied this infection model in C57BL/6J and *Tbx21*$^{-/-}$ mice and analyzed IFN-γ as well as IL-17 production by both γδ and CD4 T cells (Fig 11). To better address cytokine production we stimulated the isolated cells using two different conditions, PMA plus Ionomycin and IL-1β plus IL-23, for 4h. Using PMA plus Ionomycin we could detect IFN-γ production alone by γδ T cells, however with no increase between the naïve and infected WT mice (Fig 11A-middle panels). Moreover, under this stimulating condition there was none IFN-γ$^+$IL-17$^+$ γδ T cells (Fig 11A-middle panels). Nonetheless, by stimulating the cells with IL-1β plus IL-23 it was possible, within WT infected mice, to detect double-producers (IFN-γ$^+$IL-17$^+$) and IFN-γ single producers γδ T cell subsets (Fig 11A-lower panels-B). Interestingly both populations originated from the γδ27$^-$ T cell subset (data not shown). Surprisingly, contrary to our *in vitro* data, in the absence of T-bet there was a disappearance of the IFN-γ$^+$IL-17$^+$ coproducing γδ T cell population in proportion and cell numbers (Fig 11A-B and Sup Fig 1A). Moreover, γδ27$^-$ T cells solely producing IFN-γ were also decreased (Fig 11A-B and Sup Fig 1A). In the case of CD4 T cells, stimulated with PMA plus Iono, we could notice an increase in the production of IFN-γ between the naïve and infected WT mice (Fig 11C-D). As expected, in the absence of T-bet, IFN-γ production by CD4 T cells was abrogated (Fig 11C-D and Sup Fig 1B). Furthermore, in line with the results from the EAE model, in the absence of T-

bet there was an increase in the proportion of IL-17 producing γδ T cells, although not in numbers (Fig 11A-B and Sup Fig X).

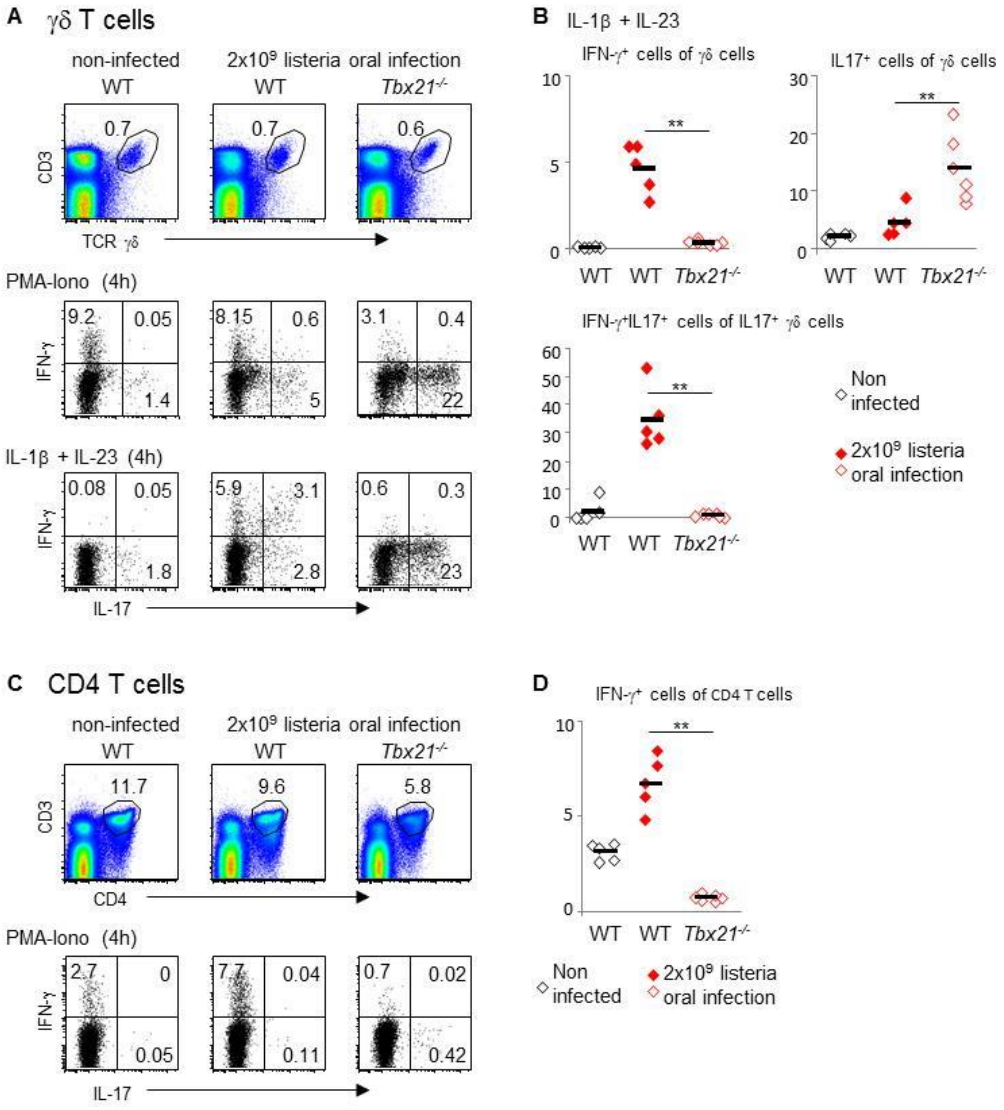


Figure 11- T-bet is absolutely required for IFN-γ production by IL-17 producing γδ T cells.
On day 8 after the *Listeria monocytogenes* infection, spleenocytes were isolated from infected and non-infected 8 weeks old *Tbx21*$^{-/-}$ and C57BL/6J (WT) mice, used as controls. **A** Representative FACS plots for total γδ T cells (on top) and intracellular cytokine staining for IFN-γ and IL-17A stimulated for 4h with PMA plus Iono (in the middle) and with IL-1β plus IL-23 (in the bottom). **B** Proportion of IFN-γ producing γδ T cells (top left), IL-17 producing γδ T cells (top right) and IFN-γ^{+}IL-17^{+} out of IL-17 producing γδ T cells (bottom) stimulated with IL-1β plus IL-23 ($WT_{non\text{-}infected}$=5,$WT_{infected}$=5 and *Tbx21*$^{-/-}$=6). **C** Representative FACS plots for total CD4 T cells and intracellular cytokine staining for IFN-γ and IL-17A stimulated for 4h with PMA plus Ionomycin (Iono). **D** Proportion of IFN-γ producing CD4 T cells ($WT_{non\text{-}infected}$=5,$WT_{infected}$=5 and *Tbx21*$^{-/-}$=6).

Taken together, our results suggest a similar transcriptional regulatory pathway between Th17 and γδ27- T cells for IFN-γ production by IL-17 producing cells.

7- RORα CONTROLS Vγ CHAIN IL-17 BIASED γδ T CELLS

The two-fold increase in the proportion of IL-17-producing γδ27$^-$ T cells in the absence of Rorα was unexpected and led us to further investigation (Fig 10A-B), since we were assuming a possible impairment of IL-17 expression. Rorα is, like Rorc, a member of the Retinoic acid-related Orphan Receptor, which has been linked to many biological processes or conditions as varied as the development of the cerebellar cortex, the control of the circadian clock, autism spectrum disorder and immune cell responses[105,170]. In the introduction we have focused on RORα cooperation with RORγt to induce Th17 differentiation[105,170]. As yet nothing is known about RORα regulating functions in γδ T cells. Thus in the second part of the project, we went on to pursue the analysis of its potential roles in γδ T cell biology.

To do our studies, we used the homozygous mutant mouse staggerer (RORα$^{sg/sg}$), that suffers a spontaneous deletion which removes an exon encoding part of the ligand binding domain of the putative receptor, leading to a generation of a non-functional RORα truncated protein[171]. Furthermore, mice engineered for the deletion of this transcription factor displayed a similar cerebellar phenotype as the staggerer mouse, demonstrating that the phenotype observed by the mutation on the staggerer mice is caused by the absence of functional RORα[172,173]. Moreover, in our hands and according to published data[174] the stagger mice displayed a decreased splenic cellularity compared to their WT litter mate controls (Sup. Fig 2). However, we noticed no difference in the total number of cells within the thymus (Sup. Fig 2).

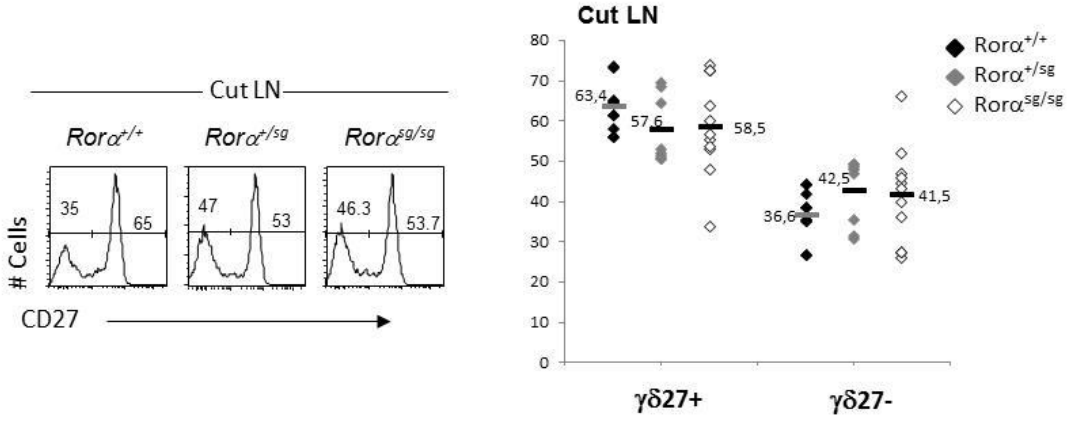


Figure 12– RORα absence has no impact in the levels of γδ27$^-$ T cell population.
γδ T cells were obtained from cutaneous lymph nodes (cut LN) of 2 to 3 weeks old Rorα$^{+/+}$, Rorα$^{+/sg}$, and Rorα$^{sg/sg}$ mice. Representative FACS plots for levels of CD27 expression levels between the three mice (Rorα$^{+/+}$, Rorα$^{+/sg}$, and Rorα$^{sg/sg}$) and γδ27+ and γδ27- cell numbers in WT litter mate control (Rorα$^{+/+}$), Rorα$^{+/sg}$ and Rorα$^{sg/sg}$. Rorα$^{sg/sg}$ n=10, control n=5.

First, we questioned if RORα govern the development of the γδ27$^-$ T cells which preferentially express IL-17. However, the proportion of γδ27$^+$ and γδ27$^-$ T cells was similar between RORα$^{+/+}$, RORα$^{+/sg}$ and RORα$^{sg/sg}$ mice (Fig 12). Nevertheless, within the γδ27$^-$ cells the subset which produces specifically IL-17 also expresses CCR6. Thus we next assessed if the proportion of CCR6$^+$γδ27$^-$ T cells, within total γδ27$^-$ T cells, were specifically increased. As shown by Figure 13A and 13B normal proportions of CCR6$^+$γδ27$^-$ T cells develop from γδ T cell compartment which lacks the functional RORα protein (in all lymphoid organs analysed thymus, cutaneous LN and spleen) (Fig 13A-B, Sup Fig 3). These results strongly contrast with the strict requirement of RORγt for the generation of the CCR6$^+$γδ27$^-$ T cell subset (Fig 13C, [139]). Altogether, these results suggest that the increased frequency of IL-17 effectors in the absence of RORα is not due to an increased development of CCR6$^+$γδ27$^-$ cells.

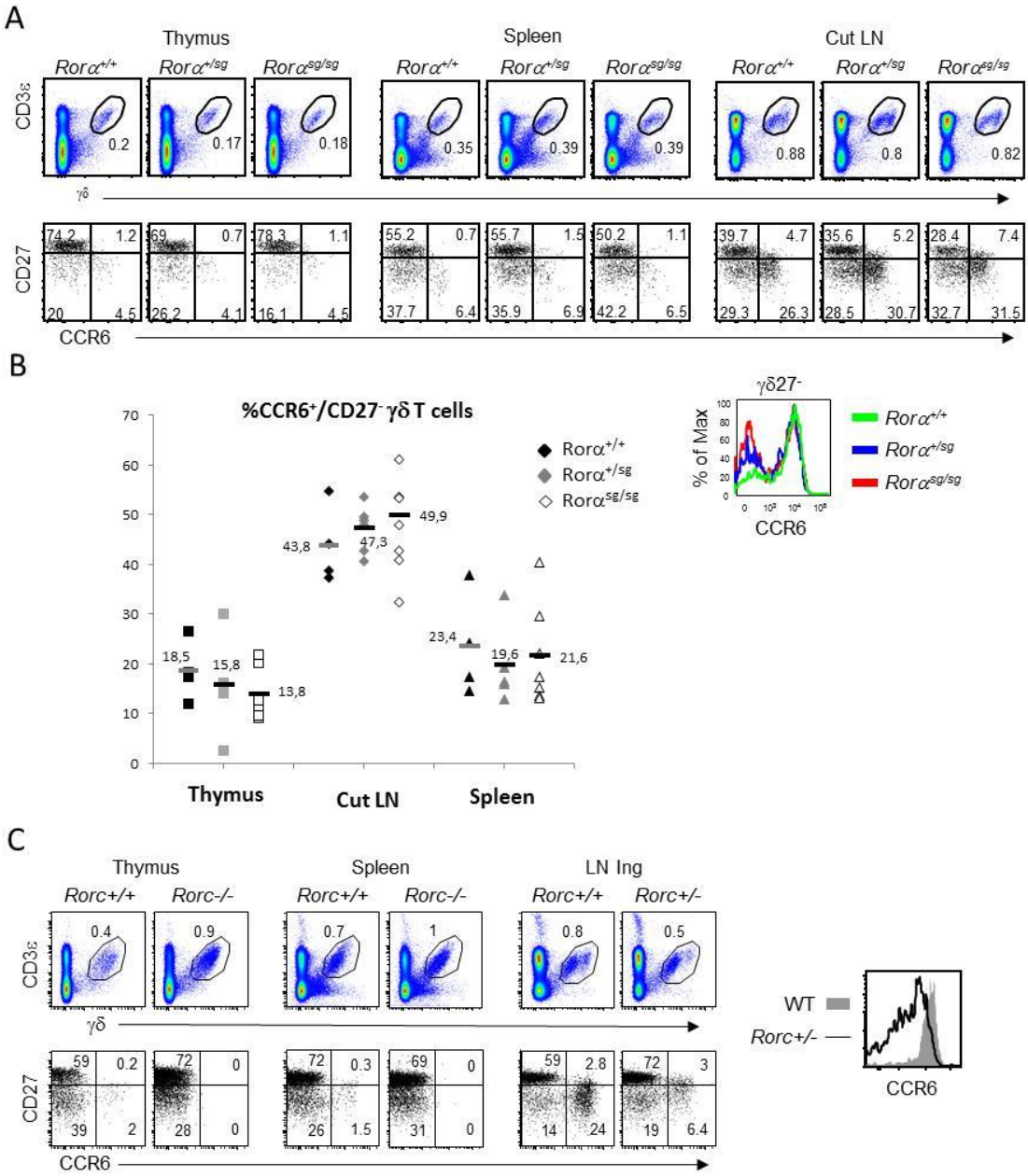


Figure 13 – Contrary to RORγτ, RORα does not control the levels of CCR6^{+}γδ27^{-} T cells.
γδ T cells were obtained from thymus, spleen and cutaneous lymph nodes (cut LN) of 8 weeks old *Rorc*$^{-/-}$ and WT litter mate control mice and 2 to 3 weeks old *Ror*α$^{+/+}$, *Ror*α$^{+/sg}$, and *Ror*α$^{sg/sg}$ mice. **A** Representative FACS plots for total γδ T cells and CD27 plus CCR6 γδ cell subsets from Rorα$^{sg/sg}$ and WT litter mate control and levels of CCR6 expression between the three mice. **B** CCR6^{+}γδ27^{-} cell percentages in WT litter mate control (*Ror*α$^{+/+}$), *Ror*α$^{+/sg}$ and *Ror*α$^{sg/sg}$. (Rorα$^{sg/sg}$ n=7, control n=4). **C** Representative FACS plots for total γδ T cells and CD27/CCR6 γδ cell subsets from *Rorc*$^{-/-}$ and WT litter mate control (*Rorc*$^{-/-}$ n=3, control n=4).

Besides CD27 and CCR6 markers, IL-17- *versus* IFN-γ-producing γδ T cells, γδ T cells can also be segregated according to the exclusive Vγ chain usage. Thus, Vγ1 T cells preferentially secrete IFN-γ whereas Vγ4 T cells favour IL-17 production[149,150,175]. However, contrary to CD27 and CCR6 segregation, this dichotomy is not absolute, as mouse Vγ4 T cells can also produce IFN-

γ[130,176,177]. Given that Vγ1$^+$ and Vγ4$^+$ are the two dominant γδ T cell subsets in peripheral lymphoid organs, we next questioned if Rorα could influence the Vγ chain usage in γδ T cell subsets.

The fraction of Vγ4 subset was reproducibly increased, at the expense of the Vγ1 subset that was decreased, in the lymphoid organs of Rorα$^{sg/sg}$ mice compared to the RORα$^{+/+}$ littermate controls (Fig 14A). Consequently, in the absence of RORα there is around a two-fold augmentation in the ratio between Vγ4/Vγ1 subsets in thymus, LN and spleen (Fig 14B). Of note, although to a lesser extent than Vγ4$^+$ cells, the frequency of Vγ1$^-$Vγ4$^-$ subset was also increased (Fig 14A). Moreover, looking at the cell number although not observed in the cut LN (Fig 14C), there was a decreased number of cells expressing the Vγ1 chain between Rorα$^{+/+}$ and Rorα$^{sg/sg}$ mice in both thymus and spleen (Sup Fig 4). Noteworthy, the effect of RORα is gene dose-dependent since the Rorα$^{+/sg}$ showed an intermediary phenotype (Fig 14B, Sup Fig 4).

Given that Rorγt is crucial for the development of a specific subset of γδ cells (CCR6+γδ27-) and that in Th17 cells it acts cooperatively with Rorα to control IL-17 production, we next aimed to determine if alteration of the frequency of Vγ4$^+$ and Vγ1$^+$ cells would also be influenced by Rorγt absence. Interestingly, we found that the ratio between Vγ4/Vγ1 subsets was not affected in the absence of Rorγt demonstrating the exclusive role of Rorα (Fig 14D-E).

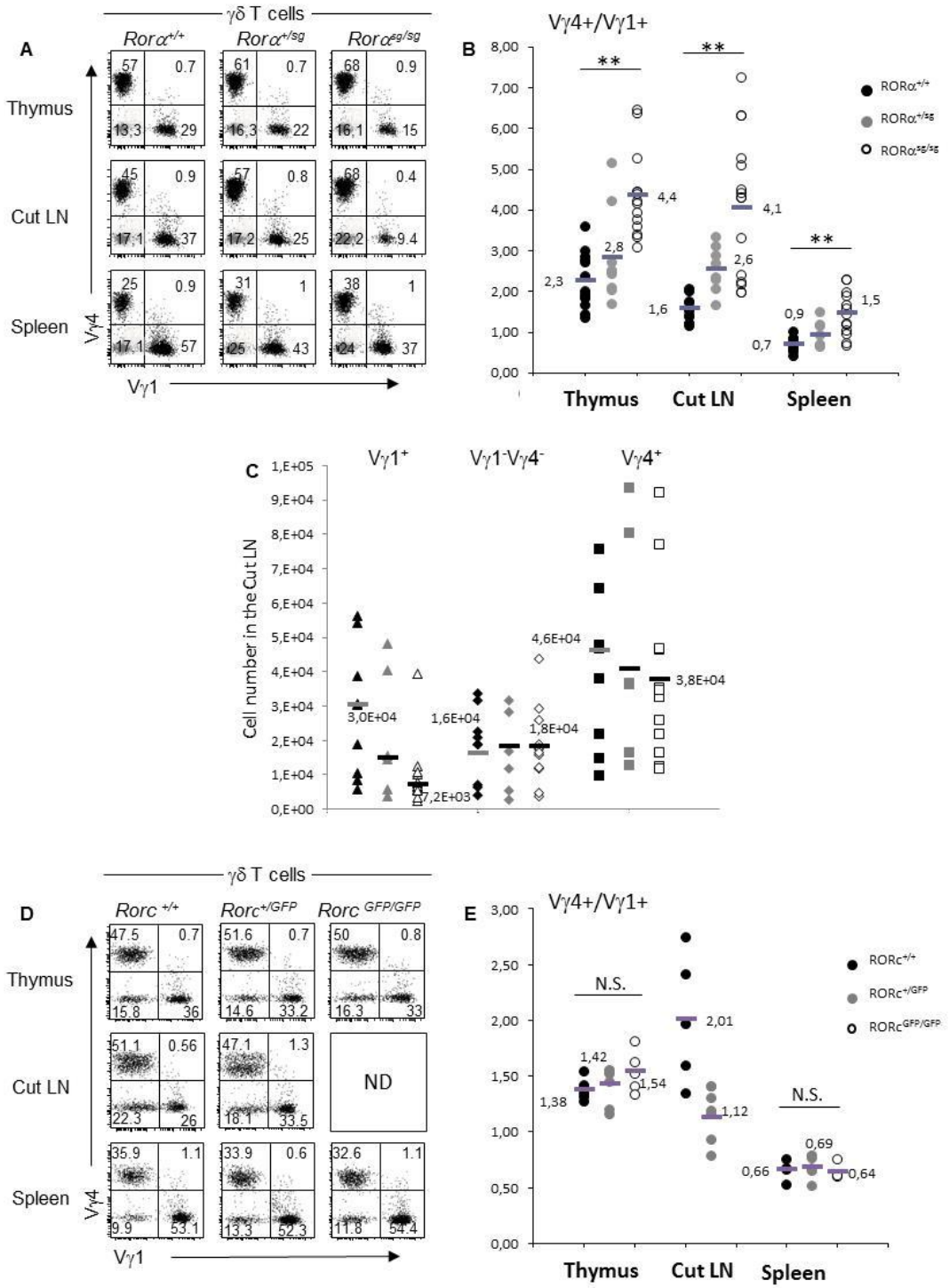


Figure 14 - Absence of Rorα leads to an increase in γδ T cells expressing the Vγ4 chain and a decrease in the Vγ1 γδ T cells.

γδ T cells were obtained from thymus, spleen and cutaneous lymph nodes (cut LN) of 8 weeks old *Rorc*$^{+/+}$, *Rorc*$^{+/GFP}$, and *Rorc*$^{GFP/GFP}$ mice and 2 to 3 weeks old *Ror*α$^{+/+}$, Rorα$^{+/sg}$, and *Ror*α$^{sg/sg}$ mice. **A** Representative FACS plots for Vγ4, Vγ1 and Vγ1-Vγ4- subsets gated on total γδ T cells from Rorα$^{sg/sg}$ and litter mate controsl. **B** Ratio numbers between Vγ4^{+} γδ T cells and Vγ1^{+} γδ T cells percentages out of total γδ T cells in thymus, cut LN and spleen of *Ror*α$^{+/+}$, *Ror*α$^{+/sg}$, and *Ror*α$^{sg/sg}$ mice (*Ror*α$^{+/+}$ n=10, *Ror*α$^{+/sg}$ n=8 and Rorα$^{sg/sg}$ n=13). **C** Vγ4^{+}, Vγ1^{+} and Vγ1^{-}Vγ4^{-} subsets cell number from cut LN in Rorα$^{+/+}$, Rorα$^{+/sg}$, and *Ror*α$^{sg/sg}$. **D** Representative FACS plots for Vγ4^{+}, Vγ1^{+} and Vγ1-Vγ4- subsets gated on total γδ T cells from from *Rorc*$^{-/-}$ and litter mate controls. **E** Ratio numbers between Vγ4^{+} γδ T cells and Vγ1^{+} γδ T cells percentages out of total γδ T cells in thymus, spleen and cut LN of *Rorc*$^{+/+}$, *Rorc*$^{+/GFP}$, and *Rorc*$^{GFP/GFP}$ mice (*Rorc*$^{GFP/GFP}$ n=5, *Rorc*$^{+/GFP}$ n=5, *Rorc*$^{+/+}$ n=5).

RORα has been associated with cell death[178] and proliferation[179–181]. For instance, RORα is downregulated at the transcriptional levels in many different types of human cancers (ovarian, breast and prostate). Conversely, RORα overexpression leasds to inhibition of cell growth[179]. With this in mind, we questioned whether γδ subset-specific alterations of the cell-death or cell-cycle rates could account for the higher ratio of Vγ4 / Vγ1 subsets in RORα$^{sg/sg}$ mice. We analysed the proportion of apoptotic cells by *ex vivo* annexin V staining, and measured the proliferative activity after *in vivo* incorporation of 5-bromodeoxyuridine (BrdU) in Vγ4, Vγ1 and Vγ1-Vγ4- subsets from RORα$^{+/+}$, RORα$^{+/sg}$ and RORα$^{sg/sg}$ mice (Fig 15A-B).

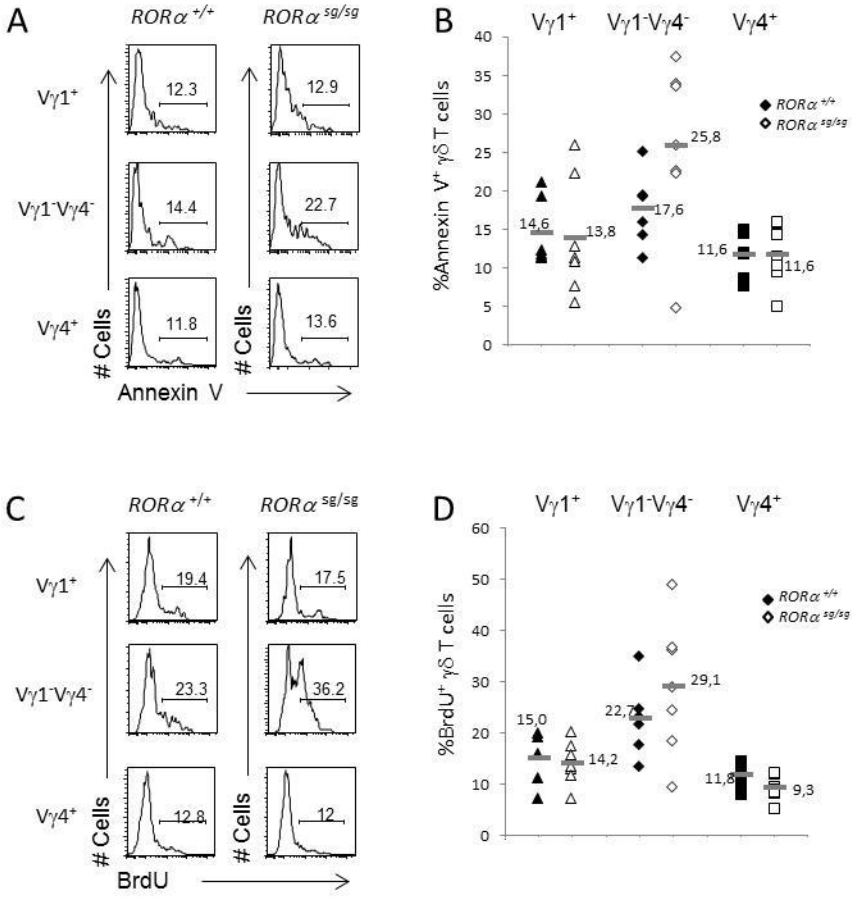


Figure 15– RORα does not control cell-cycle or cell-death in thymic Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ γδ T cell subsets.
RORα$^{+/+}$ and *RORα*$^{sg/sg}$ mice were injected i.p. with BrdU 18h before analysis. Thymocytes were then obtained and stained for BrdU for Annexin V. **A** Representative FACS plots for Annexin V stainning analysis for γδ T cell susbets (Vγ1$^-$Vγ4$^-$, Vγ1$^+$ and Vγ4$^+$). **B** Percentage of Annexin V positive γδ T cell subsets. **C** Representative FACS plots for BrdU analysis for γδ T cell susbets (Vγ1$^-$Vγ4$^-$, Vγ1$^+$ and Vγ4$^+$). **D** Percentage of BrdU positive γδ T cell subsets.

Neither the percentage of apoptotic cells (Fig 15A-B) nor the rate of proliferation (Fig 15C-D) were modified in between Vγ4$^+$ or Vγ1$^+$ γδ T cell subsets derived from mice either expressing a WT or a natural mutant form of RORα. These results were similar between thymus, cutaneous

LN and spleen (Fig 15, Sup Fig 5-6). Therefore, our results ruled out a possible role for RORα in controlling either cell-death or proliferation of the Vγ4$^+$ or Vγ1$^+$ subsets.

Taken together these results further suggest a dual role of RORα. On one hand, RORα seems to regulate the proportion of IL-17-producing γδ27- T cells, and on the other the development of Vγ4$^+$ and Vγ1$^+$ cells. At this stage of the study, it was not yet explicit if the alterations observed would represent two distinct effects of RORα or if the increased frequency of Vγ4$^+$ cells would solely account for the increase in IL-17-producing γδ27$^-$ T cells. Therefore, we pursue our experiments in order to discriminate between these two hypotheses.

First we FACS sorted Vγ1$^+$, Vγ4$^+$ and Vγ1$^-$Vγ4$^-$ cell subsets from RORα$^{+/+}$, RORα$^{+/sg}$, and RORα$^{sg/sg}$ mice and stimulated them OVN with IL-1β plus IL-23 to measure IL-17 production (Fig 16). As expected the main source of IL-17 was the Vγ4$^+$ cell subset which contained over 40% of IL-17-producing effector cells, while 15% and less than 5% were from Vγ1$^-$Vγ4$^-$ cell subset and Vγ1$^+$ cell subset, respectively. However, to our surprise, the absence of RORα led to a two-fold increase in frequency of IL-17-producers only from the Vγ1$^-$Vγ4$^-$ γδ T cell subset. This suggests that the increase in IL-17-producing effector γδ T cells results from two cumulative events. One the accumulation of Vγ4$^+$ cells and second the augmentation of the proportion of IL-17-producers within the Vγ1$^-$Vγ4$^-$ γδ T cell subset. Taken together, these data led us to hypothesize that RORα has a direct role in regulating IL-17 production by Vγ1$^-$Vγ4$^-$ cells.

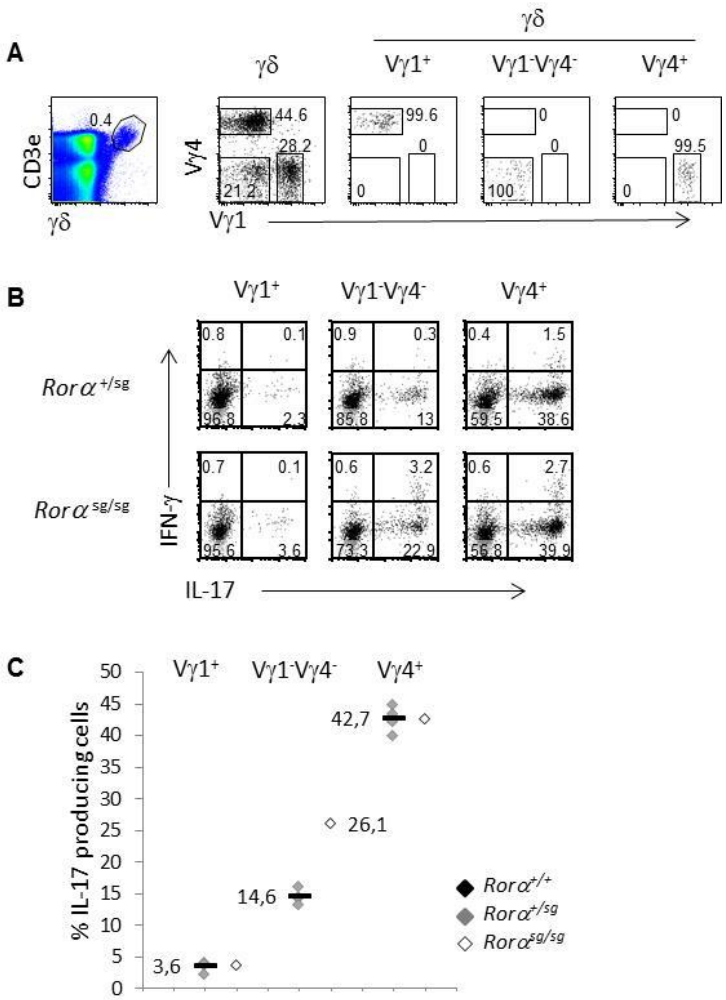


Figure 16- RORα controls IL-17 expression in Vγ1-Vγ4- γδ T cell subset.
γδ T cells were FACS sorted from pooled spleen and LNs from 2 to 3 weeks old *Rorα*$^{+/+}$, *Rorα*$^{+/sg}$ and *Rorα*$^{sg/sg}$ mice. **A** Representative FACS sorting gate strategy for sorted cell subsets (Vγ1$^+$ Vγ4$^+$ and Vγ1$^-$Vγ4$^-$) **B** Representative FACS plots of intracellular staining for IFN-γ and IL-17 in sorted cells after OVN incubation with IL-1β plus IL-23. **C** Percentages of IL-17 producing Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ γδ T cells within the *Rorα*$^{+/+}$, *Rorα*$^{+/sg}$ and *Rorα*$^{sg/sg}$ mice

RORα is known to control gene expression through both transcriptional activation and repressive activities. For instance, RORα is a transcriptional activator of *Bmal1*, which works as a key regulator of circadian clock function[182]. By contrast, once phosphorylated RORα binds to the β-catenin promoter sites and not only suppress the recruitment of transcriptional coactivators and but also the RNA Pol II, inducing the transcriptional repression of β-catenin and its targets[180]. Intriguingly, it was reported that the homozygous staggerer (sg/sg) mice have an increased of *Sox13* mRNA expression[183]. This is of particular interest since it was shown by Gray and colleagues that the absence of the TF Sox13 can cause an intrinsic and selective defect in development of IL-17-producing Vγ4$^+$ cells[150]. This is consistent with a specific role of a network consisting of a quartet of high-mobility group (HMG) box TF - SOX13, SOX4, LEF1, and TCF1 - in the development of IL-17$^+$Vγ4$^+$ cells[149,175]. Noteworthy, SOX4 and

SOX13 have been shown to regulate directly the two cell-specific genes required for IL-17-production by γδ T, RORc and B lymphocyte kinase (BLK)[184]. On the other hand, LEF1 and TCF1 counteract the SOX proteins and induce genes involved in the IFN-$\gamma^+\gamma\delta27^+$ effector subset[149].

First, we assessed the expression of *Sox13*, *Sox4*, *Lef1*, and *Tcf7* (which encode TCF1) transcripts in peripheral $\gamma\delta27^+$, $CCR6^-\gamma\delta27^-$ and $CCR6^+\gamma\delta27^-$ T cell subsets. Surprisingly, *Sox13* presented 100 times higher levels of mRNA, while *Sox4* was 10 times less expressed in $CCR6^+\gamma\delta27^-$ T cells when compared to $\gamma\delta27^+$ T cells, respectively (Fig 17A). This reveals that there are still gaps in our understanding of the role of SOX4 in $CCR6^+\gamma\delta27^-$ cells. Moreover, the expression of *Lef1* mRNA is 100-fold lower in $CCR6^+\gamma\delta27^-$ T cells than in $\gamma\delta27^+$ T cells. This is consistent with its role into inhibiting IL-$17^+\gamma\delta27^-$ T cell development, and mirrors its exclusion from γδ thymocytes fated to produce IL-17.

Sox13, *Sox4* and *Lef1* regulation is specific to γδ T cells since Th1 and Th17 cells didn't differentially expressed these genes or in the case of *Sox13* with minor differences. *Tcf7*, however, was the only one expressed about 10-times higher in Th17 compared to Th1 cells.

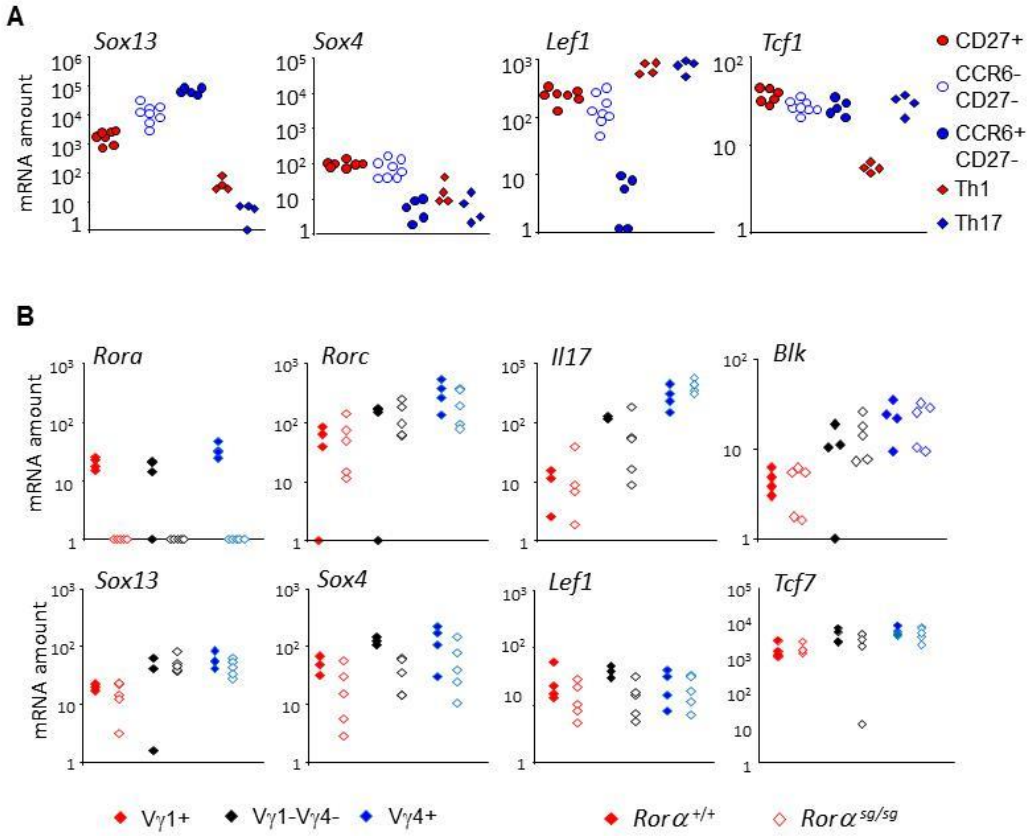


Figure 17- Expression profile of IL-17 related transcription factors in different γδ T cells subsets.
γδ T cells were FACS sorted from pooled spleen and LNs from 8 weeks old C57BL/6J mice and thymus from 2 to 3 weeks old *Rorα*$^{+/+}$ and *Rorα*$^{sg/sg}$ mice. **A** RT-PCR data for *Sox13*, *Sox4*, *Lef1 and Tcf1* expression (relative to *b2m* or *Actb*) on peripheral γδ T cell subsets : CD27$^+$ (CD27$^+$), CD27$^-$ (CCR6$^-$CD27$^-$) and CCR6$^+$ (CCR6$^+$CD27$^-$); and *in vitro*-generated CD4 Th1 and Th17 cells. **B** RT-PCR data for *Rorc, Rora, IL17, Sox13, Sox4* and *Lef1* expression (relative to *b2m* or *Actb*) on thymic γδ T cell subsets: Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ cells from *Rorα*$^{+/+}$ and *Rorα*$^{sg/sg}$ mice.

Secondly, we assessed whether RORα would impact in the expressions of these genes known to regulate IL-17 production in the γδ T cell subsets already in the thymus (Fig 17B). To do so, we FACS-sorted Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ thymocytes from *Rorα*$^{+/+}$ and *Rorα*$^{sg/sg}$ mice and assessed gene expression by real-time PCR. The primers we used for *Rorα* mRNA detection recognise part of the ligand binding domain, in γδ thymocytes from the mutant mouse we could no longer amplify a *Rorα* mRNA product which confirms the absence of a functional messenger for *Rorα* in the staggerer mouse. The lack of variation in transcripts for *Il17*, *Rorc, Blk* or Sox13 showed that RORα is dispensable for the regulation of IL-17+γδ cell-specific genes Moreover, many other targets including, but not restricted to, genes associated with Th17 cytokine production (*Il17f*, *Il22*, *Csf2*), Th17-governing TF (*cmaf*, *Ahr*), inhibitors of *Il17* transcription (*Tbox21*), receptors for cytokines regulating IL-17 production (*Il1r1*, *Il23r*) were similarly expressed by *Rorα*$^{+/+}$ and *Rorα*$^{sg/sg}$ cells (data not shown). However, *Sox4* and *Lef1* which are expressed at low levels in CCR6$^+$γδ27$^-$ T cells, are two-fold down-regulated

specifically in the in $V\gamma1^-V\gamma4^-$ cell subset that express a non-functional RORα. These results further suggest a possible role of RORα in controlling IL-17 production exclusively in $V\gamma1^-V\gamma4^-$ γδ T cell subset. Consistent with this, we observed that peripheral $V\gamma1^-V\gamma4^-$ γδ T cells express 3 and 7 times more *Rorα* mRNA than $V\gamma4^+$ and $V\gamma1^+$ cells, respectively (Sup Fig 7). In a similar way, $V\gamma1^-V\gamma4^-$ γδ T cells express 10 times more *Il17* mRNA (Sup Fig 7).

Next, we aimed to assess whether RORα would play a role at early development stage of $V\gamma4^+$ and $V\gamma1^+$ thymocytes. Given that TCR-dependent transitional maturation in early subsets of γδ thymocytes coincide with the downregulation of expression of CD24 [126] we went on to see if the Vγ4/Vγ1 cell ratio was altered in immature $CD24^+$ γδ thymocytes from $ROR\alpha^{sg/sg}$ mice (Fig 18). First of all, there was no change in the proportion between total $CD24^+$ immature and $CD24^-$ mature γδ thymocytes in the absence of a functional RORα (Sup Fig 8). In the same way, the frequency of $CD24^+$ and $CD24^-$ γδ T cells was unmodified within the $V\gamma1^+$, $V\gamma4^+$ or $V\gamma1^-V\gamma4^-$ thymocytes subsets (data not shown). Nevertheless, and most importantly, the alteration in the Vγ4/Vγ1 cell ratio was already present in the $CD24^+$ immature γδ thymocytes (Fig 18). This suggests that RORα role in favouring the development of $V\gamma4^+$ instead of $V\gamma1^+$ acts already at a post-DN3 stage however before the TCR-engagement step which leads to CD24 downregulation. Variations in the $V\gamma1^+$, $V\gamma4^+$ or $V\gamma1^-V\gamma4^-$ γδ T thymocyte subsets are then maintained after the γδ T cells exit the thymus and colonize the periphery.

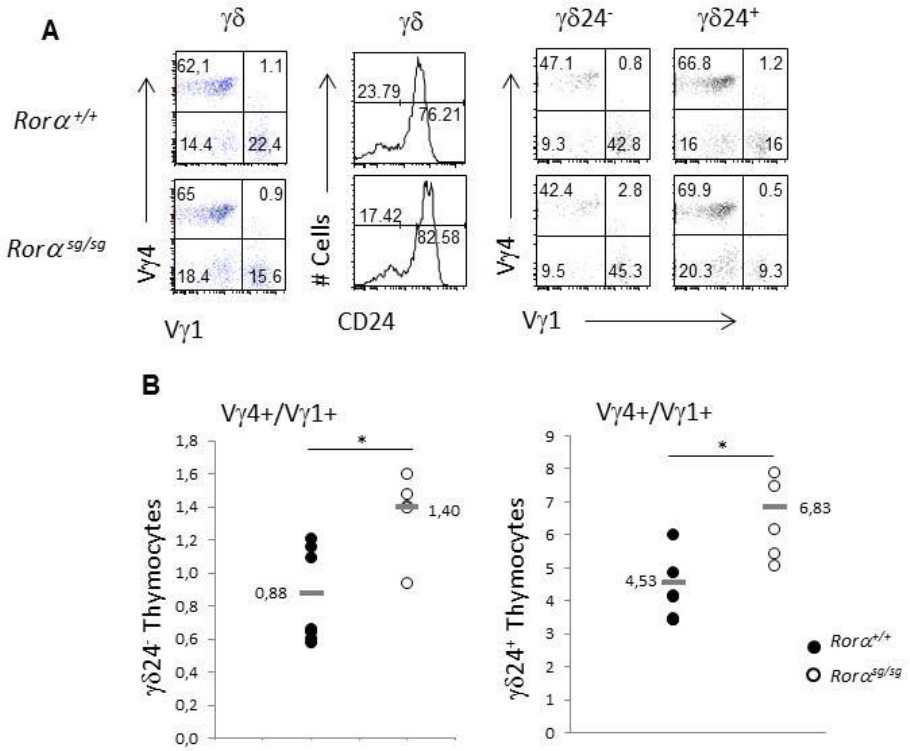


Figure 18 – Higher Vγ4/ Vγ1 is present in immature γδ24$^+$ T cells from RORα$^{sg/sg}$
γδ T cells were obtained from thymus of 2 to 3 weeks old *Rorα*$^{+/+}$ and *Rorα*$^{sg/sg}$ mice. **A** Representative FACS plots for segregation between CD24$^{+/-}$ γδ T cells within the Vγ4$^+$, Vγ1$^+$ and Vγ1$^-$Vγ4$^-$ subsets gated on CD24$^{+/-}$ γδ T cells. **B** Ratio numbers between Vγ4$^+$ γδ T cells and Vγ1$^+$ γδ T cells percentages out of total CD24$^-$ and CD24$^+$ γδ thymocytes.

Altogether, these data led us to believe that RORα presents a dual, non-overlapping, role in regulating IL-17 production strictly in Vγ1$^-$Vγ4$^-$ cells on one side and in controlling the balance between Vγ4$^+$ and Vγ1$^+$ cell development on the other. Future research will determine whether RORα is involved in a cell-intrinsic manner and during the Vγ chain rearrangement process.

VII. DISCUSSION

In the present work, we show that γδ T cells and αβ T cells present different mechanisms of cytokine regulation. It was previously shown in our laboratory[139] that γδ27$^+$ T cells only produce IFN-γ upon stimulation, resembling Th1 CD4 T cells. In line with this, a large proportion (50%) of IFN-γ producing γδ27$^+$ T cells depend on T-bet, but not on Eomes (as do CD8$^+$ αβ T cells)[72,168]. IL-17$^+$γδ27$^-$ T cells produce IFN-γ upon *in vitro* stimulation with IL-1β plus IL-23, and during tumour and EAE responses, or during Listeria infection[114,139,158,159]. Surprisingly, we found that cytokine-induced production of IFN-γ (IL-1β plus IL-23) was independent of T-bet whereas the emergence of IFN-γ$^+$IL-17$^+$γδ27- T cells during Listeria infection relied on T-bet. Therefore, γδ T cells have distinct requirements of T-bet for the production IFN-γ. The reasons behind these differences will be further discussed below. In addition, γδ27$^-$ T cells exhibit a similar phenotype to Th17 with the ability to produce a broader set of cytokines such as IL-17, IL-17F, IL-22 and GM-CSF. Consistent with previous studies[99,147,169], γδ27$^-$ T cells have different regulatory mechanisms of IL-17 production than Th17 cells. IL-17 production by γδ27$^-$ T cells although absolutely dependent on RORγt, is not affected neither by the absence of IRF4[151] nor of BATF or RORα. This supports the idea that two pathways for IL-17 production exist, which discriminates cells of the innate (γδ T cells) and adaptive (αβ T cells) arms of the immune system.

Polarisation of naïve conventional T cells relies on three types of signalling delivered by antigen presenting cells: TCR recognition, costimulation and cytokine engagement. Subsequently, memory T cells acquire the ability to respond more promptly to TCR- or cytokine-signalling to produce IFN-γ or IL-17. This property is shared within innate-like T cells and may explain the discrepancies we observed between *in vitro* and *in vivo* responses towards IFN-γ production and its dependence on T-bet. Indeed, individual stimulation analysed *in vitro* may not recapitulate the diversity of signals a cell can encounter during *in vivo* responses. Much of what is known today about T-cell signalling is based in studies carried out with conventional T cells. However these mechanisms are not always recapitulated by the innate-like T cells (γδ T and NKT cells). Below we will explain in greater detail the signalling pathways involved in IFN-γ or IL-17 production already observed in γδ T cells.

IFN-γ is a cytokine produced by a great variety of cells from the innate and adaptive immune system. It is well established that alongside with TCR stimulation, the presence of particular cytokines such as IL-12 lead naive CD4 T cells to differentiate into Th1 cells in secondary

lymphoid organs[63]. The ability of γδ T cells to spontaneously release IFN-γ strongly contrasts with the need of a long differentiation program by CD4 T cells. Stimulation of γδ T cells remains elusive *in vivo*, but *in vitro* γδTCR engagement has been shown to induce strong IFN-γ production by γδ27$^+$ T cells[139,145]. Besides TCR engagement, multiple other mechanisms can drive the differentiation of lymphoid cells, such as cytokine signalling. IL-2, IL-12 and IL-15 are able to induce IFN-γ production by γδ27$^+$ T cells, in different extents. Moreover IL-12 in synergy with IL-18 was shown to induce IFN-γ production by NK1.1$^+$ γδ27$^+$ T cells[129]. This goes along with the requirements of other innate cells (like NK cells) on IL-15 and IL-12 plus IL-18 for proper development and IFN-γ production, respectively[185,186]. Moreover, human γδ thymocytes were recently shown in our lab to acquire the capacity to produce IFN-γ upon stimulation with IL-2 or IL-15[187].

Cytokine signalling and transcriptional regulation towards cytokine production is well documented in the adaptive immune compartment. IL-12 signalling through STAT4 is essential for induction of optimal levels of T-bet which leads to IFN-γ production and commitment of CD4 T cells into a Th1 fate[73]. An amplification loop is then established controlling the expansion of IFN-γ response via STAT1 signalling and further T-bet expression[74,76]. Together with CD4 T cells, NK cells show a great dependence on STAT4 for the proper development of their responses after IL-12 stimulation[70]. The requirement of any member of the STAT family of transcription factors in γδ T cells for IFN-γ$^+$ differentiation is still a subject of debate, thus requiring further investigation. T-bet, which directly binds to the IFN-γ promoter[79], is crucial to induce the expression of this cytokine and support Th1 polarisation by diverting T cells from differentiating into other Th fates[79,188]. Eomes is, together with T-bet, another T-box transcription factor and the "master regulator" of IFN-γ production in CD8 T cells[168]. Plenty still needs to be unveiled in regard to the innate immune compartment. T-bet, along with Eomes, is known to be important for NK cell development and terminal maturation[189,190]. Moreover, in the absence of T-bet, DCs are unable to efficiently prime Th1 cell responses and NKp46$^+$ ILC3s disappear from the small intestine[191,192]. Notably, *in vitro* γδ27$^+$ T cells only partially require T-bet for their IFN-γ production and show no dependence on Eomes[145,167]. Interestingly, the signals which regulate T-bet expression in ILC3s are IL-23 dependent but IL-12 independent[193]. In Th17 cells both IL-23 and IL-12 have been reported to induce IFN-γ production, although the requirement of T-bet remains elusive to date[111,114,194–196]. As demonstrated in our work, IL-17$^+$γδ27$^-$ T cells produce IFN-γ after IL-1β plus IL-23 stimulation *in vitro* in a T-bet independent manner, suggesting that different signalling pathways may be operating between γδ27$^+$ and γδ27$^-$ T cells. Moreover, a subset of γδ27$^-$ T cells, which does not produce IL-17, was able to

respond to IL-12 stimulation and produce IFN-γ. Furthermore, we and others have shown that γδ27$^-$ T cells preferentially produce IL-17 in response to cytokines rather than TCR stimulation[139,152]. However, Sheridan and colleges have shown that γδ27$^-$ T cells respond to plate-bound α-CD3 plus α-CD28 stimulation after *in vivo* immunization with *Listeria monocytogenes*[159]. Altogether, these results suggest the existence of different signalling pathways for the production of IFN-γ by γδ T cell subsets, and underline the diversity and combination of stimulatory signals that γδ T cells integrate to produce a given cytokine *in vivo*. The importance of TCR engagement in the periphery remains unclear to date due to major gaps in our knowledge about the TCR ligands recognized by γδ T cell subsets (Vγ4 or Vγ6). *In vitro* experiments will allow us to explore the transcriptional requirement for a unique signalling cascade. To better understand the distinct inducers for IFN-γ production in γδ T cell subsets, we propose the assessment of T-bet role in cytokine signalling pathways known to induce IFN-γ expression, such as IL-12, IL-15 and IL-18 as well as IL-1β plus IL-23 in combination, or not, with TCR stimulation.

As costimulation, signalling through CD70-CD27 molecules has been shown to increase the survival and proliferation of activated T and B cells, thereby enhancing their effector function. CD70 is not only expressed by activated DCs and lymphocytes but is also constitutively expressed by APCs in the thymic medulla and by the intestinal epithelium[197]. Although not important for αβ T cell development[198], CD27-CD70 interactions were shown to drive differentiation of CD4 T cells towards a Th1 fate[199–201]. Together, γδ T cells which leave the thymus expressing CD27 (γδ27+) are essentially IFN-γ[130]. However, γδ27$^-$ T cells were shown to also have the ability to produce IFN-γ[139]. Other unconventional T cells like NKT cells , have been shown to expressed CD27[130]. A direct connection between CD27 signalling and T-bet expression is yet to be made.The developmente of γδ T cells and the molecular mechanisms underlying the differentiation between a γδ27$^-$ and γδ27$^+$ subsetare still under debate[118]. Downregulation of CD27 by γδ T cells that did not engage their TCR nor received signals through CD27 is one of the hypotheses. Recent work developed in our laboratory demonstrated that while γδ27$^+$ T cells are stably committed to the expression of *Ifng* but not *Il17*, γδ27$^-$ T cells display permissive chromatin configurations at *loci* encoding both of these cytokines[139]. This likely explains their plasticity and diversified phenotype of cytokine production, particularly IFN-γ. One unresolved issue is whether the opened chromatin configuration at the *Ifng locus*, which is imprinted in γδ27$^+$ T cells, is transmitted to a permissive state in the γδ27$^-$ T cells during their development. Further studies should

determine if changes occur at the *Ifng locus* in the more terminally differentiated γδ27^{-} T cells that up-regulate CCR6.

A myriad of studies have shown that γδ T cells are crucial providers of IFN-γ in a diversity of physiological settings[202–204]. In the case of the EAE model, it was reported that IFN-γ producing cells are important at the site of inflammation during the development of the disease [205]. In later stages of the disease the most pathogenic cells in the inflamed tissue are IL-17 producing cells[152]. Double-producing (IFN-γ^{+}IL-17^{+}) Th17 cells were also reported by several groups to be present at this stage in the CNS[107,114,158]. The majority of cells producing IFN-γ, IL-17, or both, are CD4 T cells. Nevertheless, γδ T cells are also involved in the development of this disease. In TCRδ$^{-/-}$ mice EAE was less severe, the onset of disease was delayed and the clinical scores were reduced[152]. In the case of *Listeria monocytogenes* infection, IL-17 was shown to be a critical component of early anti-listerial immunity[206–208]. Moreover, Sheridan and colleagues have shown that γδ T cells which produce IL-17 could also produce IFN-γ upon infection[159]. The molecular mechanisms which regulate the development of the double-producing cells are still poorly defined. We aimed to assess the role of T-bet in IFN-γ^{+}IL-17^{+} γδ27^{-} T cells in both *in vivo* mouse model systems just mentioned. However in our hands, EAE was readily induced without the presence of double-producers γδ27^{-} T cells. Yet, in the *Listeria monocytogenes* infection model we could induce the co-production of both cytokines (IFN-γ and IL-17) in WT infected mice, in contrast to T-bet knockouts, where there was no IFN-γ production. These results suggest the existence of two possible and distinct ways of inducing IFN-γ production by γδ27^{-} T cells, as suggested previously for the IL-17^{+}Th17 T population[111,194–196,209].

Besides the *in vitro* data acknowledging the ability of γδ27^{+} T cell subset to produce high levels of IFN-γ upon TCR stimulation, little is known about their *in vivo* behaviour. The lack of knowledge about the cognate ligands of the TCRγδ is partially accounted for this gap in information. However, the same applies to the γδ27^{-} T cell subset, which besides the ability to co-produce IFN-γ and IL-17 upon IL-1β plus IL-23 stimulation, not much more is known. In order to clarify the requirement of T-bet in the production of IFN-γ by γδT cell subsets, we intend to use, in further studies, other *in vivo* models where IFN-γ production is predominant, such as malaria (*plasmodium bergei*) or viral infection.

The capacity of γδ T cells to produce IL-17 is believed to be instructed during thymocyte development, particularly during embryogenesis[12]. Although this notion still remains controversial, some of the molecular cues needed for γδ T cell development into IL-17

producers are already known. Both TGF-β[210] and IL-7[154] are crucial for IL-17$^+$γδ thymocyte development and expansion. Moreover, IL-1β and IL-23, two cytokines secreted by macrophages and DCs, are crucial for the upregulation and maintenance of IL-17 production by γδ T cells[139,152,165]. Importantly, invariant NKT (iNKT – NK1.1$^-$CD4$^-$ NKT), another innate-like T cell subset, have been shown to produce IL-17[22] upon stimulation with both TGF-β and IL-1 β[211]. Additionally, Group 3 innate lymphoid cells (ILC3s) depend on IL-7 for their development and on IL-1β plus IL-23 to produce IL-17[212]. CD4 Th17 cells, however, require TGF-β, IL-6 and IL-1β signalling for their differentiation towards the Th17 subset while IL-23 and IL-21 are required for their maintenance[82,107]. Thus, the cytokine combinations promoting IL-17 may vary among lymphoid subsets.

Together with the external cues, downstream TFs direct γδ T cell development towards IL-17 producers. These TFs are Hes1[147], RelB[148], ETV5[213], Sox13 and Sox4[149,150] along with the kinase Blk[184]. Moreover, LTβR signalling seems to also be important for the development IL-17$^+$γδ T cells through regulation of RelB[148]. TCF1 and LEF1, on the other hand, have been pointed as negative regulators of IL-17 expression by γδ T cells[149]. In contrast, the TF that regulates type 17 differentiation in naïve CD4 T cells is RORγt[214].

During development, as a final step in their differentiation process towards a IL-17 producer, γδ27$^-$ T cells acquire the expression of CCR6. As shown previously in our laboratory[139] and by our results, CCR6$^+$γδ27$^-$ T cells are fully committed IL-17 producers. RORγt is a key TF, not onlyin the regulation of CCR6 expression in γδ27$^-$ T cells, but also for IL-17 production[139]. As we show here, there is an impairment in the development of IL-17 producing CCR6$^+$γδ27$^-$ T cells in the absence of RORγt. The same is also true for CD4 T cells: in the absence of RORγt naïve CD4 T cells cannot differentiate into a Th17 subset[99]. However, the similarities between the IL-17 expression programs of γδ27$^-$ T cells and CD4 Th17 cells are limited to RORγt involvement. As described for STAT3[147] and IRF4[151], our results showed that γδ27$^-$ T cells do not rely on either RORα or BATF for IL-17 production. In fact, in the absence of RORα we noticed an increase in the percentage of γδ27$^-$ T cells producing IL-17, which will be discussed ahead. Furthermore, both NK1.1$^-$ NKT cells and ILC3s were reported to express and depend on RORγt for IL-17 production[22,212]. The need for more players to differentiate and produce IL-17 by CD4 T cells implies an additional level of coordination that can account in part for the delayed production of IL-17 by Th17 cells.

Two distinct ways of producing IL-17 thus seem to regulate the innate and adaptive immune compartments. By relying solely on RORγt for the production of IL-17, innate-like T cells disclose their pre-disposition to produce the cytokines in the fastest way available. Noteworthy, as shown by the laboratory, γδ27$^-$ T cells display a constitutively open locus configuration in IL-17 region[139] which allows fast transcription upon binding of the transcriptional machinery.

We obtained particularly unexpected results with RORα. This TF has been linked to many immune-related and non-immune related functions. Involved in lymphocyte development[215,216], RORα is also expressed in a great variety of tissues[173,217,218]. In the immune system, RORα not only plays a role in inducing Th17 differentiation in a redundant and cooperative manner with RORγt[105,170] and in the development of ILC2s[219] but was also shown to be involved in survival and development of IgA$^+$ memory B cells[220]. Yet nothing is known about the role of RORα in γδ T cells. As we present in this work, the absence of a functional RORα leads to a specific increase in the number of IL-17 producing γδ T cells, after stimulation with IL-1β plus IL-23, particularly within Vγ1$^-$Vγ4$^-$ γδ T cells, which we believe to be Vγ6$^+$ γδ T cells. This suggests a specific regulatory role of RORα in IL-17 production at the periphery. Moreover, in RORα mutants there was also an increased ratio between the Vγ4$^+$ and Vγ1$^+$ γδ T cell subsets in the thymus, spleen and LN. These variations, however, were not due to changes in proliferation or in cell death. Interestingly, it is already possible to detect the increased ratio Vγ4/Vγ1 within the immature γδ T cells (CD24$^+$ γδ T cells) in the thymus. Thus, we hypothesize that RORα may control the development of γδ T cell subsets in accordance to their Vγ chain usage. These results suggest that RORα may have two distinct roles in γδ T cells, resembling those played by GATA3 in CD4 development and Th2 cell differentiation[221,222], at different stages of γδ T cell development.

RORα negatively impacts IL-17 production in Vγ1$^-$Vγ4$^-$ γδ T cells. This is utterly surprising in light of its role in positively controlling IL-17 production in Th17 cells. Inhibitors of IL-17 production in Th17 cells include T-bet, TCF1, GATA-3[188,223]. Various mechanisms of action have been described. For example, T-bet directly prevents RUNX1 transactivation of the gene encoding RORγt[188]. To date the mechanism by which RORα operates in the Vγ1$^-$Vγ4$^-$ γδ T cells remains to be elucidated. The increased responsiveness of the Vγ1$^-$Vγ4$^-$ γδ T cells to the IL-1β plus IL-23 stimulation could be linked to IL-1R1 since RORα regulates the expression of IL-1R1 that is crucial for the induction of IL-17 production in Th17 cells[224,225]. However, mRNA levels of IL-1R1 were similar between controls and RORα$^{sg/sg}$ mice within IL-17 biased Vγ subsets: Vγ4$^+$ and Vγ1$^-$

Vγ4$^-$ γδ T cells (data not shown). Since differences in the proportion of IL-17-producing Vγ1$^-$Vγ4$^-$ γδ T cells were more apparent in response to external cues we lean to propose that RORα contributes to peripheral signalling pathways that induce IL-17production. It remains to be shown if the molecular signalling pathway(s) downstream of IL1R (or other IL-17 inducers) are under the control of RORα. Another hypothetic mechanism is the direct binding of RORα to RORγt in order to prevent its binding to the IL-17 promoter.

The increase in the Vγ4 cell proportion and the decrease in the Vγ1 cell proportion in RORα$^{sg/sg}$ mice suggests that RORα may be involved in the development of these two γδ T cell subsets. Since the absence of RORγt and IL-1R1 did not impact the ratio Vγ4/Vγ1 (data not shown), our results propose a specific role for RORα in this process. At this stage, we cannot yet distinguish whether RORα controls Vγ1, inhibits Vγ4 development, or both. It will be of great importance to assess if the development of other γδ T cell subsets (Vγ5 and Vγ6) are also affected by the absence of RORα. Vγ5 and Vγ6 subsets, populate mostly non-lymphoid organs (epithelium), and emerge during embryonic stages (E13 to birth)[134]. In addition, IL-17-biased populations are proposed to essentially arise from embryonic precursors[12]. To address this we plan to look at γδ T cells developing in embryonic thymi from E15 onwards.

RORα broad expression throughout the organism makes it important to distinguish if its role(s) is(are) intrinsic to the γδ T cell compartment. To do so we will co-culture WT or RORα $^{sg/sg}$ γδ T cell progenitors DN2 and DN3 with a cell line, OP9-DL1, which mimics the thymic cells by expressing a Notch-ligand - DL1. In addition, we will take advantage of RORα conditional knock-out mice in order to assess the cell-autonomous impact of RORα deplection. Selective deletion of RORα in T cells will be achieved by crossing RORα floxed mice (kindly provided by Andrew McKenzie – Cambridge) with hCD2- Cre mice.

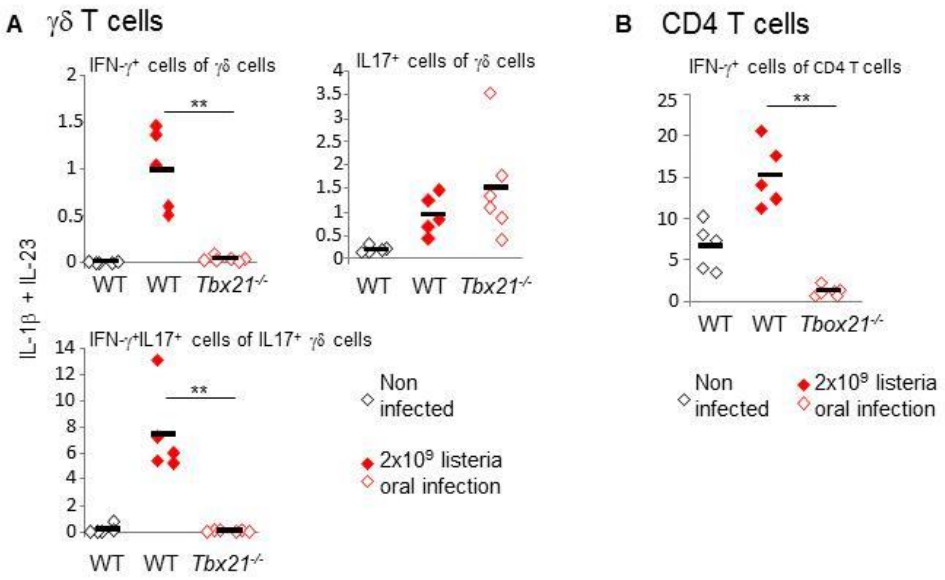
The changes in the proportion of γδ T cell subsets RORα$^{sg/sg}$ mice were already present in the immature compartment of γδ thymocytes (CD24$^+$ γδ T cells). E2A[226], Sox4 and Sox13[149,150] have been reported to be involved in Vγ4$^+$ γδ TCR expression regulation. As for Sox13, a spontaneous depletion of this TF specifically reduced the maturation of IL-17 producing Vγ4$^+$γδ T cells[150]. Similarly deletion of SOX4 results in the absence of IL-17 producing Vγ4$^+$γδ T cells[149]. We found that the expression profiles of *Sox13* and *Sox4* are different between γδ27$^+$ and CCR6$^+$γδ27$^-$ cells. S*ox4* mRNA levels 10-fold lower in CCR6$^+$γδ27$^-$ than γδ27$^+$ cells. It is plausible to suggest that SOX13 and SOX4 may have different role(s) at successive stages of γδ T cell development. This is of interest as RORα$^{sg/sg}$ mice present an increased expression of *Sox13*[183].

The absence of RORα did not affect in the expression of *Sox13* but led to a reduction of *Sox4* transcripts in γδ thymocytes. Taken this, we cannot exclude a role of RORα through modulation of expression or action of these two TF. Further work will determine the possibility that RORα directly binds on *Sox13* and/or *Sox4* promoters and/or interacts with these proteins. For instance, E2A increased the accessibility of the Vγ4 chromatin leading to an enhancement of its expression[226]. In line with these studies, we are planning to determine if RORα directly regulates Vγ chain rearrangement in γδ T cell precursors.

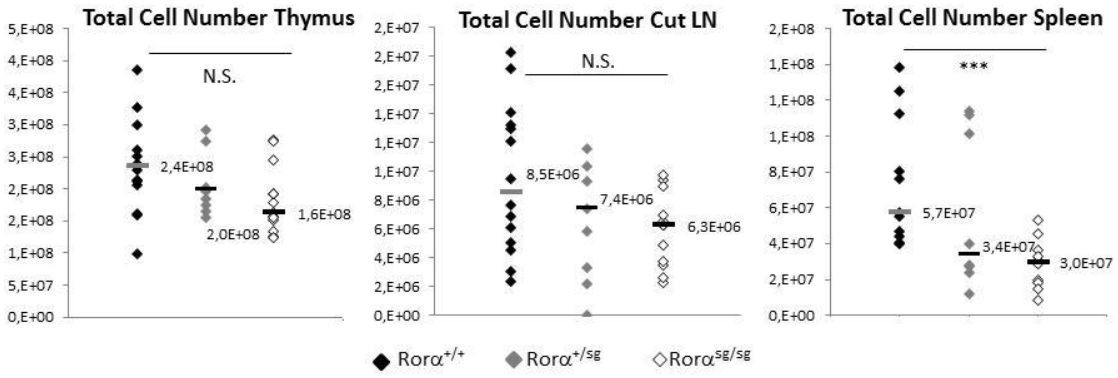
In conclusion, γδ T cells TF requirements for IL-17 or IFN-γ production here described provide a better understanding of the molecular mechanisms which direct the function of $\gamma\delta27^+$ and $\gamma\delta27^-$ T cells in the periphery. In particular, we found that $\gamma\delta27^-$ T cells which have the ability to co-produce IL-17 and IFN-γ, depend neither on RORα nor BATF for their IL-17 production. On the other hand, our results show that RORα plays a more complex and dual role in γδ T cells. RORα not only plays a developmental role by setting the Vγ4/Vγ1 balance, but also enhances the production of IL-17 in $V\gamma1^-V\gamma4^-$ γδ T cells. Further studies will disclose RORα functions and will clarify whether they are restricted to embryogenesis or occur throughout life.

VIII. ANNEXES

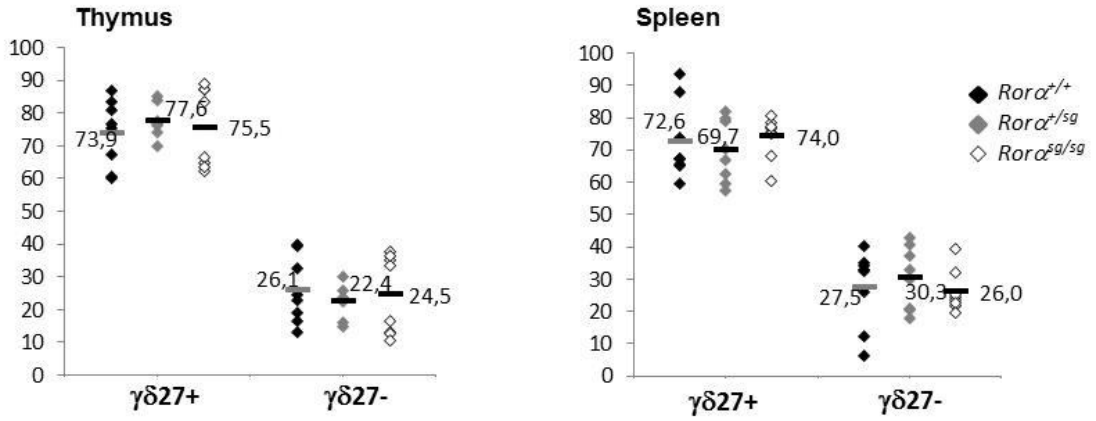
ANNEX 1 - SUPLEMENTARY FIGURES



Sup. Fig 1 – γδ, IL-17$^+$γδ and CD4 T cells depend on T-bet for production of IFN–γ
On day 8 after the *Listeria monocytogenes* infection, spleenocytes were isolated from infected and non-infected 8 weeks old *Tbx21*$^{-/-}$ and C57BL/6J (WT) mice, used as controls. **A** IFN-γ$^+$, IFN-γ$^+$IL-17$^+$ and IL-17 producing γδ T cell numbers (10^3) after stimuation with IL-1β plus IL-23 for 4h ($WT_{non\text{-}infected}$=5,$WT_{infected}$=5 and *Tbx21*$^{-/-}$=6). **B** Cell numbers (10^3) of IFN-γ producing CD4 T cells ($WT_{non\text{-}infected}$=5,$WT_{infected}$=5 and *Tbx21*$^{-/-}$=6).

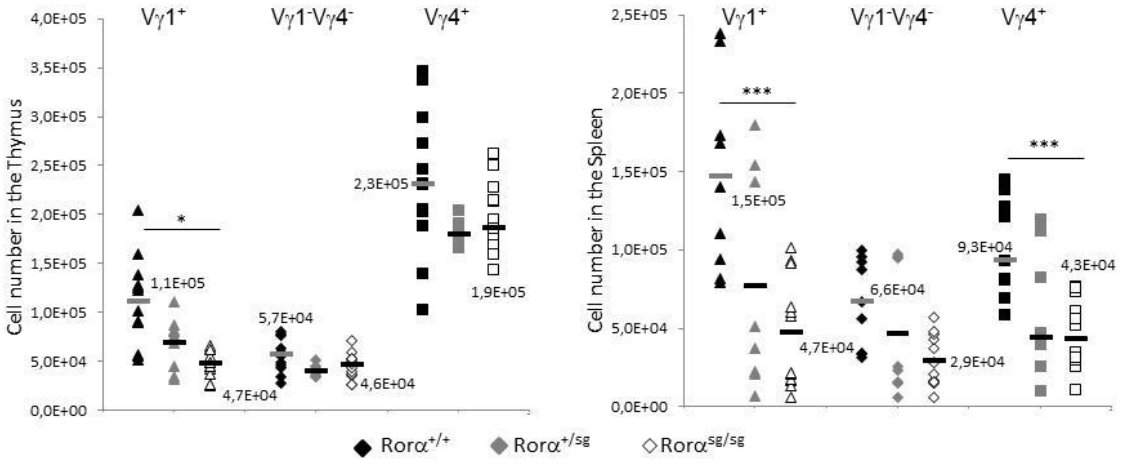


Sup. Fig 2 - RORα does not control the cellularity in other organs besides Spleen .
Cells were obtained from thymus, spleen and cutaneous lymph nodes from 2 to 3 weeks old *Ror*α$^{+/+}$, *Ror*α$^{+/sg}$, and Rorα$^{sg/sg}$ mice and counted using counting beads through FACS.



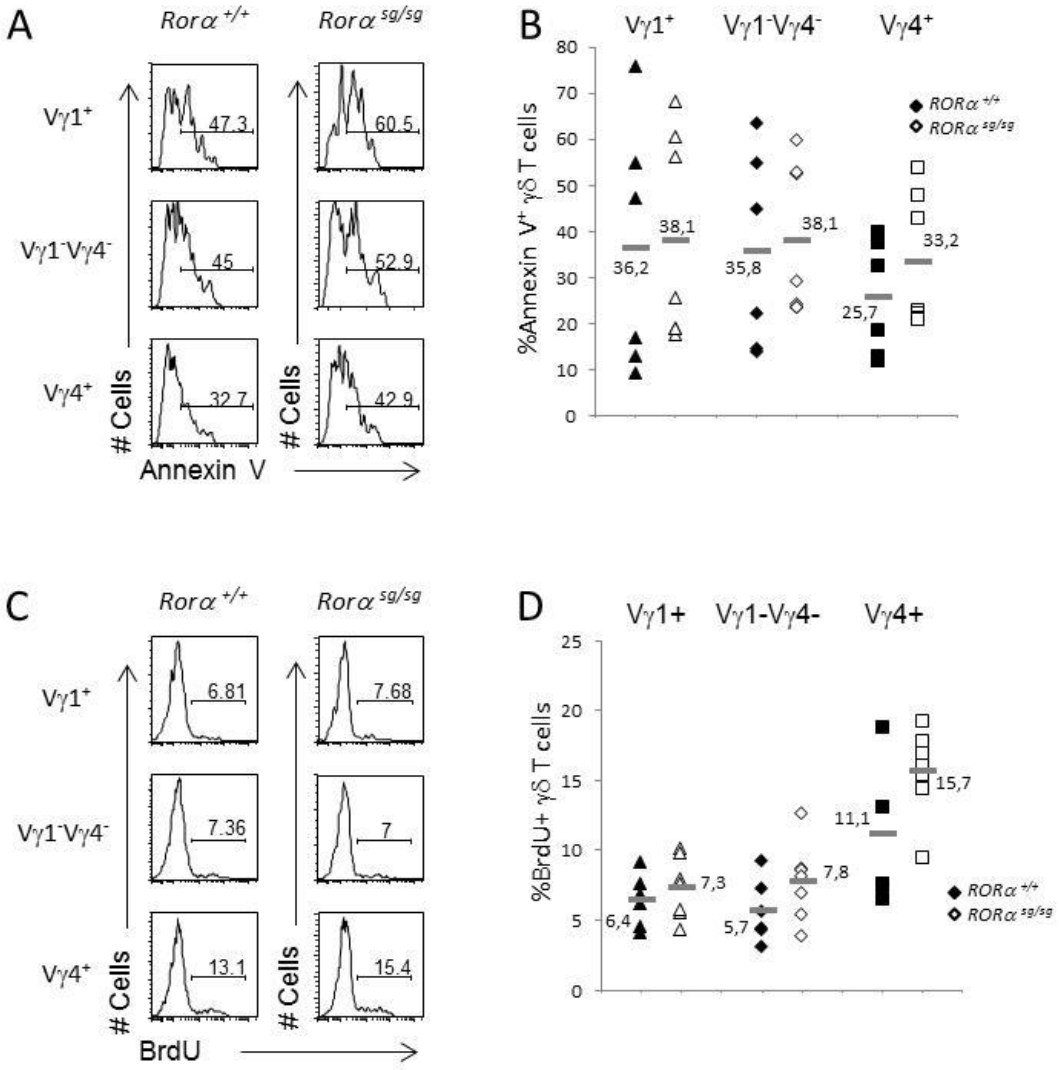
Sup Fig 3 – RORα has no impact in the thymic development or in the peripheral maintenance of γδ27-T cell population.

Cells were obtained from thymus and spleen of 2 to 3weeks old Rorα$^{+/+}$, Rorα$^{+/sg}$, and Rorα$^{sg/sg}$ mice and stained for CD27.

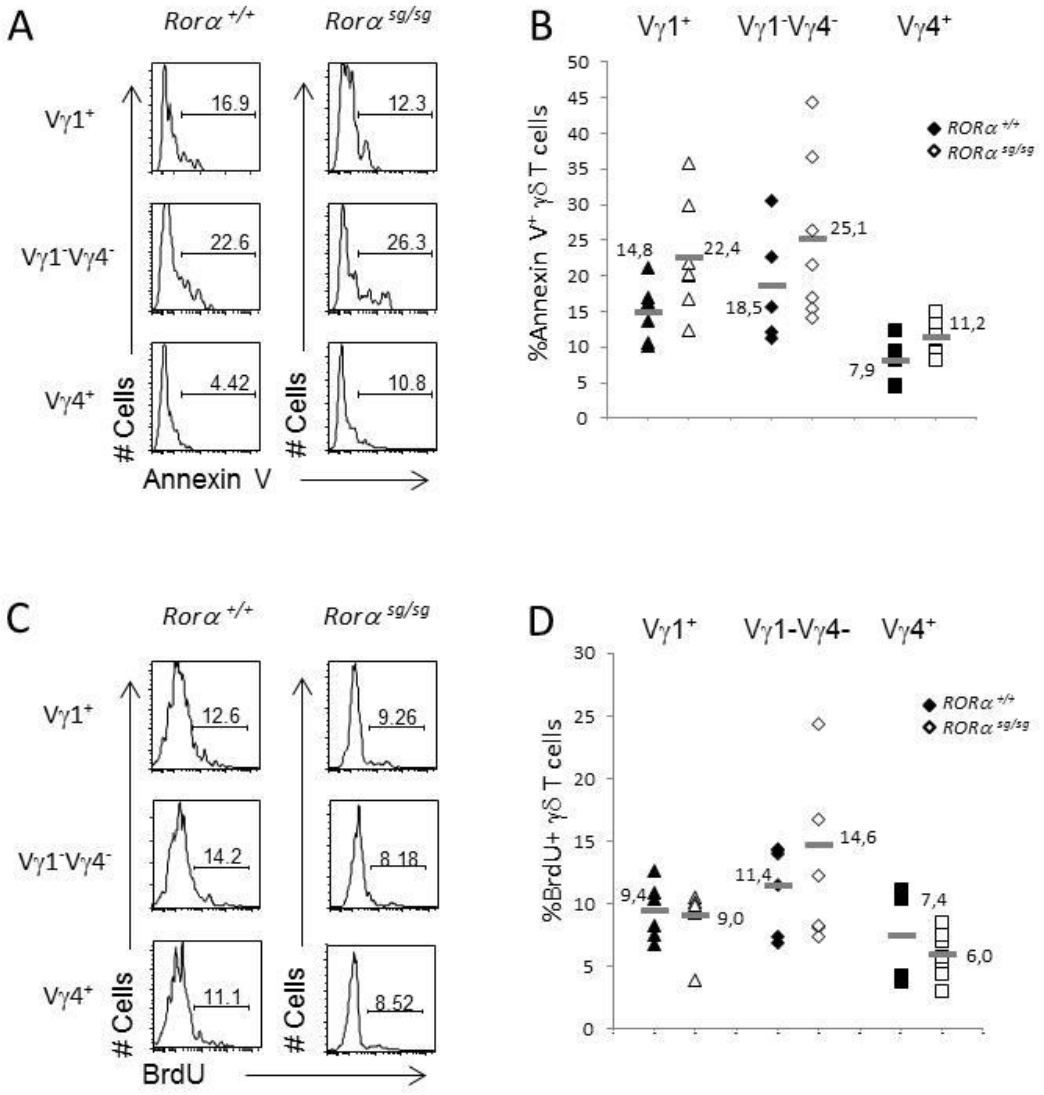


Sup Fig 4 - Vγ1^{+} decreased cell number in the absence of RORα

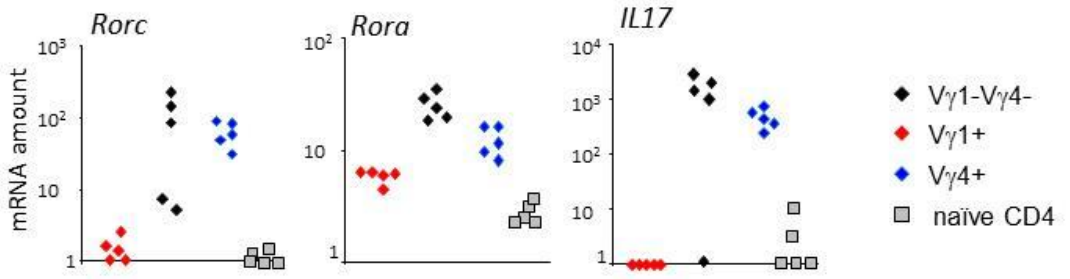
Cells were obtained from thymus and spleen of 2 to 3weeks old Rorα$^{+/+}$, Rorα$^{+/sg}$, and Rorα$^{sg/sg}$ mice and counted by using FACS counting beads.



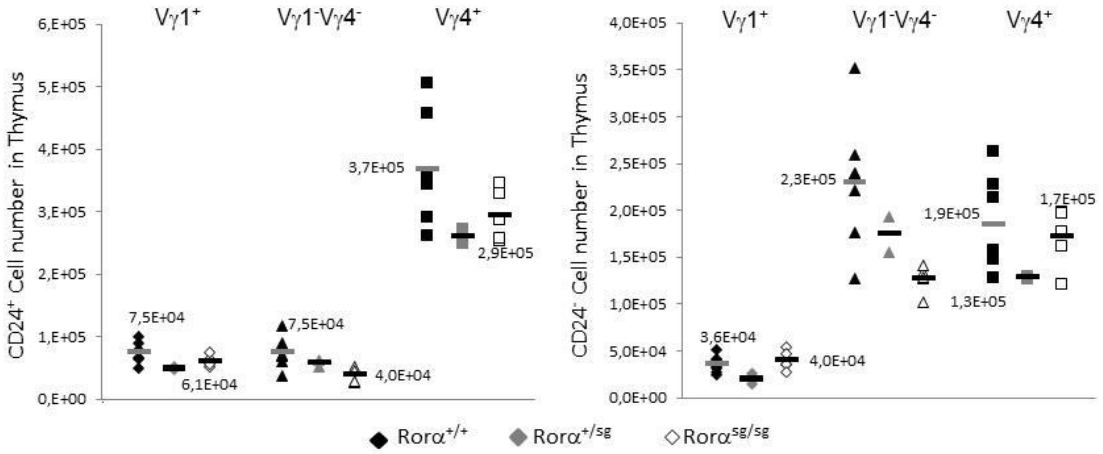
Sup Fig 5– RORα does not control cell-cycle or cell-death in cut LN Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ γδ T cell subsets.
RORα$^{+/+}$ and *RORα*$^{sg/sg}$ mice were injected i.p. with BrdU 18h before analysis. Thymocytes were then obtained and stained for BrdU for Annexin V. **A** Representative FACS plots for Annexin V stainning analysis for γδ T cell susbets (Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$). **B** Percentage of Annexin V positive γδ T cell subsets. **C** Representative FACS plots for BrdU analysis for γδ T cell susbets (Vγ1$^+$, Vγ1$^-$Vγ4 and Vγ4$^+$). **D** Percentage of BrdU positive γδ T cell subsets.



Sup Fig 6 - RORα does not control cell-cycle or cell-death in spleenic Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ γδ T cell subsets.
*ROR*α$^{+/+}$ and *ROR*α$^{sg/sg}$ mice were injected i.p. with BrdU 18h before analysis. Thymocytes were then obtained and stained for BrdU for Annexin V. **A** Representative FACS plots for Annexin V stainning analysis for γδ T cell susbets (Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$). **B** Percentage of Annexin V positive γδ T cell subsets. **C** Representative FACS plots for BrdU analysis for γδ T cell susbets (Vγ1$^+$, Vγ1$^-$Vγ4 and Vγ4$^+$). **D** Percentage of BrdU positive γδ T cell subsets.



Sup Fig 7 – Expression profile of γδ T cells subsets Vγ1$^-$Vγ4$^-$, Vγ1$^+$ and Vγ4$^+$.
γδ T cells were FACS sorted from the spleen and LN of 2 to 3weeks old *Rorα*$^{+/+}$. RT-PCR data for *Rorc, Rora* and *Il17* expression (relative to *b2m* or *Actb*) on γδ T cell subsets Vγ1$^-$Vγ4$^-$, Vγ1$^+$ and Vγ4$^+$.



Sup Fig 8 - CD24$^+$ and CD24$^-$ γδ thymocytes cell number is not affected by the absence of RORα
γδ T cells were obtained from thymus of 2 to 3 weeks old *Rorα*$^{+/+}$, *Rorα*$^{+/sg}$ and *Rorα*$^{sg/sg}$ mice. Total cell number of Vγ1$^+$, Vγ1$^-$Vγ4$^-$ and Vγ4$^+$ within the *Rorα*$^{+/+}$, *Rorα*$^{+/sg}$ and *Rorα*$^{sg/sg}$ mice out of total CD24$^+$ (left panel) and CD24$^-$ (right panels) γδ thymocytes.

ANNEX 2(A) – USED SOLUTIONS

Complete Medium: RPMI medium with 10% of Fetal Bovine Serum (FBS), 1% of HEPES Buffer, 1% Non-Essential Amino Acids, 1% Peni -strep, 1% Sodium Pyruvate, 500uL of β-mercaptoethanol and 500uL of Gentamycin all from GIBCO

Cell Culture Medium: DMEM medium with 10% of FBS and 1% of Peni-strep.

Activation medium: complete medium with PMA (SIGMA, P-8138), Ionomycin (SIGMA, I-0634) and Brefeldin A (SIGMA, B-7651)

Digestion medium: complete medium with DNAse I(Roche) and Collagenase IV(Worthington)

PBS 1X: 10% phosphate buffer saline (PBS) 10X (GIBCO) in MilliQ water.

FACS Buffer: PBS 1X with 2% FBS.

RBC Lysis Buffer: 10% Red blood cells Lysis Buffer in MilliQ water

Fix solution: Fixation buffer (eBioscience) from Kit

1X Perm Buffer: 10% Permeabilization Buffer 10X (eBioscience) from Kit in MilliQ water

Counting Beads: Nominal 10μm Latex Beads, Coulter CC size Standard L10 (Beckman Coulter, 6602796)

Genotyping Buffer: 2x My Taq Red Mix with

ANNEX 2(B) – USED CYTOKINES

Reagent	Used Concentration	Brand
mIL-12*	5ng/mL	eBioscience
Anti-IL-4	5ug/mL	eBioscience
hTGF-β**	2ng/mL	PeproTech
mIL-1β**	50ng/mL	PeproTech
mIL-6**	50ng/mL	PeproTech
IL-21	100ng/mL	PeproTech
rmIL-23***	50ng/mL	R&D systems
Anti-IFN-γ	10ug/mL	eBioscience
IL-15	50ng/mL	PeproTech
mIL-2**	50ng/mL	PeproTech
Anti-CD3	2ug/mL	Biolegend
Anti-CD28	2ug/mL	eBioscience

ANNEX 3 – USED PRIMERs SEQUENCES

Genotyping primers:

- ROR-a

oIMR1233-WT-F: 5'-TCT CCC TTC TCA GTC CTG ACA-3'
oIMR1234-WT-R: 5'-TAT ATT CCA CCA CAC GGC AA -3'
oIMR1235-mutant-F: 5'-GAT TGA AAG CTG ACT CGT TCC -3'
oIMR1236-mutant-R: 5'-CGT TTG GCA AAC TCC ACC -3'

- ROR-c

oIMR7213-common-F: 5'- CCC CCT GCC CAG AAA CAC T -3'
oIMR7214-WT-R: 5'- GGA TGC CCC CAT TCA CTT ACT TCT -3'
oIMR7215-mutant-R: 5'-CGG ACA CGC TGA ACT TGT GG -3'

qPCR primers:

- IL-17A: Reverse – TCCCTCCGCATTGACACA
Forward – CCAGAAGGCCCTCAGACTACCT

- IL-22: Reverse – CAGACGCAAGCATTTCTCAG
Forward – TGACGACCAGAACATCCAGA

- IL-17F: Reverse – ACTGGGCCTCAGCGATCTCT
Forward – CAACCAAAACCAGGGCATTT

- Irf4: Reverse – TCTGGCTTGTCGATCCCTTCT
Forward – GGAGGACGCTGCCCTCTT

- Csf2 (GM-CSF): Reverse – CCGTAGACCCTGCTCGAATATC
Forward – TGAAGAGGTAGAAGTCGTCTCTAACG

- Erg3: Reverse – TGTCCTGGCACCAGTTGGA
Forward – GACTCGGTAGCCCATTACAATCA
(probe: CGAGCTCTTTCCAGCCAGCCCC)

- Ifng: Reverse – GAGATAATCTGGCTCTGCAGGATT
Forward – TCTTCTTGGATATCTGGAGGAACTG

- Tcf-1: Reverse – ACTGGCTTCTTAGCCTCCTTCTCT
Forward – CTTGATGCTGGGATCTGGTGTAC

- Lef-1: Reverse – CGACATTCGCTCTCATTTCTTTC
Forward – CAGCTATTGTAACACCTCAGGTCAAA

- Sox13: Reverse – TCCCAGAAACCTCTCCTTCCA

Forward – GAGCAGTGGGTCCCCAGAA

- Sox4: Reverse – TGCCAATGCTCCCCTAAGC
Forward – TCCAGCGTGCCCCATCT

- Rorc: Reverse – TGCGCTGCCGTAGAAGGT
Forward – GTCCAGACAGCCACTGCATTC

- Rora: Reverse – GGAAGGTCTGCCACGTTATCTG
Forward – TCCCCTACTGTTCCTTCACCAA

-Hlx: Reverse – GCTTGTATGTCTGTGGCATGGT
Forward – AGCTCCAACCCAAGAAATTCTGT
(probe: ACACATTTCCAGGTCCCTATGCTGTGCTC)

- Tbx21: Reverse – AACTTCCTGGCGCATCCA
Forward – ATGCCAGGGAACCGCTTATA

- Eomes: Reverse – TCAGGGTTTTTCCTTAAGTGTG
Forward – TGGAGATATTCTGTCCACTTCG

- Batf: Reverse – GCGGAGAGCTGCGTTCTG
Forward – CTGGCAAACAGGACTCATCTGAT

- Actin: Reverse – TGGTACGACCAGAGGCATACAG
Forward – CGTGAAAAGATGACCCAGATCA

- beta2-microglobulin: Reverse – ATCACATGTCTCGATCCCAGTAGA
Forward – CATACGCCTGCAGAGTTAAGCA

ANNEX 4 – SCORE RATE OF DISEASE SEVERTY IN EAE

5 point scale:

1- Tail atony; Loose end of tail tonus, but when hold by the tail the mouse legs are open in a V shape and mobile

2- Hind limb weakness; Loose end of tail tonus, and when hold by the tail one of the mouse legs is parallel to the body and mostly immobile (l\).

3- Hind limb paralysis; Loose end of tail tonus, and when hold by the tail the mouse legs are parallel (ll) and mostly immobile, the when in the cage although the mouse keeps one (or two) of the paralyzed leg behind. The mouse scrawls by pulling its body with front mobility.

4-Quadrapligea; Loose end of the tonus, and when hold by the tail the mouse legs are parallel (ll) and mostly immobile, then when in the cage although keep 2 paralyzed leg behind. Loose the ability to pull its body forward with paralysis coming up to the front and creating weakness.

5- The mice is moribund

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