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Role of Ikaros in the regulation of GM-CSF expression and in the pathogenicity of CD4$^+$ T cells

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Part I

Resumé

Rôle d'Ikaros dans l'expression du GM-CSF et dans la pathogénicité des lymphocytes T CD4$^+$

INTRODUCTION :

La différenciation des cellules T CD4$^+$ en cellules T effectrices auxiliaires (TH) est initiée par l'engagement de leur récepteur T (TCR) et des molécules costimulatrices (CD28), ainsi que des signaux induits par des cytokines produites au cours de la réponse immunitaires contre des pathogènes spécifiques. Parmi les cellules TH, les lymphocytes TH17 ont comme fonction primaire l'élimination de pathogènes spécifiques qui ne sont pas ciblés par d'autres cellules TH, comme les lymphocytes TH1 ou TH2. Cependant, les cellules TH17 ont aussi un fort potentiel inflammatoire et ont été impliquées dans des pathologies autoimmunes et inflammatoires (sclérose en plaque, psoriasis).

Les cellules CD4$^+$ TH17 sont caractérisées par l'expression entre autres du facteur de transcription programmateur RORγt et de la cytokine IL-17A. *In vitro*, les cellules TH17 conventionelles (cTH17) sont différenciées en présence de TGFβ1 et IL-6 et produisent de l'IL-17A et la cytokine anti-inflammatoire IL-10. Les cellules cTH17 sont décrites comme non-pathogéniques et sont importantes dans les infections contre certains pathogènes et le maintien de l'homéostasie intestinale. Cependant, les cellules T CD4$^+$ naïves peuvent aussi être différenciées en cellules TH17 pathogénique (pTH17) en présence de TGFβ1, IL-6 et IL-23 ou IL-6 et TGFβ3 (Lee et al.2012). Dans ce cas, les cellules pTH17 expriment l'IL-17A et de fortes quantités de GM-CSF et d'IL-22. Les cellules pTH17 peuvent aussi être différenciées en présence d'IL-1β, IL-6 et IL-23 et produisent alors de l'IL-17A et de l'IFNγ. Le potentiel pathogénique des lymphocytes TH17 a été défini par leur capacité à induire une pathologie sévère dans le modèle murin de la sclérose en plaques (Experimental Autoimmune encephalomyelitis ; EAE), après transfert adoptif. Cependant, les facteurs qui régulent cette pathologie restent à identifier.

Le facteur de transcription Ikaros joue un rôle critique dans la différenciation des cellules T immatures et matures. Récemment, il a été montré qu'Ikaros est essentiel au développement des cellules TH17, et favorise l'expression de RORγt et de l'IL-17A (Wong et al, 2013). Cependant, le devenir de ces cellules et la nature des mécanismes moléculaires intervenant dans cette régulation restent incomprises. Afin d'avoir une vision globale des gènes pouvant être affectés par Ikaros dans la différenciation cTH17, mon laboratoire d'accueil a effectué une analyse transcriptomique. Alors que l'expression de l'*Il17a* diminue en absence d'Ikaros, environ 250 gènes sont fortement exprimés de manière ectopique dans les cellules TH17 Ikaros^{ko} et surtout comprennent des gènes caractéristiques des cellules pTH17, dont l'*Infg* et le *Csf2*, codant pour le GM-CSF. A l'inverse, certains gènes décrits comme régulateurs (*Il10*, *Il9*) sont diminués en absence d'Ikaros. Ces résultats suggèrent que dans des conditions non-pathogéniques, les cellules TH17 Ikaros^{ko} perdent leur profil régulateur et basculent vers un phénotype TH17 pathogénique suggérant qu'Ikaros est nécessaire pour polariser correctement les cellules T naïves vers les programmes cTH17.

OBJECTIFS DE MA THESE:

Notre hypothèse est qu'Ikaros est un régulateur clé de la fonction des cellules T CD4$^+$ et en particulier des cellules TH17 en limitant leur potentiel pathogénique ce qui aurait pour conséquence de modifier leur activité inflammatoire.
Les deux objectifs principaux de mon projet de thèse sont :

1-Déterminer les mécanismes moléculaires par lesquels Ikaros influence le phénotype pathogénique des lymphocytes T CD4$^+$

2-Tester la fonction des lymphocytes TH17 déficients en Ikaros dans un modèle de pathologie inflammatoire

RESULTATS :

Pour étudier le rôle d'Ikaros dans le développement des cellules T CD4$^+$, nous avons utilisé deux modèles murins dans lesquels Ikaros est absent de façon tissu-spécifique ou inductible. Les deux modèles sont basés sur le système Cre/Lox : (1) des souris conditionnelles déficientes en Ikaros spécifiquement dans les cellules T (Ikaros$^{f/f}$ x CD4-Cre, appelées TKO) et (2) des souris Ikaros$^{f/f}$ croisées avec des souris où la recombinase Cre est fusionnée au domaine de liaison du ligand du récepteur à l'oestrogène (Cre-ER) dont l'ADN a été inséré dans le locus Rosa (Ikaros$^{f/f}$ x Cre-ER), permettant une expression ubiquitaire. Dans ce cas, la délétion d'Ikaros est induite par traitement au tamoxifène. Dans mon rapport, ces souris sont appelées IKO.

Objectif 1- Déterminer les mécanismes moléculaires par lesquels Ikaros influence le phénotype des lymphocytes T CD4$^+$

A-Impact d'Ikaros dans la production du GM-CSF

L'expression du GM-CSF en absence d'Ikaros en absence de cytokines polarisantes
Nous avons voulu déterminer si la présence des cytokines polarisantes pour générer les cellules TH17 était nécessaire à l'expression du GM-CSF en absence d'Ikaros. En effet, il a été démontré que le TGFβ1 inhibe l'expression du GM-CSF (El-Behi et al 2011). De manière intéressante, j'ai observé que les cellules T mutantes expriment plus fortement le GM-CSF quand elles sont activées par les anticorps anti-CD3 et anti-CD28 sans ajout de cytokines polarisantes (condition TH0). Ces données indiquent que la signalisation via le TCR et son corécepteur CD28 est suffisante pour induire une forte expression du GM-CSF en absence d'Ikaros.

Corrélation directe entre la délétion d'Ikaros et l'expression du GM-CSF
L'expression du GM-CSF pourrait être une conséquence directe de la délétion d'Ikaros ou le résultat d'un programme de pré-activation déjà présent dans les cellules naïve T CD4$^+$ à cause de la délétion d'Ikaros. Pour répondre à cette question, nous avons trié des cellules naïves T CD4$^+$ de la lignée IKO qui n'ont pas reçu d'injection de tamoxifène et sont donc équivalentes à des cellules WT. Après activation des cellules par des anticorps anti-CD3 et anti-CD28, la délétion d'Ikaros a été effectuée *in vitro* en utilisant du 4-Hydroxitamoxifen ou de l'éthanol comme contrôle. Nous avons observé que l'expression du GM-CSF augmente en relation directe avec la délétion d'Ikaros en condition TH0 et TH17. De plus, la diminution de l'expression de l'IL-17A en condition TH17 est aussi corrélée à la délétion d'Ikaros. Cette

expérience confirme que l'expression du GM-CSF est la conséquence directe de la perte d'Ikaros et non la conséquence d'un programme de pré-activation des cellules T CD4$^+$.

La réintroduction d'Ikaros dans les cellules KO diminue la production du GM-CSF
Pour confirmer le rôle d'Ikaros sur l'expression de GM-CSF, nous avons réintroduit Ikaros dans les cellules T CD4$^+$ TKO. Pour cela, les cellules naïves T CD4$^+$ de la lignée TKO ont été triées, activées avec des anticorps anti-CD3 et anti-CD28 pendant 24h et infectées avec un rétrovirus exprimant Ikaros et GFP ou un rétrovirus contrôle. La surexpression d'Ikaros, détectée grâce au marqueur fluorescent GFP, dans les cellules TKO réduit d'environ 2 fois le pourcentage de cellules exprimant le GM-CSF par rapport à la condition contrôle. Ces résultats confirment qu'Ikaros est un répresseur de l'expression de GM-CSF dans les cellules T CD4$^+$.

B-Mécanismes moléculaires de régulation de l'expression du GM-CSF dans les cellules T CD4$^+$ KO

L'expression du GM-CSF dans les cellules T CD4$^+$ dépend de l'activation via le TCR.
Une question importante était de déterminer si l'engagement du TCR (CD3) était nécessaire et si seul l'engagement du TCR (CD3) était nécessaire ou si la co-stimulation avec le CD28 était aussi importante pour induire l'expression du GM-CSF. Les cellules T CD4$^+$ naïves ont donc été activées par PMA et Ionomicine ou par des anticorps anti-CD3 en présence ou absence d'anticorps anti-CD28. J'ai démontré, par cytometrie de flux, que dans les cellules IKO, l'engagement de CD3 est nécessaire et suffisant pour induire l'expression du GM-CSF (50%). Cependant, l'ajout d'une activation du corécepteur CD28 augmente l'expression de GM-CSF (70.1%).

La production du GM-CSF en absence d'Ikaros est indépendante de la voie de signalisation Fas/FasL
Un article récent a démontré un nouveau rôle de la voie de signalisation Fas-FasL dans la régulation de l'expression de protéines pro-inflammatoires. Après engagement de cette voie, la voie NF-κB est activée et la production de certaines cytokines pro-inflammatoires, comme le GM-CSF, est augmentée (Cullen et al, 2013).

Au cours de nos expériences de culture cellulaire, nous avons observé une augmentation de la mort cellulaire chez les cellules KO. Les analyses transcriptomiques montrent une augmentation de l'expression de *Fasl*, sans changement dans l'expression de autres gênes anti- ou pro-apoptotiques. Nous avons donc émis l'hypothèse que la voie Fas-FasL était plus engagée dans les cellules IKO augmentant potentiellement la production de GM-CSF. Pour tester cela, les cellules T CD4$^+$ naïves ont été triées et mise en culture TH0 en présence ou absence d'anticorps neutralisant anti-FasL. Après 3 jours de culture, la neutralisation de FasL entraîne une diminution du GM-CSF dans les cellules T CD4$^+$ WT en accord avec la publication de Cullen et al. Cependant, l'expression du GM-CSF dans les cellules KO n'est pas affectée par la neutralisation du FasL. Ces résultats indiquent que la production du GM-CSF en absence d'Ikaros ne dépend pas de la voie Fas-FasL.

Le RNA-SEQ et l'ATAC-SEQ confirment Ikaros comme régulateur de l'expression du GM-CSF
Au vu de l'importante expression du GM-CSF déjà dans la condition TH0 et du rôle connu de Ikaros dans la modification de la chromatine, nous avons décidé de mieux étudier le profil d'expression des gènes en relation à l'aspect de la chromatine dans les cellules WT et TKO en absence de cytokines polarisantes. L'ATAC-SEQ et le RNA-SEQ ont été effectué dans des cellules WT et TKO naïves et après et 1 et 2 jours de culture en présence de seules anti-CD3 et anti-CD28. L'analyse de RNA-SEQ nous a permis de confirmer, dans les cellules TKO, une augmentation de l'expression de certains gènes codant pour des cytokines pro-

inflammatoires, comme *Ifng, Csf2, Eomes* and *Tnf.* Ce que on a pu observer en regardant les modifications de la chromatine c'est que Ikaros semble réguler environ le 10-15% des gènes déjà dans les cellules naïves. L'intégration des analyses de ATAC-SEQ et du RNA-SEQ nous permet de définir que Ikaros a un rôle prévalente comme répresseur de la transcription : Dans les cellules en absence de Ikaros, les gènes que sont le plus exprimés par rapport au WT sont les gènes qui ont une accessibilité à la chromatine plus importante. Les gènes plus exprimés et les peaks plus modifiés dans les cellules TKO appartiennent aux voies de signalisation STAT5 et NF-κB.

L'expression du GM-CSF en absence d'Ikaros semble dépendre de NF-κB et de STAT5
Au vu de l'importance de la voie de signalisation NF-κB et STAT5 dans l'expression de cytokines pro-inflammatoires et de la présence d'un site de liaison des sous-unités NF-κB et STAT5 sur le promoteur du *Csf2*, nous avons décidé de poursuivre l'étude de ces deux voies. Les cellules $CD4^+$ T naïves ont été triées et mises en culture en condition TH0 en présence d'un inhibiteur spécifique d'IKKβ (IKK2 I), pour inhiber la voie NF-κB, ou d'un inhibiteur de STAT5 (STAT5i) pour empêcher l'activation de la voie STAT5, ou en présence de DMSO comme control. De façon intéressante, l'ajout d'IKK2I ou de STAT5i diminue très fortement l'expression de GM-CSF dans les cellules IKO par rapport au control. Concernant la voie NF-κB, ces résultats ont été confirmé avec une autre approche expérimentale : les cellules WT et IKO ont été infectés avec un rétrovirus expriment une forme constitutivement active d'un inhibiteur intrinsèque de la voie NF-κB (SR-IκB).

<u>Objectif 2-Tester la fonction des lymphocytes TH17 déficients en Ikaros dans un modèle de pathologie inflammatoire</u>
Les cellules T $CD4^+$ mutantes expriment peu ou pas d'IL-17A. Cependant, étant donné la forte expression du GM-CSF et de l'IFNγ, les cellules TH17 déficientes en Ikaros possèdent un caractère pro-inflammatoire prononcé. Afin de déterminer si ces cellules ont une activité pro-inflammatoire plus importante, nous avons décidé d'étudier la progression du modèle murin du psoriasis dans laquelle l'importance des cellules TH17 est démontrée.

Pour cela, nous avons induit un psoriasis sur des groupes de souris WT ou KO appartenant à la lignée TKO (dans lesquelles la délétion d'Ikaros est chronique), suite à l'application quotidienne d'une crème, Aldara, sur les oreilles. Nous avons suivi l'évolution de la maladie pendant une semaine, puis à terme, nous avons analysé les réponses des lymphocytes T, afin de déterminer l'efficacité de la réponse immunitaire mise en jeu et l'impact d'Ikaros sur ces processus in vivo.

De manière inattendue, nos résultats montrent que la progression du psoriasis est comparable entre les souris WT et TKO alors que l'analyse de ganglions drainants les oreilles par cytometrie de flux confirme la diminution d'expression d'IL17A par les cellules T $CD4^+$ TKO. Cependant, l'expression du GM-CSF entre les cellules T $CD4^+$ KO et WT semble augmenté. Les lymphocytes T γδ sont connus pour jouer un rôle primordial dans le développement de la maladie. Dans nos souris TKO, ces lymphocytes T γδ ne subissent pas de délétion d'Ikaros et expriment de l'IL-17A, ce qui pourrait expliquer la progression similaire de la pathologie entre les souris WT et TKO.

CONCLUSION :

Ikaros est un important facteur de transcription pour la différentiation des lymphocytes T $CD4^+$. Son absence détermine des changements au niveau de la chromatine et de l'expression des gènes qui sont indépendants de la polarisation des lymphocytes T. Ikaros semble être important pour réprimer un certain numéro des gènes codifiant pour des cytokines pro-inflammatoire. La voie de signalisation NF-κB et STAT5 semblent avoir un rôle très important dans la régulation de l'expression du GM-CSF in absence de Ikaros. Nous n'avons pas encore compris quel est le rôle de Ikaros dans ces deux voies de signalisation, mais il semblerait que Ikaros agisse surtout en modifiant l'accessibilité à la chromatine en masquant à STAT5 et NF-κB l'accès au promoteur de *Csf2*. Dans le model in vivo, le model de Psoriasis ne nous a pas permis de comprendre le rôle de Ikaros in vivo. Le rôle de Ikaros et la relation avec la production des cytokines pro-inflammatoires reste encore à définir et est actuellement en cours d'étude dans un modèle de EAE.

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Part II

Introduction

Chapter 1

T lymphocyte activation

Contents

In both adult humans and mice, hematopoietic and progenitor stem cells (HPSCs) can mature and progressively differentiate. These processes are finely regulated and take place in ordered steps starting from the bone marrow (BM). T cells derive from HSPCs that have migrated to the thymus (Shah and Zúñiga-Pflücker, 2014). The T cell development is described by complex, specific and organized steps happening in the thymus where many factors can interplay. In this work I will not detail more the T cell differentiation process in the thymus because it is not subject of my research work, but I will rather focus on T cell activation and polarization.

1.1 TCR-CD3 complex structure

Once T cell differentiation has been completed in the thymus, T cells express a complete and functional TCR-CD3 complex and the co-receptor CD4 or CD8. The TCR-CD3 complex is composed by different components that can be schematized as in the Figure 1.1. The TCR itself is formed by an heterodimer: $\alpha\beta$ for 95% of the T cells and $\gamma\delta$ for the 5% left. Each chain contains a constant region and a super-variable region, that is unique for each T cell and has the role to recognize a specific antigen presented by the APCs in the form of short peptides bound to Major Histocompatibility Complex molecules (pMHC). The TCR specificity is a complex matter of discussion in research and its understanding acquires a crucial role in the use

of new immunotherapies such as CAR T cells. It seems that in a mouse we can identify about 2x10^6 different non-stimulated αβ TCR clones and each clone can be composed by about 100 cells (Casrouge et al., 2000). The TCR does not contain an intracellular signaling and it is constitutively and noncovalently associated to the CD3 protein complex. The CD3 complex is formed by six chains: two ε chains, one δ, one γ chain and two ζ chains. Each chain of the CD3 complex contains Immunoreceptor Tyrosine-based Activation Motif (ITAM), in its intracellular part, that can be phosphorylated leading to the activation of the cells (Figure 1.1) (Mariuzza et al., 2020). Many concepts about the TCR-CD3 complex are considered as a dogma, however many questions about how this complex works remain still unanswered. It has to be considered that the human TCR-CD3 complex has been reconstituted in a very elegant way by cryo-electron microscopy only last year (Dong et al., 2019) allowing to have an high resolution model of this complex that is an hallmark of our immune response.

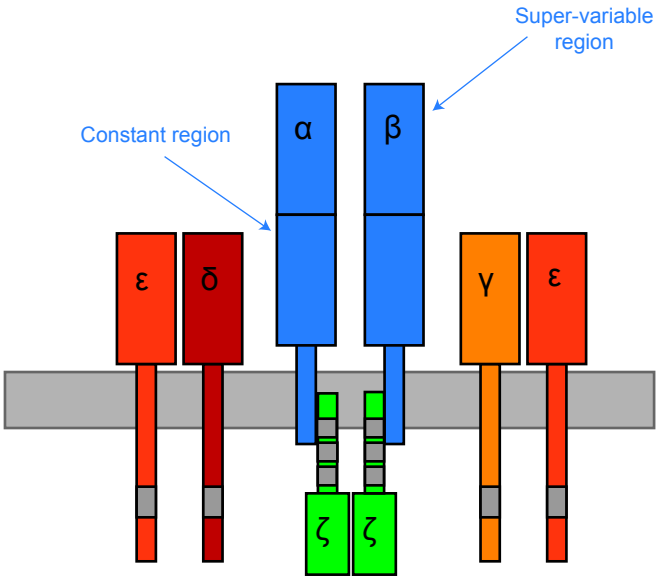

Figure 1.1: **The TCR-CD3 complex.** The TCR is formed by an αβ heterodimer. Each chain contains a constant and a super-variable regions. The CD3 is formed by six chains: two ε chains, one δ, one γ chain and two ζ chains. In grey: ITAMs.

In the mouse, at the end of T cell maturation, about 90% of cells die in the thymus during the differentiation process and only 2-4% of them leave the thymus. Mature T lymphocytes egress the thymus at the level of the cortico-medullary junction (CMJ). Many factors can control this process and S1PR1 (Sphingosine-1-Phosphate Receptor 1) is one of them. Indeed, its expression by T cells is required to lead T cells

to egress from the thymus to the periphery (Matloubian et al., 2004). T cells enter the Secondary Lymphoid Organs (SLOs) through specialized post-capillary venules in a multistep adhesion process where CD62L, CCR7, CXCR4 and LFA1 seem to have a crucial role (Girard et al., 2012). The exact mechanism regulating the T cell migration across these venules remains still unclear. Technologies as intravital imaging may help in studying these mechanisms through their ability to study and visualize living cells and their dynamic behavior. If T cells do not encounter the right pMHC complex in the SLOs, they return to the circulation through the thoracic duct and start again circulating through the bloodstream.

1.2 Early T cell activation

T cells in the SLOs randomly interact with pMHC presented by APCs. A T cell that has not been activated by encountering its pMHC is called naive. Upon recognition of its specific antigen, a T cell arrests the migration process and extends the APC/T cell interaction leading to the formation of an immunological synapse (IS). Upon full activation, $CD4^+$ T cells proliferate and differentiate in specialized effector cells, the helper T cells (Th). In order to be fully activated, T cells need 3 main signals transmitted by: 1) the TCR-CD3 complex upon recognition of a specific pMHC complex, 2) the co-stimulatory molecules, such as CD28 and 3) the cytokines present in the environment (Figure 1.2).

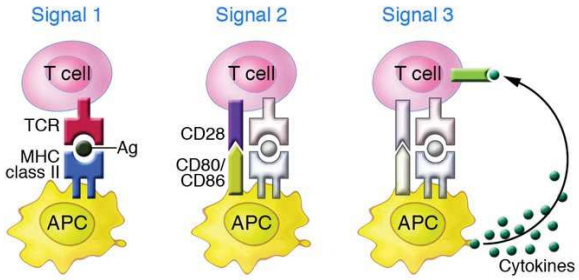

Figure 1.2: **Within the immune synapse formed between APCs and T cells, three signals are required for antigen-specific T cell activation**. Signal 1 comprises the presentation of antigen peptide, in the context of MHC class II molecules, which is recognized by the antigen-specific TCR. Signal 2 involves the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. CD80/CD86 on APCs interact with their receptor, CD28, on T cells to generate activatory signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals (not shown). Signal 3 is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to polarize them toward an effector phenotype. Ag, antigen (Gutcher and Becher, 2007).

1.2.1 The first signal: the TCR triggering

The first step to T cell activation is the TCR engagement with a pMHC on the surface of APCs. The affinity and the duration of the interaction between the TCR and pMHC are really important in shaping the immune response. When there is a match between the variable region of the $\alpha\beta$TCR and the pMHC presented by APCs, the time of the TCR-pMHC interaction increases and leads to the triggering of the TCR-CD3 complex through a multi-step process, however how exactly the TCR-pMHC interaction can trigger the TCR activation still is a matter of debate (Courtney et al., 2018).

Proximal signaling events downstream the TCR-CD3 complex activation

When a TCR-pMHC interaction occurs, CD4 or CD8 co-receptors can seal, "zip", the interaction. This association brings Lck and Fyn, src tyrosine kinase proteins that are bound to the cytoplasmic part of the co-receptors, close to the cytoplasmic tails of the TCR-CD3 complex. Thanks to their tyrosine-kinase activity, Lck and Fyn can now phosphorylate the ITAM motifs present on the CD3 intracytoplasmique chains determining a conformational change on the CD3 complex (Figure 1.3) (Palmer and Naeher, 2009). Zap-70, a member of the syk kinases, is recruited by the phosphorylated ITAMs from the cytosol to the plasma membrane where it is fully activated by the phosphorylation of Lck. The signaling cascade continues with Zap-70 phosphorylating the transmembrane adapter protein LAT and the cytosolic adapter protein SLP-76. At this point, LAT and SLP-76 permit the assembly of a "signalosome" that is able to recruit a variety of effector proteins and thus transmit the signal (Roncagalli et al., 2014).

Following the TCR-CD3 complex trigger, among other proteins, the phospholypase C (PLCγ1) is activated. PLCγ1 hydrolizes the membrane lipid phosphatidyl inositol 4,5 bisphosphate producing the two messengers: IP_3 and DAG. DAG triggers the activation of two main pathways through the mediation of Ras, a guanine nucleotide-binding protein, and PKCϑ, a PKC family member. Ras leads to the activation of the mitogen-associated protein kinases (MAPKs) extracellular signal-regulated kinase 1 and 2 (Erk1/2) and thus to the activation, among other transcription factors (TFs), of STAT3 and AP1. PKCϑ is known to mediate the activation of the NFAT and NF-$\varkappa$B pathways. The activity of the proximal signalings is crucial to transmit the activation signal to downstream pathways (Smith-Garvin et al., 2009; Hwang et al., 2020). This description of proximal signaling events downstream the TCR-CD3 complex activation progressing in a linear way is clearly an oversimplification of a signaling cascade that is much more complex and still remains enigmatic.

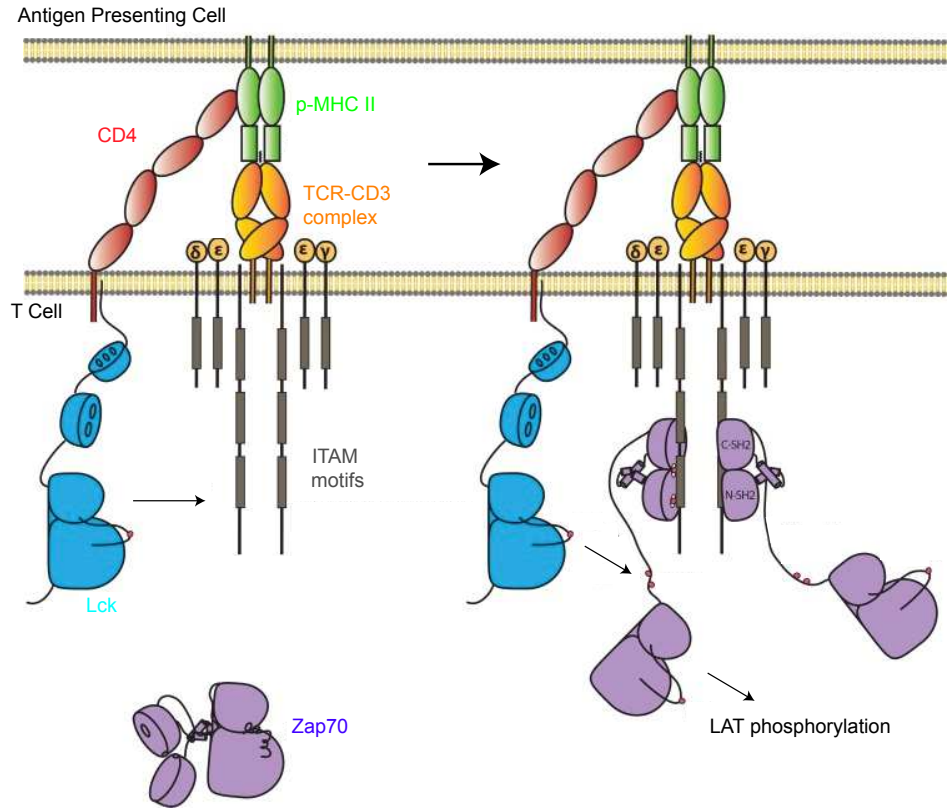

Figure 1.3: **Phosphorylation of ZAP70 is required to initiate TCR signaling**. TCR signaling is initiated by two tyrosine kinases: Lck and ZAP-70. Activated Lck phosphorylates ITAMs in the intracellular tails of CD3 chains. Doubly phosphorylated ITAMs recruit ZAP-70 from the cytosol to the plasma membrane. ZAP-70 is phosphorylated and fully activated by Lck. ZAP-70 is able to phosphorylate two scaffold proteins, LAT and SLP-76, leading to the recruitment of effector proteins that stimulate T cell activation (Figure adapted from Yan et al., 2013).

The tonic signaling

Because of the positive and negative selection in the thymus, naive T cells can weakly interact with self-pMHC. When this weak interaction happens, a low-affinity binding occurs and a basal signal, the "tonic signaling", begins. The TCR-self-pMHC binding does not last and simply leads to a basal level of phosphorylation of ITAMs. The tonic signaling contributes to naive T cell homeostasis and survival (Myers et al., 2017).

1.2.2 The second signal: co-stimulatory molecules

Early work showed that the TCR-CD3 complex activation alone induces T cell anergy, and that the co-stimulatory signals were necessary to prevent T cell unresponsiveness (Jenkins et al., 1988; Frauwirth et al., 2000). The binding of co-stimulatory molecules, like CD28 or ICOS, to their ligands is a key event not only to amplify

the TCR-CD3 complex activation, but also to activate some characteristic pathways within T cells (Sanchez-Lockhart et al., 2011). The formation of the IS helps the CD28 binding to its ligand, CD80 (B7-1) or CD86 (B7-2), expressed on the surface of APCs. CD28 is constitutively expressed on mouse T cells, while ICOS is expressed upon TCR-CD3 complex activation. CD28 stimulation leads to the activation or sustains a variety of downstream signalings, such as the PIK3/Akt pathway which is crucial for T cell activation. Other co-stimulatory molecules with an inhibitory activity exist such as CTLA4 or PD1, but I will not further develop their role in the next chapters (Esensten et al., 2016).

1.2.3 The third signal: cytokines

The third and last signal is given by the cytokines that are present in the environment. APCs are the main producers of cytokines, but T cells themselves can also produce them and influence their own polarization. I will discuss more in detail this part in the next chapter.

Chapter 2

Signal transduction in T cells

Contents

The activation of a T cell in response to an antigen triggers a variety of downstream pathways and some of them are able to induce gene expression modifications. As example, in human T cells, the T lymphocyte stimulation by anti-CD3 and anti-CD28 antibodies leads to the activation or inactivation of about 3000 genes (Diehn et al., 2002). In this chapter I will detail two of the main signaling cascades that can influence gene expression within the activated T cell: 1) the pathways downstream the TCR-CD3 complex and 2) the pathways activated by cytokines. These two signaling pathways are not independent and can interplay between each other.

2.1 Distal signaling events downstream the TCR-CD3 complex

Downstream the TCR-CD3 complex stimulation, many pathways are activated, here I will describe only some of them, the ones that are important for my research work: the calcium signaling, NFAT and NF-$\varkappa$B pathways and GSK-3 signaling cascade.

2.1.1 Calcium signaling

Following the TCR-CD3 complex engagement and the activation of proximal events, that I discussed in the chapter above, the calcium signaling is one of the main second messengers that is rapidly occurring. Indeed, the IP_3, generated by the PLCγ1 activation, can directly bind to the IP_3R located in the endoplasmic reticulum (ER) membrane triggering the release of Ca^{2+} store from the ER (Figure 2.1). The sensors STIM1 and STIM2 sense the Ca^{2+} ER store depletion and cause the opening of Calcium-Release-Activated Calcium (CRAC) channels allowing to sustain the influx of Ca^{2+} from the extracellular space (Berridge, 2016). In a short term, within seconds or minutes after the calcium entry, Calmodulin (CaM) and Calcineurin (CN) are activated. In a longer term, Ca^{2+} ions lead to the activation of several downstream pathways and TFs, such as NFAT and NF-$\varkappa$B and in this case the consequences of the calcium signaling activation are mostly associated to de novo gene expression (Oh-hora and Rao, 2009; Berridge, 2016). In T cells, the importance of the calcium signaling for the NFAT activation is well established, while its role in the regulation of NF-$\varkappa$B is not well defined yet, however it would seem that the dynamic Ca^{2+} signals play a key role in its activation (Berry et al., 2018).

Calmodulin and Calcineurin

CaM (Calcium-modulated protein-Calmodulin) is a protein whose activation depends on calcium. In resting T cells, the four calcium-ion binding sites present in CaM are not fully occupied. After T cell activation, the influx of Ca^{2+} allows the occupation of those four sites enabling CaM to undergo an important conformational change: CaM can now bind and activate the CN.

CN is a calcium/calmodulin dependent serine/threonine protein phosphatase that is composed by a catalytic subunit, CN A, and a regulatory subunit, CN B. Ca^{2+}/CaM binds to the CN B subunit leading to the activation of its phosphatase activity. The activated CN can now activate downstream pathways and TFs, including the Nuclear Factor of Activated T-cells (NFAT) proteins (Oh-hora and Rao, 2009). CN seems to be implicated also in the activation of the NF-$\varkappa$B pathway, indeed the use of Cyclosporin A (CsA), a CN inhibitor, determines the reduction of NFAT and NF-$\varkappa$B activities (Mattila et al., 1990).

2.1.2 NFAT signaling

The NFAT family is composed of five members (NFAT1 to 4 and NFAT5). NFAT1 was first identified in T cells in 1988 for its ability to regulate the IL-2 expression (Shaw et al., 1988). NFAT1 (Nfatc2) and NFAT2 (Nfatc1) are the ones mostly ex-

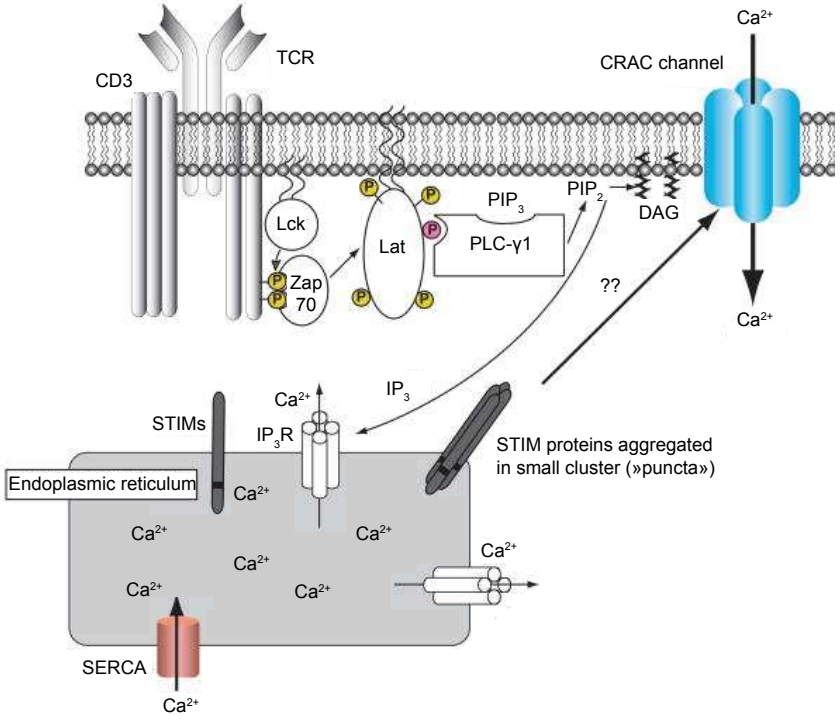

Figure 2.1: **Store-operated Ca2+ entry in T cells**. The binding of pMHC to the TCR triggers the activation of protein tyrosine kinases, such as LCK and ZAP70, which eventually results in tyrosine phosphorylation and activation of PLCγ1. PLCγ1 hydrolyzes the membrane phospholipid PIP_2 to IP_3 and DAG. IP_3 open IP_3R, which permits Ca^{2+} efflux from ER Ca^{2+} stores. The ER Ca^{2+} sensors STIM1 and STIM2 sense the resulting reduction of ER Ca^{2+} stores. STIM proteins aggregate into small clusters ("puncta") in the ER membrane and trigger store-operated Ca^{2+} entry via the CRAC channel. Ca^{2+} iflux elevates intracellular Ca^{2+} concentration (Figure adapted from Oh-hora et al 2010).

pressed in peripheral T lymphocytes. NFAT5 (TonEBP) is almost ubiquitously expressed and is mostly induced by the osmotic stress (Neuhofer, 2010; Lee et al., 2019). The NFAT family is well studied mostly in T cells where they regulate the expression of *Il2*, *Tnf*, *Il4*, *Csf2* and *Fasl*, important for cell activation, differentiation and apoptosis (Latinis et al., 1997; Hogan et al., 2003; Johnson et al., 2004a). In resting cells, NFAT proteins are located in the cytoplasm in an inactive/phosphorylated state. Following the calcium signaling induction, the activated CN dephosphorylates several serine residues in the nuclear localization sequences (NLS) of NFAT proteins. The NLS is not masked anymore, NFAT is now activated and can translocate in to the nucleus in order to bind to its target genes (Figure 2.2). NFAT5 is the only member of the family whose activation does not depend on Ca^{2+} ions. NFAT proteins bind to DNA mostly as monomers, however often they are described to bind in cooperation with other partners, such as AP-1 or NF-$\varkappa$B.

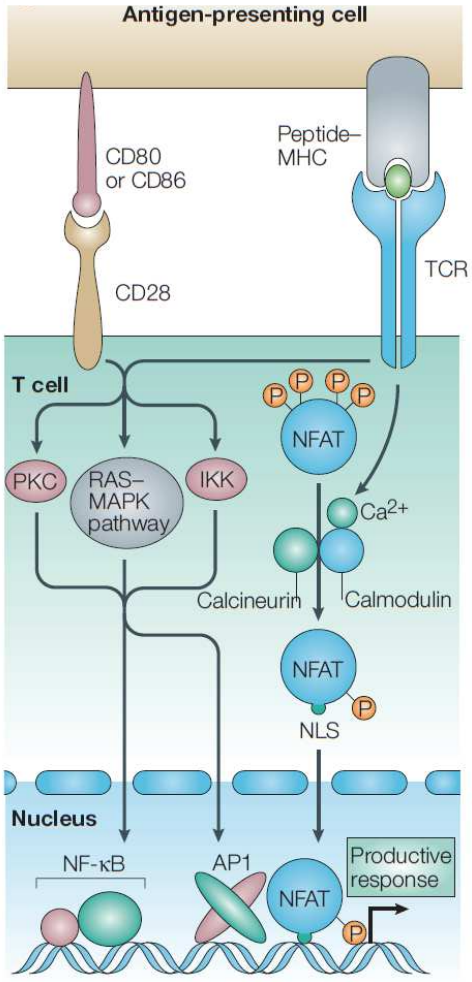

Figure 2.2: **NFAT-activated program**. Signals that are delivered by the engagement of the T-cell receptor (TCR; signal 1) and co-stimulatory molecules (such as CD28; signal 2) induce different signalling pathways that result in the activation of several transcription factors. In the nucleus, nuclear factor of activated T cells (NFAT) proteins cooperate with activator protein 1 (AP1) and other transcription factors to induce a program of gene expression that is characteristic of a productive immune response (Macian, 2005).

The cycle of activation/inhibition of NFAT is finely regulated. After activation, to be relocated and kept inactive/phosphorylated into the cytosol, NFAT can be the substrate by different kinases: Glycogen synthase kinase-3β (GSK-3β) is the most described, while CK1 has been shown to be important to promote the phosphorylation of NFAT1 (Crabtree and Olson, 2002; Okamura et al., 2004). Pharmacologic inhibitors of NFAT nuclear translocation include Tacrolimus (FK506) and CsA. In humans, these drugs are used for their immunosuppressive action to improve graft survival and to treat autoimmune diseases. The molecular mechanism used by the two drugs is similar and it is characterized by the inhibition of the CN activity (Wang and Heitman, 2005; Liu, 2009).

2.1.3 NF-κB signaling

The TF NF-κB was described for the first time in 1986 as a nuclear factor that could bind to the enhancer of the immunoglobulin kappa light chain of activated B cells: Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (Sen and Baltimore, 1986). Over the years, it was discovered that NF-κB was implicated in many more signaling cascades and that it could be activated in response to a variety of internal and external stimuli (Zhang et al., 2017). NF-κB is described to modulate the expression of genes that are crucial for inflammation, cell development, cell cycle, proliferation and cell death. In particular, NF-κB signaling can control many T cell functions like proliferation, through the regulation of cyclines D1 and D2 expression, survival, through the positive regulation of some anti-apoptotic proteins, such as Bcl-2, or inflammation, trough the expression of pro-inflammatory cytokines (Catz and Johnson, 2001). The deregulation of this pathway can be associated to cancer and inflammatory diseases and a great variety of mutations of different factors implicated in the NF-κB pathway have been described also in humans (Zhang et al., 2017). The NF-κB family is composed of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) that can associate with each other to form active homo- or hetero-dimers. The Rel protein family members can associate to form up to 15 different dimers, however the existence of all these associations has not yet been confirmed (Figure 2.3) (Oeckinghaus and Ghosh, 2009). All the members of the family share an N-terminal Rel-homology domain (RHD), which is responsible for the DNA binding and for the homo- or hetero-dimerization. Only p65/Rela, RelB and c-Rel contain a carboxy-terminal transactivation domain (TAD) and are capable to permit the activation of the transcription of the target genes (Oeckinghaus and Ghosh, 2009). The activity of the members of the family can be influenced by post-translationally modifications. Here, I will describe more in detail the function of each subunit.

a) p65/RelA

RelA seems to be one of the most important members of the family. RelA-deficient mice show a dramatic phenotype characterized by the death in the embryo (Beg and Baltimore, 1996). RelA activity can be modulated by different mechanisms: 1) by its binding with inhibitor proteins, such as IκB; 2) by its own phosphorylation by a variety of kinases 3) or by its acetylation (Oeckinghaus et al., 2011; Hoesel and Schmid, 2013). The phosphorylation of p65 is mostly linked to the activation of the subunit itself, however the function of all different p65 phosphorylations described in the literature is not clear, and it is possible that some specific phosphorylation could inhibit the p65 activity. GSK-3β expression has been shown to be important to positively regulate RelA activation, however its role is not yet to-

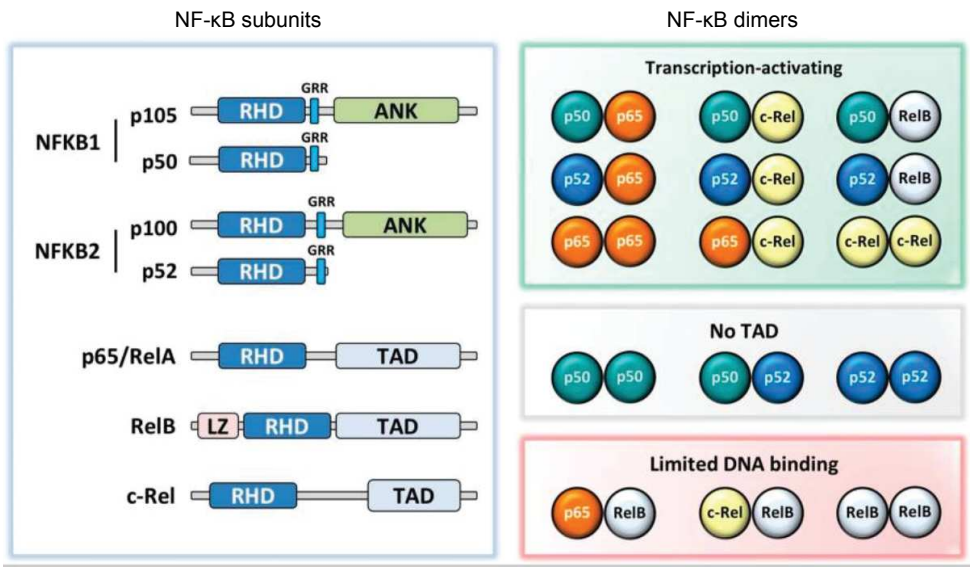

Figure 2.3: **NF-κB subunit structures and dimeric combinations**. All subunits contain a Rel homology domain (RHD). NF-κB1 and NF-κB2 have a glycine rich region (GRR), followed by an Ankyrin repeat domain (ANK) in the precursors p105 and p100. p65, RelB, and c-Rel contain a transactivation domain (TAD), with RelB additionally containing a leucine zipper (LZ) motif. While most dimers activate transcription, p50:p50, p50:p52, and p52:p52 dimers lack a TAD, and therefore repress transcription in the absence of co-activating factors and p65:RelB, c-Rel:RelB, and RelB:RelB dimers are thought to have limited DNA binding (Concetti and Wilson, 2018).

tally understood (Hoeflich et al., 2000). p65 can also be acetylated at different sites. For example, the acetylation of lysines 218, 221 and 310 promotes the activation of p65, while the acetylation on lysines 122 and 123 inhibits its activity (Oeckinghaus and Ghosh, 2009). The p50/p65 hetero-dimer represents the most abundant Rel dimer present in cells.

b) RelB

RelB is an unusual member of the family that it is constitutively expressed in lymphocytes. RelB-deficient mice survive in the uterus, but die some days after birth because of the presence of a systemic inflammation (Weih et al., 1995). In resting T cells, RelB activity is inhibited because it is sequestered in the cytosol by the subunits p105 or p100. Upon T cell activation, p105 and p100 can be processed to their active subunits p50 and p52, respectively, releasing RelB from its inhibition (Yang et al., 2019). RelB degradation can be mediated by its phosphorylation by different kinases, among them GSK-3β (Abd-Ellah et al., 2018). RelB forms hetero-dimers mostly with the p100/p52 subunit.

c) c-Rel

c-Rel is mostly expressed in hematopoietic cells and its expression is regulated by the IκB proteins. It is known to have a role in IL-2 expression (Visekruna et al., 2012). In c-Rel-deficient mice T cell proliferation is impaired (Köntgen et al., 1995).

d) NF-κB1 (p50) and NF-κB2 (p52)

p105 and p100 are the only members of the family that are synthesized as pro-forms and then proteolically processed to p50 and p52, respectively (Figure 2.3). p105 and p100 contain a C-terminal domain containing ankyrin repeats that when phosphorylated are degraded in an ubiquitin-proteosome dependent manner. The degradation of this inhibitory C-terminal domain leads to the activation of p50 and p52. In contrast to the other members of the family, p50 and p52 do not contain a transactivation domain, thus they need to form an hetero-dimer with one of the other members in order to act as transcriptional activators (Cartwright et al., 2016). Nfkb1- or Nfkb2-deficient mice display an impaired T cell activation (Köntgen et al., 1995).

After almost 35 years from its discovery, the NF-κB signaling cascade still remains an enigmatic pathway. So far, two main pathways leading to the activation of NF-κB have been described: 1) the classic (canonical) and 2) the alternative (non-canonical) pathways. These two pathways interact and can interplay within the same cell (Figure 2.4). Here I will detail the main mechanisms regulating their activation.

1) The canonical pathway

The canonical pathway has been demonstrated to be activated by some inflammatory cytokines such as TNFα or IL-1β and by the stimulation of the TCR or the BCR (B Cell Receptor). The most important players in the canonical signaling are c-Rel, RelA and p50. In resting T cells, NF-κB subunits are hold in the cytoplasm, as inactive forms, by inhibitory proteins, such as the typical IκBs (Figure 2.4). In the literature, among the typical IκB proteins, IκB-α is the most well described. In more detail, the typical IκB proteins bind the RHD domain of the NF-κB subunits thus masking the NLS sequence and preventing them to enter the nucleus. Upon T cell activation, the phosphorylation of IκB-α at Ser32/36 by the IκB kinase complex (IKK) leads to the degradation of IκB-α and thus the release of the NF-κB subunits that are now free to translocate in to the nucleus and act on their target genes (Figure 2.4). The IKK complex plays a central role in the activation of the NF-κB canonical and non-canonical pathways and it is composed of three subunits:

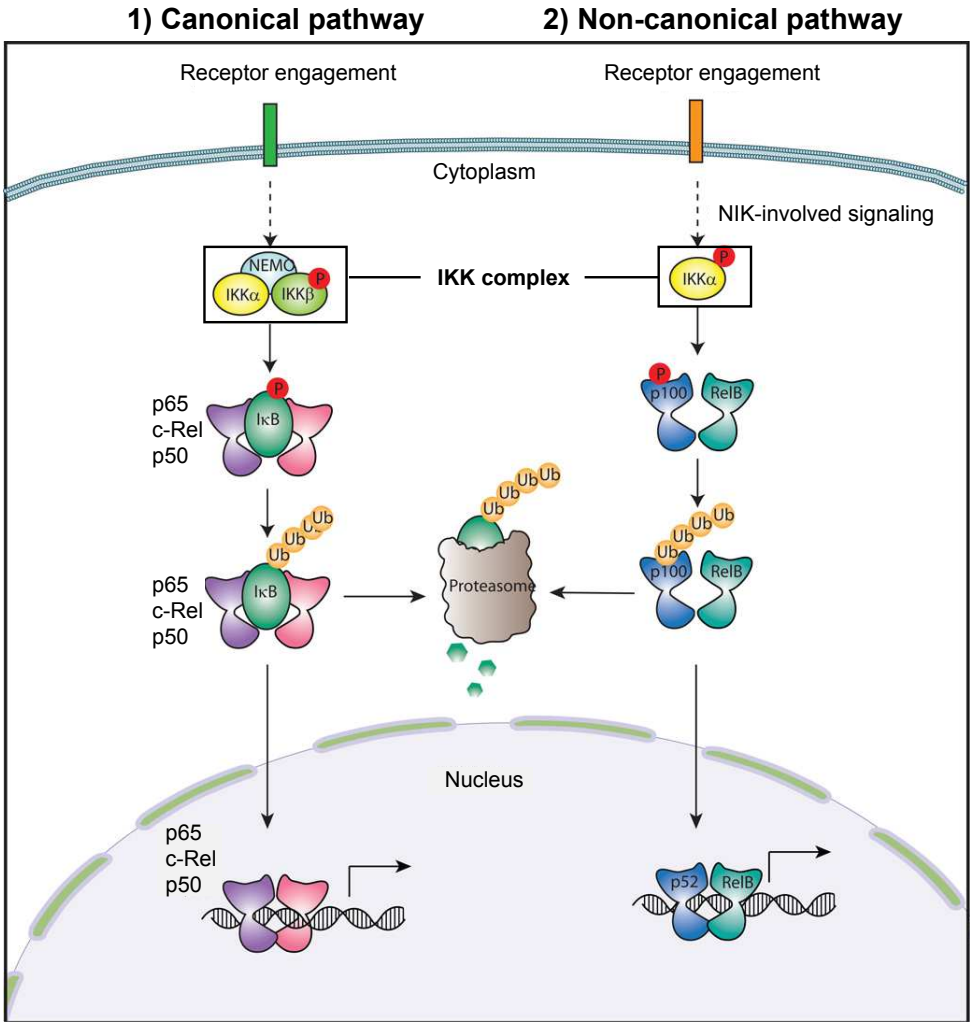

Figure 2.4: **Canonical and non-canonical pathways of NF-κB activation**. NF-κB activation occurs through two different pathways: canonical and non-canonical. In the canonical pathway, the IKK complex, composed of IKKα, IKKβ, and the regulatory subunit NEMO, phosphorylates IκB, leading to its ubiquitination and proteosomal degradation. A NF-κB dimer, which is comprised of p50, p65, or c-Rel, is released from IκB and translocates into the nucleus, where it binds to a κB-binding site for regulation of gene expression. In the non-canonical pathway, IKKα phosphorylates p100 resulting in its ubiquitination and partial processing into p52. A heterodimer of p52 and RelB translocates into the nucleus and binds to it binding sites. P, phosphorylation; Ub, ubiquitination (adapted from Oh and Ghosh, 2013).

two catalytic subunits, IKKα (IKK1), IKKβ (IKK2) and a regulatory subunit IKKγ (NEMO). The mechanisms required for the activation of the IKK complex are not fully understood, but NEMO ubiquitination, a mechanism that is independent from the proteosome (Chen, 2012), and IKKα or IKKβ phosphorylation seem to be critical. The crucial step necessary for the activation of the canonical NF-κB pathway is the activation of the IKKβ kinase. The signaling cascade that determines the activation of the IKKβ can be different depending on which receptor is engaged and

starts the signal. I will describe here two main signaling cascades that lead to the activation of the canonical pathway in T cells: the TCR-CD3 complex and the TNFR activation.

TCR-CD3 complex activation. The activation of NF-$\varkappa$B by the TCR-CD3 trigger is regulated by the activation of a complex network of signaling cascades that is still not totally understood. Upon T cell activation, after the calcium release, the PKCϑ is activated and seems to be crucial in the activation of the IKK complex via the CARMA1-BCL10-MALT1 (CBM) complex (Figure 2.5). Only two years ago, cryo-electron mycroscopy was performed to study the structure of the CBM complex and gave more insight about the dynamics for the assembly and the interactions between the three components of the complex (Schlauderer et al., 2018; David et al., 2018). CARMA1 (also called CARD11) can be directly phosphorylated by PKCϑ, but other proteins seems to be able to mediate this process, such as Akt, IKKβ itself and CaM (Thome et al., 2010; Roche et al., 2013). The phosphorylated CARMA1 changes its conformation and recruits BCL10 and MALT1 to form the CBM complex. The BCL10 phosphorylation by GSK-3β and the phosphorylation of MALT1 seem to be important to enhance the activation of the pathway (Abd-Ellah et al., 2018; Gehring et al., 2019). The CBM complex can now be ubiquitinated by TRAF6, an adaptor protein with E3 ubiquitin ligase activity, that facilitates two main processes: 1) the IKKγ (NEMO) recruitment and 2) the activation of the kinase TAK1. These two actions seem to be crucial for the activation of the IKK complex and thus of the canonical pathway. The presence of a negative feedback in the NF-$\varkappa$B pathway is critical to determine a fine regulation of the pathway itself. IKKβ can also contribute to the NF-$\varkappa$B pathway negative regulation through the phosphorylation of BCL10 in its C-terminal domain that triggers its degradation (Wegener et al., 2006); MALT1, with its proteolytic activity, cleaves the NF-$\varkappa$B inhibitors such as A20 (also Tnfaip3) and CYLD thus allowing to maintain the activation of the pathway, however MALT1 can cleave also BCL10 itself permitting thus to decrease the pathway activity (Coornaert et al., 2008). These data suggest that the NF-$\varkappa$B pathway triggered by the TCR-CD3 complex activation is a complex network with many guardians where the same protein, such as the kinase IKKβ, can be responsible to promote or to inhibit the activation of the pathway itself. Indeed, the NF-$\varkappa$B signaling cascade remains still an enigmatic pathway.

TNFR activation. Another signaling pathway that activates the NF-$\varkappa$B canonical pathway and can be present also in T cells is the TNF-Receptor (TNFR) signaling. The TNFR superfamily contain many receptors and all of them are able to trigger the activation of the NF-$\varkappa$B canonical pathway, only some of them (CD40, LTβR RANK, BAFF-R) trigger specifically the NF-$\varkappa$B non-canonical pathway. Here, in this section, I will detail two of the ones implicated in the canonical pathway:

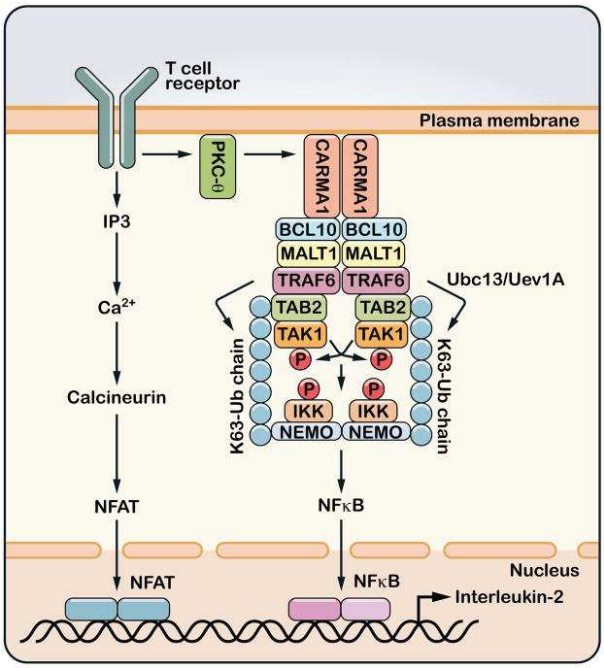

Figure 2.5: **The CBM pathway**. Upon engagement of MHC-bound peptides,TCR triggers a cascade of tyrosine phosphorylation events that lead to the activation of PKCϑ. PKCϑ then phosphorylates the membrane-associated protein CARMA1, which in turn recruits BCL10 and MALT1. MALT1 binds to TRAF6 and perhaps other ubiquitin E3 ligases. The binding of MALT1 to TRAF6 induces TRAF6 oligomerization and activates its E3 ligase activity, which then catalyzes K63 polyubiquitination to activate TAK1 and IKK. T cell receptor signaling also activates the calcineurin – NFAT pathway through increasing the intracellular concentration of calcium. NFAT, NF-χB and other transcription factors cooperate in the nucleus to induce the production of interleukin-2 (IL-2) (Chen, 2012).

TNFR1 and Fas.

The TNFR1 stimulation can trigger the activation of the canonical pathway by a specific signaling cascade (Figure 2.6). The binding of the TNFα to its receptor, TNFR1, triggers the recruitment of some adapter proteins, such as TRADD, RIPK1, TRAF2/5 and cIAPs. cIAPs are responsible for the ubiquitination of RIPK1 that facilitates the recruitment of LUBAC, an ubiquitin ligase complex, and of TAK1, a kinase, that mediate the activation of the IKK complex. If the NF-χB pathway is not engaged, cells undergo cell death through the formation of the Complex II (Figure 2.6) (Hayden and Ghosh, 2014). I will detail better the Complex II function in the "Termination of the T cell activation" chapter. Because of its action on the NF-χB signaling, the neutralization of TNFα is an important therapeutic option in many inflammatory diseases, however it can also lead to exacerbation of the inflammation, underlining the complex and not totally understood role of this pathway (Mehta et al., 2018). Moreover, a really interesting characteristic of the TNFα

pathway is that a reverse signaling in $CD4^+$ T cells has been described, indicating that the signaling can be transmitted in the cell that expresses the ligand TNFα in its membrane-bound form (Vudattu et al., 2005).

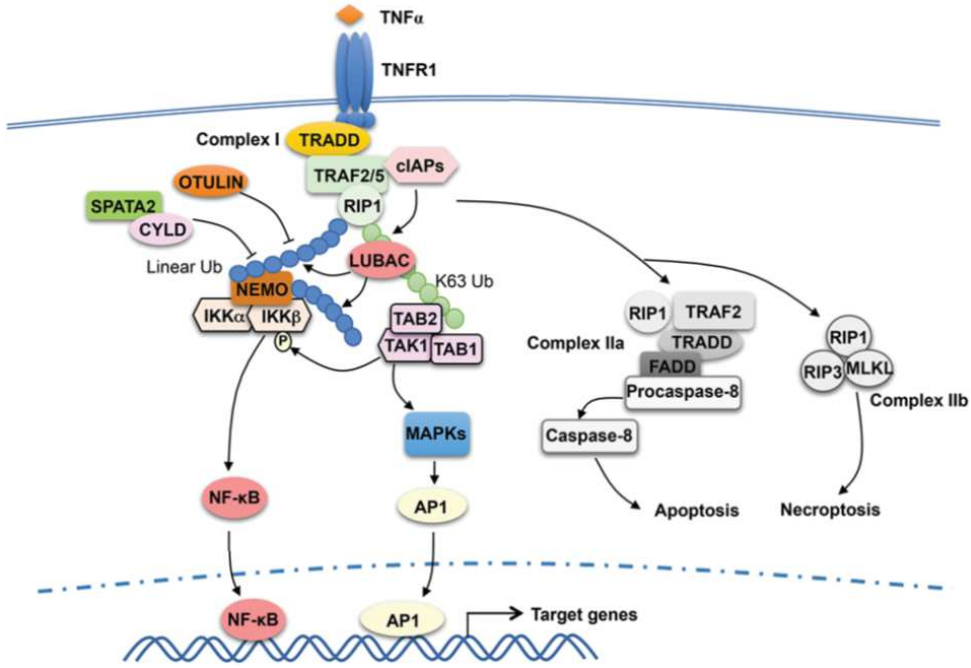

Figure 2.6: **The TNFR1 pathway**. TNFα binding to TNFR1 triggers the assembly of a TNFR1-associated signaling complex, complex I, which is composed of TNF receptor-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIP1), TNF receptor-associate factor 2/5 (TRAF2/5), and the E3 ubiquitin ligases cIAP1 and cIAP2 (cIAPs). Upon activation, cIAPs conjugate K63-linked ubiquitin chains to RIP1, which facilitates recruitment and activation of the kinase transforming growth factor beta-activated kinase 1 (TAK1) as well as the recruitment of the linear ubiquitin chain assembly (LUBAC). LUBAC conjugates linear ubiquitin chains to RIP1 and, thereby, facilitates the requirement of IκB kinase (IKK) via the linear ubiquitin-binding function of NF-κB essential modulator (NEMO). Subsequent ubiquitination of NEMO by LUBAC, along with TAK1-mediated IKKβ phosphorylation, results in IKK activation. The activated IKK and TAK1 mediate activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK)/AP1 signaling pathways that promote cell survival. TRAF2 also participates in the subsequent formation of a cytoplasmic complex, complex IIa, which mediates apoptosis induction. When caspase-8 is inhibited, TNFR1 signaling also leads to formation of complex IIb, leading to necroptosis. DUBs, including OTULIN and CYLD, negatively regulate signaling functions of LUBAC by cleaving linear ubiquitin chains. SPATA2, as a high-affinity binding partner of CYLD and LUBAC, facilitates CYLD function by recruiting CYLD to LUBAC (Adapted from Shi and Sun, 2018).

Another interesting member of the TNFR family that triggers the NF-κB canonical pathway is Fas. Fas/FasL signaling is mostly an apoptosis inducer, but a very

elegant study associates its activation also to the secretion of pro-inflammatory cytokines (Cullen et al., 2013). Fas (CD95) is a death receptor localized on the surface of most cells, while FasL is expressed on the surface of some immune cells, including activated T cells. When Fas is activated by the FasL binding, Fas receptors oligo-dimerize and the Fas cytosplasmic domain can activate the downstream cascades (Figure 2.7). The activation of Fas leads to the recruitment of adapter proteins, such as FADDs. FADDs can recruit TRAF2, caspase-8, cIAPs and RIPK1. cIAPs are responsible for the ubiquitination of RIPK1 that allows the formation of a protein complex, called FADDosome, formed by FADD, caspase-8 and polyubiquitinated RIPK1. The FADDosome is responsible for the apoptosis process and for the activation of the canonical NF-$\varkappa$B pathway. Cullen and colleagues describe two functions as crucial for the pro-inflammatory cytokine production: 1) the scaffold function of RIPK1 and caspase-8. Indeed, the kinase activity of RIPK1 that is inhibited by the necrostatin is not able to influence the pro-inflammatory cytokine production, whereas the RIPK1-knock-down reduces drastically the pro-inflammatory cytokine expression through the inhibition of the NF-$\varkappa$B activation. On the same principle, only the caspase 8-knock-down is able to reduce the pro-inflammatory cytokine production (Cullen et al., 2013). 2) The ubiquitination of RIPK1 by cIAPs. By using IAP neutralizing molecules, Cullen and colleagues were able to suppress the Fas-induced cytokine production in HeLa cells (Figure 2.7) (Cullen et al., 2013). In the article, after stimulation by anti-Fas Abs the production of RANTES has been observed increased also in murine primary thymocytes, however it would be interesting to confirm the increased expression of other inflammatory cytokines such as IL-6, IL-8 or GM-CSF and apply these results also in peripheral T cells (Cullen et al., 2013).

This specific signaling via the TNFR underline the ability of the TNFR superfamily to be implicated in two important signaling events such as cell death through the activation of apoptosis or necroptosis and inflammation through the activation of the canonical NF-$\varkappa$B pathway.

2) The non-canonical pathway

The non-canonical pathway can be activated by some receptors such as B-cell activation factor (BAFF-R), CD40, receptor activator for nuclear factor kappa B (RANK) or lymphotoxin β-receptor (LTβR) (Figure 2.4). The members of the NF-$\varkappa$B family implicated in this pathway are mostly RelB and p52. In the non-canonical pathway, after the binding of the ligand to its receptor, a specific kinase, the NF-$\varkappa$B-inducing-kinase (NIK), activation seems to be crucial to directly phosphorylate IKKα, predominantly on S176. The activated IKKα can now phosphorylate p100 on S866 and

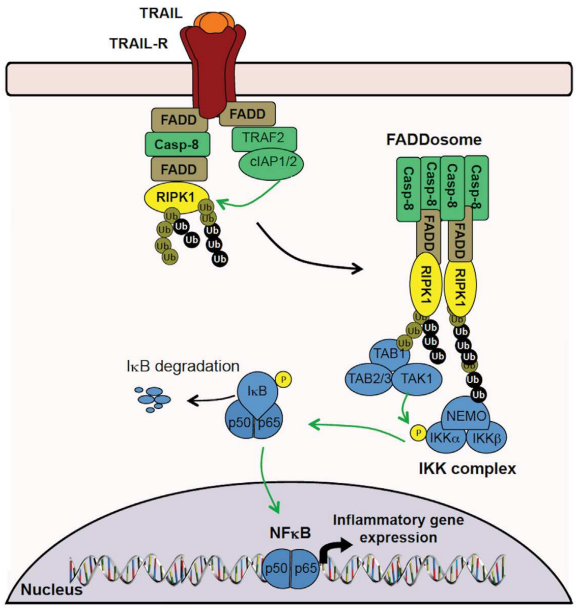

Figure 2.7: **Fas and the NF-χB signaling**. TRAIL receptor/Fas engagement induces apoptosis and NF-χB-dependent expression of multiple pro-inflammatory mediators. Caspase-8 participates in the TRAILR/Fas-induced inflammatory signaling by serving as a scaffold protein for assembly of a Caspase-8-FADD-RIPK1 "FADDosome" complex that leads to NFkB-dependent inflammatory genes expression (Adapted from Henry and Martin, 2017).

S870 which results in its proteosomal processing to p52. p52 can form an heterodimer with RelB and translocate into the nucleus (Oh and Ghosh, 2013). Although the upstream signaling determining the IKKα activation remains unclear, it seems that NIK is required for the IKKα activation. Under resting conditions, in the cytoplasm NIK is bound by the cIAPs-TRAF2-TRAF3 complex and degraded through its ubiquitination by cIAPs (Figure 2.8 A). Upon receptor activation, cIAPs ubiquitinate TRAF2-3 instead leading to their degradation and avoiding the degradation of NIK that now accumulates in the cytosol and activates the non-canonical pathway (Figure 2.8 B) (Sun, 2011). As in a negative feedback loop, the activated IKKα phosphorylates NIK leading to its own degradation (Figure 2.8 C). In mice deficient for cIAPs, NIK is not degraded and the non canonical NF-χB pathway is over-activated inducing the secretion of pro-inflammatory cytokines (Giardino Torchia et al., 2013). Recent studies using NIK deficient mice and *Nfkb2*$^{\text{Lym1}}$ mice, that express a non-processable p100, have demonstrated a crucial role of the non-canonical pathway in the induction of the neuroinflammation model experimental autoimmune encephalomyelitis (EAE) (Yu et al., 2014; Li et al., 2016b; Jin et al., 2009).

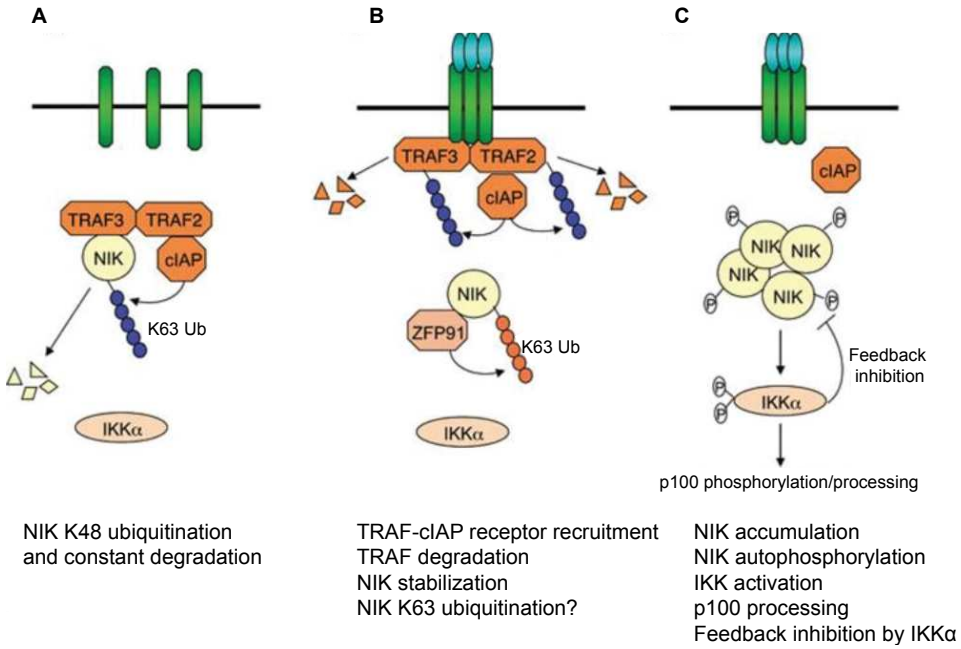

Figure 2.8: **NIK stabilization as a mechanism of non-canonical NF-$\varkappa$B signaling**. (A) Under normal conditions, NIK is bound by TRAF3 and recruited to the cIAP1/2 ubiquitin ligase via TRAF3 dimerization with TRAF2. The TRAF3-TRAF2-cIAP E3 complex mediates constant ubiquitination and proteasomal degradation of NIK, thus preventing non-canonical NF-$\varkappa$B activation. (B) In response to receptor crosslinking, TRAFs and cIAP1/2 are recruited to the receptor, where cIAP1/2 ubiquitinates TRAF2 and TRAF3 and stimulates their degradation. ZFP91 mediates K63 ubiquitination of NIK, which may promote stability and catalytic activity of NIK. (C) Accumulated NIK activates IKKα, which in turn phosphorylates p100, leading to p100 processing. IKKα also phosphorylates NIK to promote NIK degradation, a feedback mechanism that may control the magnitude of NIK activation (Sun, 2011).

Nuclear action of the NF-$\varkappa$B pathways

Both pathways (non-canonical and canonical) can interact and be activated within the same cells through the stimulation of different signaling cascades. For example, within a T cell, the canonical pathway can be activated via the TCR-CD3 complex engagement and at the same time, the non-canonical pathway can be activated via the CD40 stimulation. Inside the nucleus, the NF-$\varkappa$B proteins bind target genes on specific consensus sequences containing approximately 10bp: GGGRNWYYCC (N=any base, R=purine, W=adenine or thymine and Y=pyramidine) (Zhang et al., 2017). The action of the NF-$\varkappa$B family members has been suggested to be characterized by two waves: the first one occurs rapidly after the cell stimulation and leads to the binding of the NF-$\varkappa$B members on some target genes that are already in an open chromatin region, among them *Tnf* and *Ifng*; the second wave happens later and allows the NF-$\varkappa$B proteins to bind genes that before were inaccessible by closed chro-

matin structures, among them *Il2* (Saccani et al., 2001). In order to bind specific DNA sequences, the NF-κB family members interact between each other's, but they can also interact with many different TFs or co-factors such as Sp1, AP-1, STAT3 and NFAT (Oeckinghaus et al., 2011). Moreover, it has been suggested that the NF-κB proteins can modify the chromatin landscape by recruiting co-activators, such as the p300/CBP histone acetyltransferase complex, or repressive complexes (Zhong et al., 1998; Bhatt and Ghosh, 2014). The NF-κB signaling is tightly regulated by itself with negative feedback loops, in fact NF-κB not only up-regulates IκB-α that enhances the NF-κB subunits removal from the nucleus, but also up-regulates some negative regulators of the signaling as CYLD or A20 that can promote the degradation of TRAF6 and IKKγ and thus the inhibition of the pathway. However, this is not the only mechanism leading to the control of the NF-κB activation, many proteins are involved and the mechanisms regulating their activities are not always known (Renner and Schmitz, 2009).

2.1.4 GSK-3 signaling

GSK-3 is a serine/threonine kinase ubiquitously expressed that is highly conserved from yeast to mammals. GSK-3 consists of a family of two members: GSK-3α and GSK-3β. GSK-3 has mainly been studied in the glucose metabolism, but it can regulate many processes, including cell signaling, transcription factor activity and cell death. Its deregulation, mostly its hyperactivation, seems to be associated to diseases such as diabetes mellitus, inflammation, tumorogenesis and neurological disorders. GSK-3 regulates the activity of a variety of substrate by phosphorylation and GSK-3 activity itself is regulated by a phosphorylation/dephosphorylation process: the phosphorylation of the serine residue at position 21 in GSK-3α or 9 in GSK-3β by a variety of kinases (Akt, PKC or PKA) leads to its inhibition, while the dephosphorylation leads to its activation. GSK-3β can intervene in the modulation of the activity of different TFs. In the "NFAT" section, I already discussed its role in the inhibition of the NFAT signaling. GSK-3β can also play a role in the NF-κB pathway. It seems that, the phosphorylation of NF-κB by GSK-3β can regulate the NF-κB signaling cascade in two opposite ways depending on the context. On one hand, the phosphorylated NF-κB can promote the dock of some repressor complexes that mediate the inhibition of the transcription of the NF-κB proteins themselves, but on the other hand, the phosphorylated NF-κB seems to promote its binding on target genes inducing their expression (Hoeflich et al., 2000; Steinbrecher et al., 2005; Martin et al., 2005; Ougolkov et al., 2007; Beurel et al., 2010). The drastic phenotype described in mice lacking GSK-3β, characterized by the embryonic lethality, is really similar to the one observed in mice deficient for IKKβ or RelA

(components of the NF-$\varkappa$B signaling). These results highlight the fact that there is no agreement about the role of GSK-3 on the NF-$\varkappa$B activity and probably there is an important influence determined by the context and the cell type. In more detail, it seems that a fine regulation of GSK-3 expression is crucial for the T cells function. In T cells, GSK-3 activity seems to negatively correlate with the T cell activation. Indeed, in resting T cells, GSK-3 is constitutively active, but upon TCR-CD3 complex activation, the Lat signalosome switches on pathways, including Akt or the Mapk/Erk, that lead to the phosphorylation and inactivation of GSK-3. Moreover, if the TCR-CD3 trigger is associated to the activation of the CD28 signaling, the GSK-3 inhibition is even stronger (Garcia et al., 2008; Beurel et al., 2011; Maurer et al., 2014). However, GSK-3 activity seems to be implicated also in activated T cells and more in detail it has been described during the Th1 and Th17 differentiation. Indeed, GSK-3 positively regulates STAT1 and STAT3 activation in Th1 and Th17 cells, respectively. Moreover, in an EAE mouse model the induction of the disease is much less important in mice treated by lithium (inhibitor of GSK-3) and the IL-17 and IFNγ production are also strongly reduced. In T cells, these results suggest that GSK-3 is first inactivated after TCR-CD3 complex/CD28 stimulation, but still it is activated again during Th polarization, however the mechanisms regulating the reactivation of GSK-3 remain unknown (Beurel et al., 2011; Beurel et al., 2013). GSK-3 seems to also play a role in T cell proliferation (Patterson et al., 2018). Indeed, the inactivation of GSK-3 in naive human $CD4^+$ T cells increases the proliferation of cells, while the expression of a constitutively active form of GSK-3β in mice inhibits the proliferation of T cells (Garcia et al., 2008). Finally, the inhibition of GSK-3 promotes survival of T cells, indicating that GSK-3 activity is required for T cell death (Sengupta et al., 2007).

2.2 Cytokine signaling

As I already mentioned in the first chapter, cytokines have a crucial role in T cell activation and differentiation because they can shape the polarization of naive T cells towards a specific T cell subtype. As an example, low doses of TGFβ1 and IL-6 are crucial for the Th17 cell differentiation, while high doses of TGFβ1 determine a Treg cell differentiation. Cytokine receptors can be subdivided in different groups, however the two major groups are: 1) the type I and 2) type II receptors (Leonard and Lin, 2000). Among the cytokines, the ones binding to type I receptors are: IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, GM-CSF and G-CSF. While type II receptors are bound by cytokines like IFNs and IL-10. These receptors lack intrinsic tyrosine kinase activity and they signal through the Jak/STAT pathway that is one of the main signaling pathway used by a variety

of cytokines and ligands. A deregulation of this pathway can result in different immune disorders (O'Shea et al., 2015).

Jaks (Janus kinases) are kinases that are constitutively bound to the cytoplasmic regions of type I and II cytokine receptors. The binding of the cytokine to its receptor leads to the multimerization of the receptor subunits permitting to bring two Jaks in close proximity (Figure 2.9). The activated Jaks phosphorylate specific ty-

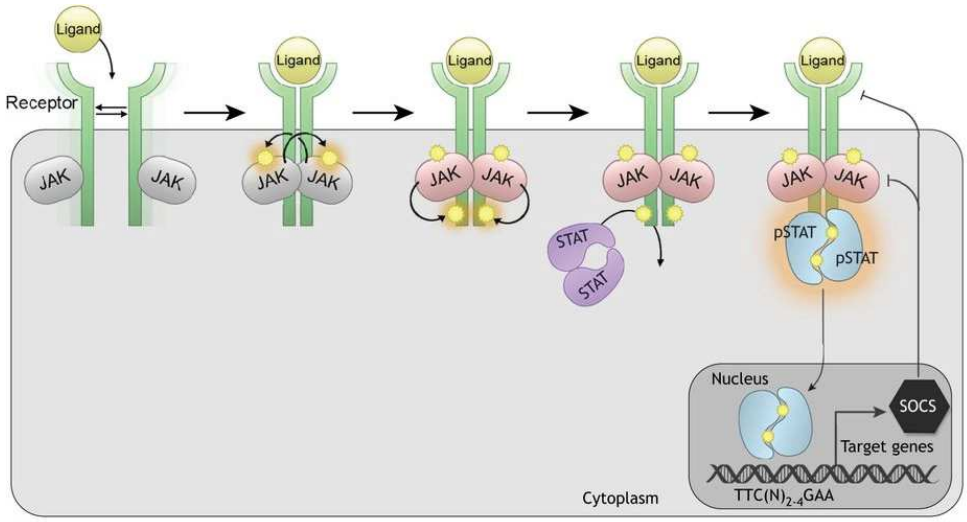

Figure 2.9: **The Jak/STAT pathway**. An extracellular ligand (yellow) binds to a transmembrane cytokine receptor (green), which lacks intrinsic kinase activity and instead constitutively associates with JAKs (gray). Ligand binding to receptors causes a conformational switch, leading to JAK activation by transphosphorylation. The activated JAKs (pink) then phosphorylate the receptor on tyrosine residues in the cytoplasmic domain. Inactive STAT dimers (purple) are recruited to the receptor at those phospho-tyrosine sites and are phosphorylated by activated JAKs. The phosphorylated STAT (pSTAT) dimers (blue) assume an activated dimer conformation, translocate to the nucleus, bind specific DNA sequences in target genes and alter gene expression. SOCS genes, which are targets of the JAK/STAT pathway, encode inhibitory proteins (black) that promote degradation of the cytokine receptor and JAKs, thereby providing a negative-feedback loop. Tyrosine phosphorylation events are indicated by orange halos (Herrera and Bach, 2019).

rosine residues leading to the recruitment of cytoplasmic TFs STATs, that were discovered studying the transcriptional activation in response to IFNs (Darnell et al., 1994). The STAT family (Signal transducer and activator of transcription) is composed by seven members in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. The two STAT5 are encoded by two linked genes, *STAT5a* and *STAT5b* that share about 90% of homology. STAT5 is expressed in all lymphocytes and can be activated by a variety of cytokines, including IL-2, IL-3, IL-7, IL-15 and GM-CSF. In more detail STAT5 is necessary for lymphocyte development and can have different roles, such as example STAT5 can inhibit the Th17 cell differenti-

ation while it can promote the Treg cell polarization (Owen and Farrar, 2017). A deregulation of the STAT5 pathway can result in cancer cell proliferation and survival and it has been described in many hematological diseases (Wingelhofer et al., 2018). Each STAT contains four main domains: 1) a N-terminal-domain important for STAT function; 2) a DNA-binding domain involved in the DNA binding; 3) the SH2 domain important for the recruitment to the cytokine receptor at the level of the tyrosines phosphorylated by Jaks and 4) the transcriptional activation domain on the C-terminal region (Figure 2.10) (Mitchell and John, 2005). STATs normally are unphosphorylated (inactive) and lie in the cytoplasm, but when they are tyrosine phosphorylated by the activated Jaks, they can dimerize and translocate to the nucleus to bind to their target genes (Figure 2.9). The STAT signaling can be negatively regulated. Among different inhibitors of the pathway, there are the suppressor of cytokine signaling (SOCS). Within T cells, the IL-7 and IL-2 signaling play a crucial role at different steps of the lifetime of a T cell such as survival, proliferation and differentiation. These two cytokines bind to a type I cytokine receptor that use a common γ chain (IL-2Rγ), common for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptor complexes. IL-2Rγ_c expression is constitutive in T cells. Here I will focus on the IL-7 and IL-2 role.

Figure 2.10: **Structure characteristics of STATs**. CCD, coiled coil domain; LD, linker domain (Adapted from Wu et al., 2019).

2.2.1 The IL-7 signaling

The IL-7 cytokine is constitutively produced by stromal cells and fibroblastic reticular cells in the T regions of SLOs (Link et al., 2007). Among other functions, in the periphery IL-7 mediates the survival and homeostasis of naive and memory T cells. The IL-7R is a heterodimer formed by IL-7Rα and IL-7Rγ_c. Following the IL-7 binding to the IL-7Rα, the common γ_c receptor is recruited, leading to the formation of a high affinity complex. IL-7R signals mostly through the Jak/STAT5 pathway (ElKassar and Gress, 2010). A distinctive feature of the IL-7Rα is that it is expressed by naive and memory resting T cells, but it is downregulated after TCR-CD3 complex activation. In more detail, in naive CD4$^+$ cells, IL-7Rα expression is maintained by the TF Foxo1 that is activated and can directly bind on the *Il7r* enhancer. However, after the TCR-CD3 complex activation, different kinases are activated, including

Akt, and phosphorylate/inactivate Foxo1 leading to a downregulation of IL-7Rα expression (Kerdiles et al., 2009).

2.2.2 The IL-2 signaling

IL-2 secretion is important for proliferation of activated T cells. Upon the TCR-CD3 complex stimulation, the NFAT pathway is one of the first signaling that is activated promoting the IL-2 secretion rapidly after T cell activation (Sojka et al., 2004). In IL-2-deficient mice, an aberrant lymphoproliferation is described underling its importance as a regulator of the immune response (Chastagner et al., 2002). IL-2 receptor is composed of three subunits: IL-2Rα, IL-2Rβ, and γ_c. The IL-2Rβ and IL-2Rγ_c are considered intermediate-high affinity receptors, because of the association of Jak kinases to their cytoplasmic domains, and they are the only ones capable to transduce the intracellular signal. IL-2Rγ_c is expressed by all T cells, whereas IL-2Rα is not expressed by naive $CD4^+$ T cells, but it is strongly induced only upon TCR-CD3 complex activation. IL-2Rβ is expressed at low levels on naive cells and its expression increases after TCR-CD3 complex activation (Smith et al., 2017). IL-2 binds to its high affinity heterotrimeric receptor activating three main signaling cascades: the Jak/STAT, the PI3K-Akt and MAPK pathways. However, the STAT5 pathway is the most well described downstream of the IL-2R. The IL-2/STAT5 signaling has a crucial role in differentiation and proliferation of many subtypes of T cells: it plays a role in both Th1 and Th2 differentiation by inducing IL-12Rβ2 and IL-4Rα expression, respectively (Liao et al., 2011); in $CD4^+$ T cells it leads to the inhibition of Th17 cell differentiation permitting STAT5 to compete with STAT3 for the binding to the *Il17* promoter, while it positively regulates Treg cell differentiation (Yang et al., 2011; Ross and Cantrell, 2018; Noster et al., 2014).

Chapter 3

$CD4^+$ T cell polarization

Contents

When a T cell encounters the specific pMHC complex and receives the activation stimuli by the 3 signals, it transits from a naive state to an activated state. Depending on the different cytokines present in the environment, naive T cells can polarize towards different subtypes. As my research focus on $CD4^+$ T cells, in this chapter I will only describe the $CD4^+$ cell differentiation focusing in particular on Th17 cells.

3.1 Naive T cells

T cells are called naive when they have not yet encountered their pMHC complex and, in mice, they express some specific markers as CD62L, have a low/negative expression of CD44 and a negative expression of CD25. In mice raised in Specific Pathogen Free (SPF) conditions, they represent about 80% of the T cell population. The tonic and the IL-7 signaling pathways are crucial for the survival of these cells.

3.2 T cell subtypes

Once our organism receives a threat by a pathogen, naive $CD4^+$ T cells can encounter their pMHC II complex and be activated in different T cell subtypes, depending on the nature and tropism of the threat: the Th and the Treg cells. The activated T cells undergo clonal expansion for 3 to 5 days and, when the threat is defeated, the most part of these cells die. From the first description of the Th1 and Th2 populations defined in 1986 (Mosmann et al., 1986), many other subtypes were discovered, each of them with a specific phenotype and role (Figure 3.1 describes four main subtypes) (Carbo et al., 2013). In this section, I will talk about two populations of Th cells, Th1 and Th2, and I will also briefly describe the Treg population. I will discuss in more detail the Th17 cell population in a separated section.

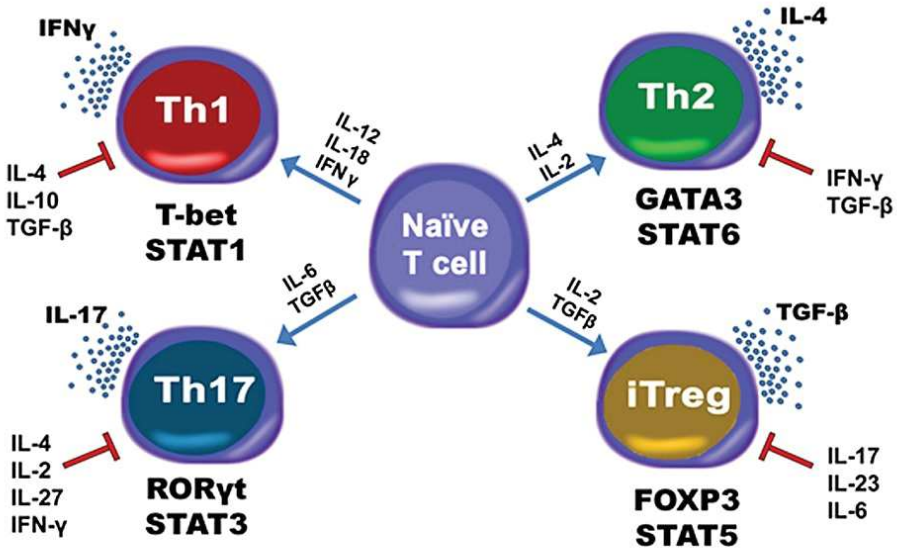

Figure 3.1: **Schematic representation of the cytokines and transcription factors controlling the main $CD4^+$ T effector cell differentiation**. (Carbo et al., 2013).

Th1 cells

IL-12 and IFNγ are the critical cytokines to initiate the Th1 cell differentiation. Th1 cells can secrete IFNγ, but also IL-2 and TNFα. The master regulator of Th1 development is the TF T-bet, but STAT4 and STAT1 are also important. Indeed, T-bet positively regulates the expression of IL-12Rβ2, a subunit of the IL-12R, and with the help of STAT4, it promotes the expression of IFNγ leading to a positive feedback loop for the Th1 differentiation (Romagnani, 1999). These cells mediate the immune response against intracellular pathogens such as bacteria and viruses.

Abnormal Th1 activation can play a role in some inflammatory and autoimmune disease such as Multiple Sclerosis (MS) and Rheumatoid Arthritis (RA) (Fletcher et al., 2010; Chen et al., 2012).

Th2 cells

Th2 cells protect the host from extracellular pathogens. They are programmed by the master TF GATA3 and mainly produce the cytokines IL-4, -5 and -13 that will help to recruit eosinophils and stimulate the production of IgE by B cells. However, an uncontrolled and exagerated Th2 response can also lead to the development of some pathologies, such as allergic diseases and asthma (Romagnani, 1999; Mitson-Salazar and Prussin, 2017).

Treg cells

Treg cells constitute a specialized and heterogeneous population either generated in the thymus by the negative selection (tTreg) or in the periphery (iTreg). iTreg cells differentiate in presence of high doses of TGFβ1 and they strongly express CD25 (IL-2Rα). They are unable to produce IL-2, thus they are dependent on the IL-2 produced in the environment: the IL-2/STAT5 signaling pathway is considered as crucial for the Treg cells development as it induces the expression of Foxp3, a critical TF in Treg cells (Chinen et al., 2016). Through the secretion of IL-10 and TGFβ1, these cells are able to inhibit T cell proliferation and cytokine production leading to the suppression of most immune cells and preventing auto immunity (Shevyrev and Tereshchenko, 2020). A dysregulation in the Treg population can lead to autoimmune diseases.

3.3 Th17 cells

The presence of IL-6 and low doses of TGFβ1 in the environment can provide the third signal necessary for the conventional Th17 cell (cTh17) differentiation (McGeachy et al., 2007). A variety of cells, such as macrophages, DCs and T cells themselves are able to secrete IL-6 and TGFβ1. cTh17 cells mostly protect the host against extracellular pathogens like *Klebsiella pneumoniae* and fungal infections like *Candida albicans*. Th17 cells produce a critical cytokine, IL-17, the pro-inflammatory cytokine that gives the name to this Th population. However IL-17 production is not restricted to Th17 cells and can be secreted by a variety of other cells, including $CD8^+$ cells (Tc17), $\gamma\delta$ T cells, NK cells and neutrophils. IL-17 binds to the IL-17 receptor complex that, among humans, is expressed by a variety of tissues and can be detected in vascular endothelial cells and B and T lymphocytes (Moseley et al.,

2003). Th17 cells can produce other inflammatory mediators, such as IL-6, IL-8, TNFα, IL-21, IL-22 but they are also characterized by the secretion of IL-10, an important anti-inflammatory cytokine that is involved in the balance between the pro-inflammatory and the regulatory function of the Th17 cells (Wu et al., 2018; Xu et al., 2020). When the equilibrium is lost and the balance is on the side of the inflammatory function, Th17 cells are considered pathogenic Th17 (pTh17). In this chapter I focus on cTh17, however, I will precise more the function of pTh17 in the next chapter.

3.3.1 The regulation of the cTh17 cell differentiation

Many TFs and cytokines are able to influence the cTh17 cell differentiation and sometimes the exact mechanism promoting the cTh17 polarization is not totally understood. Here I organize many of these factors in two main categories: 1) the positive regulators that promote the cTh17 cell differentiation and 2) the negative ones that prevent the cTh17 cell differentiation (Figure 3.2) and I detail the main regulators of the IL-17 expression in the Figure 3.3.

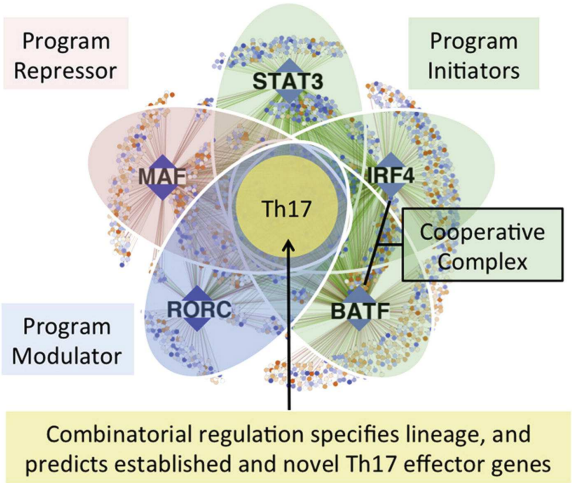

Figure 3.2: **The regulatory network for Th17 cells.** Many transcriptional factor can intervene in the shaping of Th17 cells (Ciofani et al., 2012).

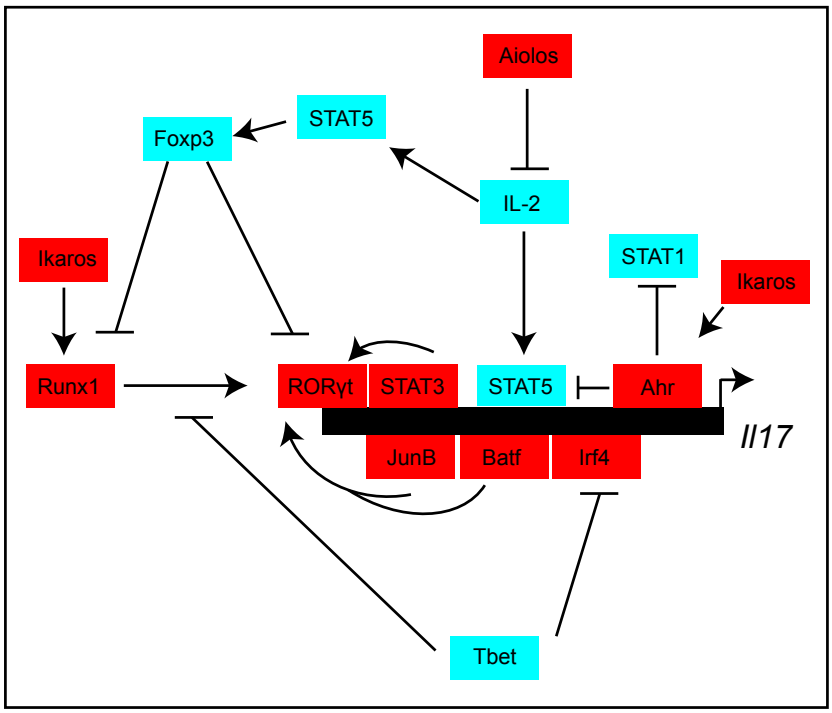

Figure 3.3: **Model for the regulation of the IL-17 expression**. The positive and negative regulators of IL-17 expression are shown in red and blue, respectively. RORγt, STAT3, Batf and Irf4 can directly bind to the *Il17* promoter (Ciofani et al., 2012). STAT3 can act directly on the *Il17* promoter or via its regulation of RORγt expression (Durant et al., 2010). Batf can also positively regulate RORγt expression (Schraml et al., 2009) and at the same time form an heterodimer with JunB to positively regulate the IL-17 expression (Yamazaki et al., 2017). Ikaros can directly regulate the expression of Ahr and Runx1 promoting the expression of RORγt and IL-17 (Wong et al., 2013). Indeed, Ahr has been described to directly regulate the IL-17 expression by binding on the *Il17* promoter (Veldhoen et al., 2008; Cui et al., 2011) or indirectly through the regulation of STAT1 and STAT5 activities (Kimura et al., 2008). T-bet can inhibit Irf4 expression by binding to its promoter (Gökmen et al., 2013) or can interact with Runx1 preventing its association with RORγt (Lazarevic et al., 2011).

1) Positive regulators

cTh17 cells are mainly characterized by the IL-17 secretion and are mostly programmed by the TF retinoic acid-related orphan receptor (RORγt), a nuclear hormone receptor. An interesting aspect of RORγt is that it is characterized by a ligand binding domain on its C-terminal part that is fundamental for its transactivation and a N-terminal domain that permits RORγt to bind to the DNA. The particular structure of RORγt signifies that many ligands with liphofilic features, such as hormones, vitamins, steroids and fatty acids, can interact and transactivate the RORγt protein. In Th17 cells, RORγt is expressed after the T cell stimulation by IL-6 and TGFβ1 and it positively regulates the IL-17 expression by directly binding to the

Il17 promoter (Ivanov et al., 2006; Yang et al., 2008; Ciofani et al., 2012). RORγt functions alone but also in collaboration with other TFs such as STAT3. Moreover, STAT3 can also regulate the RORγt expression. Indeed, upon the activation of the IL-6 signaling, the STAT3 pathway is activated and it positively regulates the RORγt and IL-17 expression by directly binding on their regulatory regions. An important impairment of the cTh17 phenotype and function is observed if the STAT3 signaling is defective (Laurence et al., 2007; Chen et al., 2006b). Another interesting TF that is important for the RORγt function and thus for Th17 cells is HIF1α (hypoxia-inducible factor1α). It is known that naive T cells produce ATP (Adenosine triphosphate) through a catabolic metabolism, while when they are activated and become effector T cells, their metabolism switches to glycolysis in order to respond to the increased energetic demands. HIF1α is able to up-regulate the glycolytic activity and it is strongly expressed in Th17 cells. HIF1α is described also to directly regulate the *Rorc* promoter and to associate directly with RORγt to activate its function. Indeed, in mice deficient for HIF1α an impaired Th17 differentiation and an enhanced Treg cell differentiation are observed suggesting that HIFα is essential to control the Th17/Treg balance (Shi et al., 2011; Dang et al., 2011).

Batf and Irf4 are considered important in the cTh17 differentiation, as they directly bind to the *Il17* and *Il21* promoters and they seem able to facilitate the recruitment of other TFs implicated in cTh17 cell differentiation, via remodeling the chromatin landscape (Mudter et al., 2011; Ciofani et al., 2012). For instance, Batf is so important for the Th17 cell polarization and IL-17 production that, in *Batf*$^{-/-}$ mice, the IL-17 expression is absent in CD4^{+} T cells polarized under cTh17 conditions, even if RORγt is over-expressed by retroviral infections (Schraml et al., 2009). These results suggest that the absence of Batf does not allow the chromatin modifications at the level of the *Il17* promoter that are necessary for the activity of RORγt.

The TF Ikaros has also been described as a positive regulator of IL-17 expression, however its role is not clearly understood yet. Indeed, in mice deficient for Ikaros, the cTh17 cell differentiation is impaired with a reduced expression of IL-17. It would seem that Ikaros can not directly regulate the IL-17 or RORγt expression, but it can indirectly regulate it by binding and activating the *Ahr* and *Runx1* promoter (Wong et al., 2013).

2) Negative regulators

Foxp3 is a TF involved in the negative regulation of cTh17 cells. Foxp3 expression is increased by high doses of TGFβ1. It can directly interact with RORγt or Runx1 and suppress the IL-17 expression (Zhang et al., 2008). Indeed, the expression of RORγt

via STAT3 activation or Foxp3 via STAT5 activation regulates the differentiation towards Th17 or Treg cells, respectively (Chen et al., 2006b). Also the IL-2 signaling is able to activate STAT5 that can compete with STAT3 for the binding on the *Il17* promoter, leading to the inhibition of the IL-17 expression (Laurence et al., 2007). On the other hand, cTh17 cells are able to regulate themselves and can limit the IL-2 production through the expression of Aiolos, a TF of the Ikaros family, which can directly bind on the *Il2* promoter inhibiting its transcription (Quintana et al., 2012).

c-Maf is not considered a simple negative regulators, it is considered indeed an important TF able to modulate the Th17 cell differentiation. Its role has not been yet totally understood, however it would seem that it can regulate the balance between the differentiation towards cTh17 or pTh17 cells (Imbratta et al., 2020).

The coordinated and controlled Th17 response is fundamental for a functional host defense against infections, and the cTh17 cell differentiation is regulated by a complex transcriptional network, which if deregulated can induce inflammatory and autoimmune diseases.

3.3.2 Plasticity of Th17 cells

The stimuli sent by the environment to the different T cell subtypes can modify the chromatin state leading to a plasticity of some $CD4^+$ T cells that can differentiate towards different T cell subtypes in a context-dependent manner. However not all T cells are plastic. Th17 cells are known to be highly plastic, able to modulate the immune response of the organism with a bias towards a regulatory (plasticity towards Treg cells) or an inflammatory pattern (bias towards Th17/Th1 cells) (Figure 3.4) (Muranski and Restifo, 2013). Th17 cells show plasticity via two main mechanisms: 1) they can directly switch off/on their specific Th17 gene program or/and 2) they can express at the same time genes and cytokines associated to different effector subsets. The first mechanism is well described in the literature: low doses of TGFβ1 (with IL-6) leads to the differentiation of Th17 cells, but high doses can switch off the Th17 gene program and switch on the Treg program within the cells modulating the type of immune response towards a regulatory response (Figure 3.4) (Zhou et al., 2008). Whereas, as example of the second mechanism, Th17 cells can differentiate towards the Th17/Th1 cell subtype that shares characteristics of Th17 and Th1 cells. In an *in vivo* model of EAE using IL-17 fate mapped mice, a cell population co-expressing both IL-17 and RORγt, marker regulators of Th17 cells, and IFNγ and T-bet, master regulators of Th1 cells, was described defining this population as 'ex-Th17' or 'non-classical Th1 cells' (Figure 3.4) (Hirota et al.,

2011). However, the mechanisms regulating the Th17 cell plasticity are not fully understood. Indeed, Th17 cells are not always plastic and in some circumstances a separate production of different Th cells is necessary: for instance after a *Candida albicans* infection, two different populations are described to shape the immune response, the Th17 cells producing IL-17 and the Th1 cells expressing IFNγ (Hirota et al., 2011). Thus, when a threat is presented to the organism, it would seem that the messages sent to the cells are crucial to define and shape our immune response.

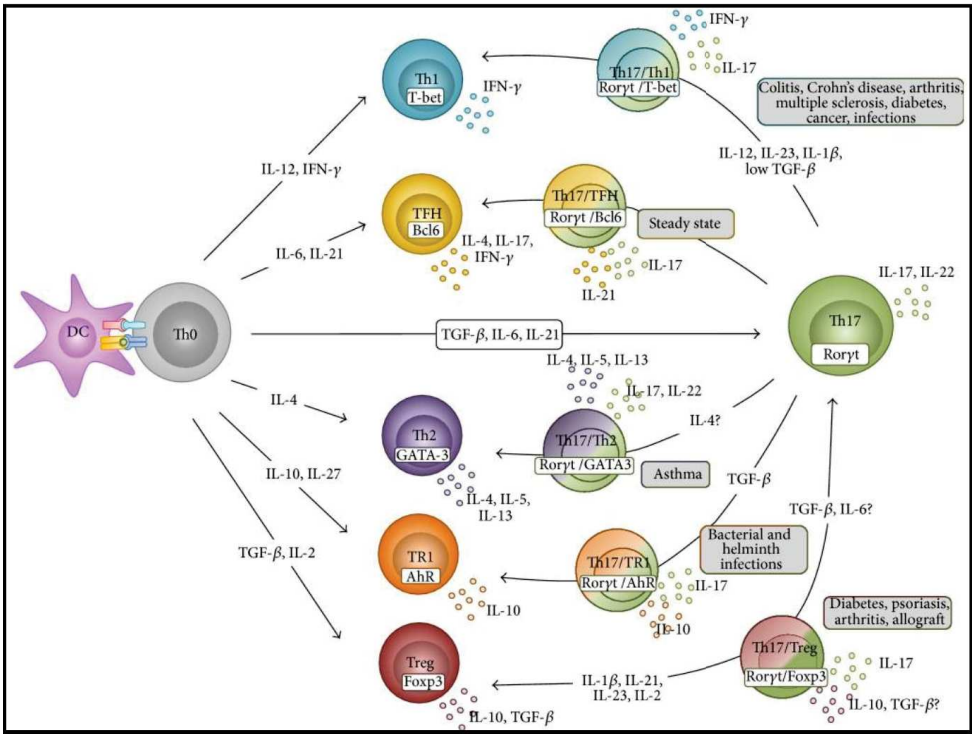

Figure 3.4: **Th17 cell plasticity**. T helper cells differentiate from naive T cells. Th17 cells are endowed with the capacity to convert toward different other lineage subsets, depending on the microenvironment. Upon steady state Th17 cells constantly convert toward TFH and participate in the development of IgA-secreting germinal center B cells. In addition, Th17 cells acquire pathogenic functions by converting toward Th1 cells during autoimmunity, cancer, and infections or toward Th2 cells during asthma. Alternatively, Th17 cells gain immunosuppressive functions by converting toward Foxp3$^+$ Treg cells or TR1 cells in the context of autoimmune diseases or infections. TR1: T regulatory type 1; TFH: Follicular T Helper (Guéry and Hugues, 2015).

3.4 Memory T cells

After the danger has been eliminated in the organism, the majority of the CD4$^+$ effector T cells die, while a minor part can survive, forming the memory T cell pool. Memory cells give immediate protection to future threats that have the same Ag.

These cells in fact can respond to a weaker TCR-CD3 complex stimulation compared to naive T cells and they are able to transduce a signaling within 1-2 hours (Jameson and Masopust, 2018). These cells highly express CD44 and IL-7Rα in mice. The pool of memory cells decline through time in mice and humans.

Chapter 4

Pathogenic CD4 $^{+}$ T cells

Contents

If the balance in the amount and type of cytokines secreted in the environment by the different members of the immune response is deregulated, Th cells can acquire a pathogenic phenotype and, thus, can contribute to the pathogenesis of autoimmune and chronic inflammatory diseases. T cells can be referred to as pathogenic if after adoptive transfer they are able to induce the EAE. EAE is considered the mouse model for the MS (Multiple Sclerosis) in humans. MS is an inflammatory and neurodegenerative disease of the central nervous system that is characterized by demyelination and neuronal degeneration provoked by inflammatory leukocytes that are able to damage the blood-brain barrier (BBB). Once infiltrated in the Central Nervous System (CNS), these pathogenic cells secrete inflammatory cytokines and maintain the pro-inflammatory phenotype. MS/EAE is a complex disease where many factors can interplay, including chemokines, cytokines, T and B cells, DCs, macrophage, neutrophils and nervous system cells (Figure 4.1). $CD4^{+}$ T cells seem to play a crucial role in the MS pathogenesis (Wagner et al., 2020). Th17 and Th1 cells with a pathogenic function (pTh17 and pTh1 cells) are the main populations implicated in the MS induction, however the identification of a specific phenotype responsible for the pathogenicity is really challenging. It would seem that pTh17 cells promote the loss of integrity of the BBB via the production of IL-17 and pTh1 cells recruit leukocytes from the periphery and positively regulate adhesion molecules expression via the production of IFNγ (Wagner et al., 2020). GM-CSF, TNFα, IL-17

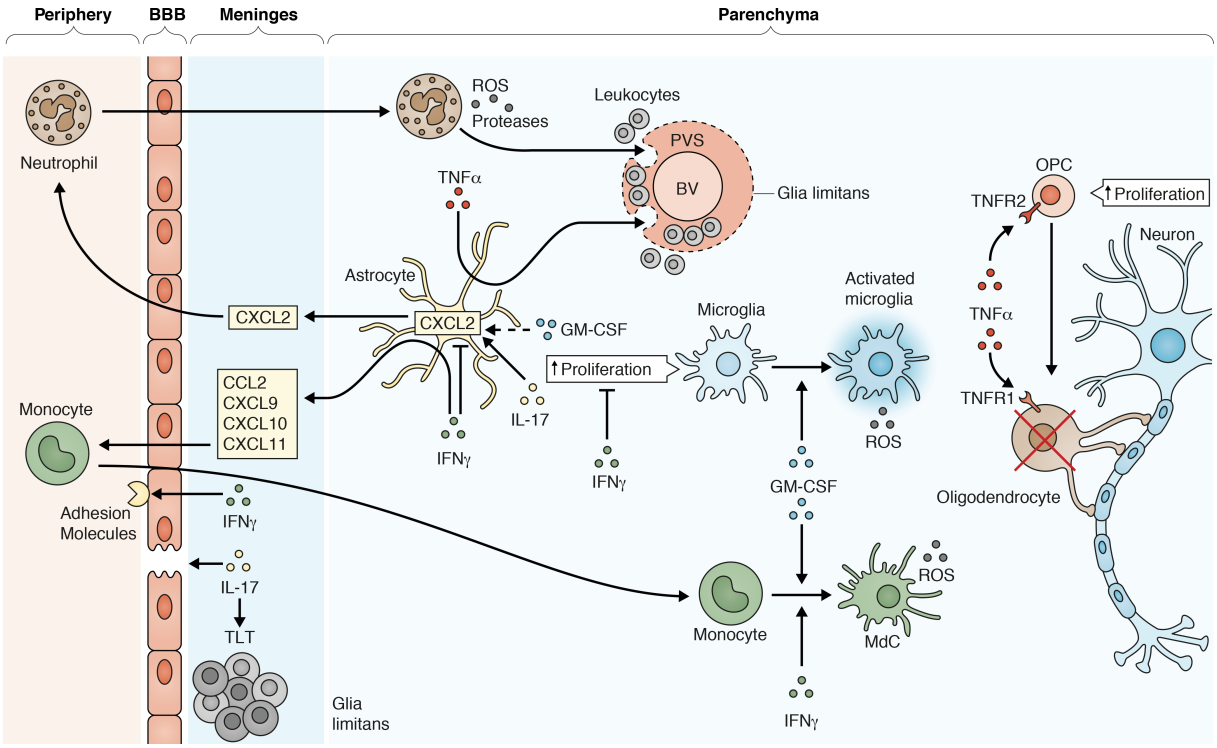

Figure 4.1: **Myelin-specific CD4⁺ T cells that cross the BBB and are reactivated within the meninges produce pathogenic cytokines that orchestrate the inflammatory response in EAE**. IL-17 contributes to loss in the integrity of the BBB by breaking down tight junctions between endothelial cells, and acts on astrocytes in the brain to produce CXCL2, a chemokine that recruits neutrophils. Neutrophils produce ROS that can damage axons. They also produce proteases that help break down the glia limitans surrounding blood vessels (BV), allowing leukocytes to escape from the perivascular space (PVS). IL-17 also promotes stromal cell remodeling within the meninges, which results in the formation of tertiary lymphoid tissue (TLT). IFNγ promotes recruitment of leukocytes from the peripheral circulation by increasing the expression of adhesion molecules on the endothelial cells of the BBB. IFNγ also induces astrocytes to secrete chemokines that attract monocytes, e.g., CCL2, CXCL9, CXCL10, and CXCL11, and facilitates the maturation and activation of monocytes into inflammatory monocyte-derived cells (MdCs) that produce ROS. Additionally, IFNγ exerts inhibitory effects on CNS inflammation by decreasing microglial cell proliferation. In the brain, IFNγ inhibits expression of CXCL2 in astrocytes, which decreases recruitment of neutrophils. In contrast, IFNγ promotes neutrophil recruitment to the spinal cord (not shown). GM-CSF enhances neutrophil recruitment to the brain and spinal cord, promotes microglia activation, and activates monocytes to acquire an inflammatory phenotype. TNFα acts on astrocytes to produce factors that promote migration of leukocytes from the PVS into the parenchyma. TNFα also enhances oligodendrocyte cell death via signaling through TNFR1 but promotes oligodendrocyte progenitor cell (OPC) proliferation via TNFR2 signaling (Wagner et al., 2020).

and IFNγ are important cytokines for the MS pathogenesis and they are found at high levels in the Cerebrospinal fluid (CSF) and blood of MS patients (Ghezzi et al., 2019; Galli et al., 2019). More recently, two new populations were described in the CSF of MS patients that may have a role in the MS pathogenesis: the $CD4^+$ T cells with a cytotoxic phenotype and the Follicular Th subtype. However their role in the development of the disease remains to be determined (Schafflick et al., 2020).

Given the complexity of MS, a variety of drugs, with different targets, are used to treat the disease. INFβ therapy is among the first line treatments for the MS patients, its action is not totally understood, but it seems that INFβ modulates the inflammatory cytokines production, as example it suppresses GM-CSF secretion by T cells (Rasouli et al., 2015). Secukinumab, a monoclonal antibody directed against IL-17, reduces the lesion activity in MS patients, highlighting the importance of IL-17 secretion in the induction of the disease (Havrdová et al., 2016). Genome-wide association study (GWAS) was used in order to find candidate genes that could be associated to the disease. As an example, a polymorphism in the *IL2RA* gene is considered a genetic risk factor for MS (Consortium, 2007) and, in the literature, high IL-2 secretion is described to induce the GM-CSF expression through the STAT5 signaling in human T cells (Noster et al., 2014). Daclizimab, a monoclonal antibody against the IL-2Rα showed promising results in MS patients (Bielekova, 2019). In view of the growing evidence for the critical role of GM-CSF in MS, targeting this cytokine appears to be a choice strategy, MOR103, a recombinant human monoclonal antibody against GM-CSF, has been proved safe for MS patients and need to be tested for clinical relevance (Constantinescu et al., 2015).

Defining the characteristics of cells crucial for the MS induction, by using the murine model EAE, is an important issue to better understand the mechanism of this pathology and to find therapies that can be transferred to humans. Here I will define three T cell populations that are described in the literature to have a pathogenic phenotype and to have a role in the pathogenesis of the EAE: the pathogenic Th17, the pathogenic Th1 and the Th producing GM-CSF (Th-GM) cells.

4.1 The pathogenic Th17 cells

4.1.1 pTh17 cell generation

cTh17 cells, when transferred in recipient mice, can induce only very mild symptoms of EAE, while Th17 cells that acquire a pathogenic function are able to induce a severe form of EAE in mice and, in this case, they take the name of pathogenic Th17 (pTh17) (McGeachy et al., 2007; Ghoreschi et al., 2010). Until now, two main ways to drive the pTh17 cell differentiation have been described: 1) Th17 cells cul-

tured with a specific cocktail of cytokines different from the conventional one and 2) a modification in the cTh17 metabolism.

1) The role of specific cocktails of cytokines

In the literature, three main culture cytokine cocktails able to balance the differentiation towards the pTh17 are described: 1) IL-6 + TGFβ1 + IL-23, 2) IL-1β + IL-6 + IL-23 and 3) TGFβ3 + IL-6. IL-23 is the cytokine mostly responsible in driving the pTh17 phenotype. It can be secreted in the environment by DCs, APCs and macrophages. IL-23R is not expressed by naive $CD4^+$ T cells, but its expression is rapidly increased when IL-6 is present (Langrish et al., 2005). Thus, the conventional cytokine cocktail (IL-6 and TGFβ1) can promote the differentiation of naive $CD4^+$ T cells into cTh17 cells, but adding IL-23 to the cell culture leads to a shift towards the pTh17 phenotype and function (Figure 4.2) (Ivanov et al., 2006; McGeachy et al., 2007; Lee et al., 2012). The combination of IL-1β + IL-6 + IL-23

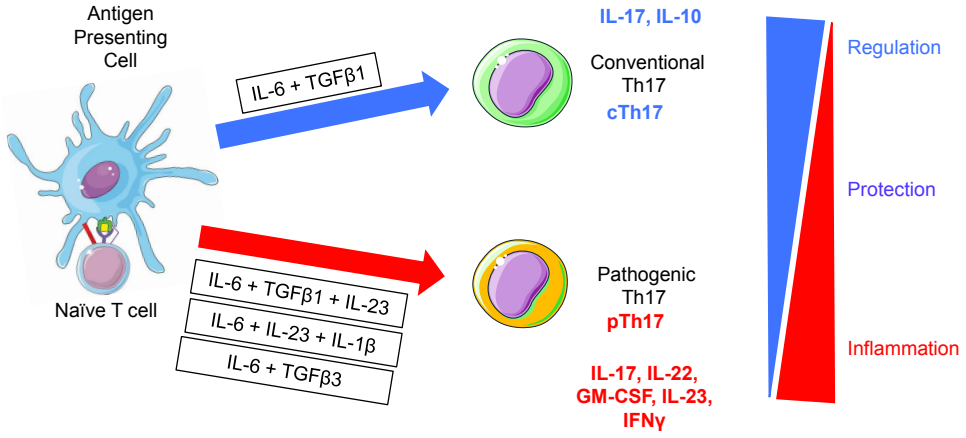

Figure 4.2: **Model of the development of a spectrum of effector Th17 cells**. Naive T cells receiving antigen-specific stimulation in the presence of TGFβ1 (from Treg cells or autocrine T cell sources) and IL-6 (from activated dendritic cells) upregulate IL-17 and IL-10 production. Upon stimulation with TGFβ1 + IL-6 + IL-23, or IL-6 + IL-23 + IL-1β or TGFβ3 + IL-6, these cells continue to produce IL-17, but also upregulate factors such as IL-22 and GM-CSF or IFNγ. These highly pro-inflammatory cells are more pathogenic to the host. It is likely that a spectrum of Th17 cell states is induced and the predominant phenotype will determine homeostasis versus immunopathology (Idea for the figure taken and adapted from McGeachy et al., 2007).

has also been shown to promote the development of pTh17 cells (Ghoreschi et al., 2010). In this case, the TGFβ signaling is not anymore necessary for the Th17 cell differentiation and STAT3 is activated through the IL-6 and IL-23 signalings. The third and last cytokine cocktails described in the literature and able to drive pTh17 differentiation is composed by TGFβ3 and IL-6 (Lee et al., 2012). TGFβ3 is strongly

induced in pTh17 exposed to IL-6 + TGFβ1 + IL-23. Thus, these studies highlight a critical role of the IL-23 axis in the development of pTh17 cells. Consistent with this, IL-23 deficient mice are resistant to EAE and the use of antibody neutralizing specifically the IL-23 signaling could block both the acute phase and the EAE relapse (Cua et al., 2003; Lee et al., 2012; Chen et al., 2006a).

2) The role of the metabolism

The generation of pTh17 cells can also be dependent on metabolic changes. CD5L (AIM-Apoptosis inhibitor of macrophages) is a soluble protein, member of the scavenger receptor cysteine-rich superfamily, that promotes the survival of cells, in particular macrophages. It is normally expressed by T cells and in particular by cTh17. CD5L/AIM regulates the cholesterol composition and thus restrains the production of endogenous RORγt ligands regulating and limiting the RORγt binding on the *Il17* and *Il23* regulatory regions. CD5L/AIM expression decreases in cTh17 after the IL-23 stimulation and CD5L/AIM down-regulation is considered as a pTh17 marker. Consistently, CD5L-deficient mice develop a severe form of EAE (Wang et al., 2015).

In the recent years, the place of the diet in the generation of pTh17 cells is gaining interest. Indeed, really interestingly, two elegant studies show that adding salt *in vitro* in the cell culture of human or murine $CD4^+$ T cells or in mice *in vivo* in the diet promotes the differentiation of pTh17, exacerbating the EAE induction. The osmotic stress created by the increased NaCl concentration intake activate the NFAT5 pathway and the kinase SGK1 that promote the differentiation of pTh17 cells (Kleinewietfeld et al., 2013; Wu et al., 2013).

4.1.2 pTh17 cell phenotype

pTh17 cells have a complex phenotype, different from the one characteristic of cTh17 cells, however they still express some typical markers of cTh17 such as IL-17 even if its expression is neither necessary nor sufficient for the development of EAE (Hofstetter et al., 2005; Codarri et al., 2011). pTh17 cells can express T-bet, IFNγ and IL-23R (Lee et al., 2012). Normally cTh17 cells have a low expression of GM-CSF, however pTh17 cells usually express high amounts of GM-CSF. GM-CSF produced specifically by T cells was described to have a crucial role during the effector phase of the EAE (El-Behi et al., 2011; Codarri et al., 2011; Komuczki et al., 2019). T-bet- or GM-CSF-deficient mice are resistant to the induction of EAE, while mice deficient for IFNγ or for the specific subunit of the IL-12R could develop an even more aggressive disease (McQualter et al., 2001; Becher et al., 2002; Bettelli et al., 2004;

Codarri et al., 2011; El-Behi et al., 2011). I will describe more in detail the fundamental role of GM-CSF in the pathogeneicity of T cells in the "GM-CSF" chapter.

Normally the differentiation of T cells is regulated by an equilibrium between their regulatory and inflammatory functions. It would seem that the presence of IL-23 in the environment and/or the GM-CSF production by T cells can bias the T cell differentiation towards an inflammatory function (Figure 4.2). These cytokines can act not only in T cells themselves but also in APCs leading to the spread of the inflammatory response and to the interaction between innate and adaptive immune response.

4.2 Pathogenic Th1 cells

The deregulation of the transcriptional program in Th1 cells towards a pathogenic phenotype can promote the EAE induction. A clear definition of pTh1 or a clear cocktail of cytokines that drive pTh1 cell differentiation is not well defined. However, pTh1 cells are described to express GM-CSF, IFNγ and T-bet (Stienne et al., 2016). Indeed, T-bet and GM-CSF seem to have a crucial role not only in pTh17 cells but also in pTh1 cells. In pTh1 cells, GM-CSF expression seems to be regulated by Foxo3, a member of the FOXO subfamily of forkhead TF, in an Eomesodermin (Eomes)-dependent way. Foxo3-deficient T cells have an impaired ability to produce GM-CSF, but over-expressing Eomes could rescue the GM-CSF expression (Stienne et al., 2016). STAT4, another Th1 associated TF, seems to be essential for the EAE induction and it is described to be able to directly regulate GM-CSF expression by binding to the *Csf2* promoter (McWilliams et al., 2015).

4.3 Th-GM cells

In 2014 another population with a strong pathogenic potential in EAE induction was described, the Th-GM cells. The pathogenic potential of Th-GM cells was mostly determined by the expression of GM-CSF. Microarray analyses show that Th-GM cells are clearly a different population than Th1 or cTh17 cells (Sheng et al., 2014). In more detail, IL-7 and IL-2 were described as the fundamental cytokines able to induce GM-CSF expression through the activation of STAT5. The induction and the severity of EAE in STAT5-deficient mice was lower when compared to WT mice(Sheng et al., 2014). The IL-2 signaling pathway was not more detailed, the study focuses mostly on the IL-7 signaling. The IL-7 signaling pathway was already mentioned as part of the pTh17 profile described by Lee et al. and IL-7Rα seems to be highly expressed on T cells at the peak of the EAE. Moreover,

IL-7Rα blockade leads to the amelioration of EAE (Arbelaez et al., 2015; Lee et al., 2012). Thus, the IL-7 signaling pathway may be important for the pathogenicity of T cells. In humans, a similar cell population was described and, in this case, GM-CSF could be secreted in absence of polarizing cytokines or in presence of IL-2 through the activation of the STAT5 pathway (Noster et al., 2014; Hartmann et al., 2014).

It is clear that pTh17, pTh1 and Th-GM cells are important for EAE development. However, it is still not clear if these cells contribute to activate a common pathway leading to the production of inflammatory cytokines, such as GM-CSF, and in this case a possible common pathway candidate could be the STAT5 signaling, or if they act in a synergistic manner in order to induce the pathogenesis of EAE. The etiology of MS and the mechanisms behind it remain unclear, probably because of the great complexity of the disease. The close interaction between mice and humans studies associated to the characterization of the different pathogenic $CD4^+$ T cells in mouse model of EAE (pTh17, pTh1 and Th-GM) is fundamental. Indeed, the identification of the key role of GM-CSF in the EAE pathogenesis and its possible regulation by STAT5 via IL-2 and IL-7 is closely associated to the discovery of the *IL2RA* polymorphism and its association to the GM-CSF production also in MS patients (Noster et al., 2014; Hartmann et al., 2014). This type of interplay helped to the therapeutic validation of the monoclonal antibody against the IL-2Rα in MS patients. INFβ is a drug efficiently used in MS patients from 1993, but its mechanism is only partially known. It was only recently that its ability to reduce the production of GM-CSF, cytokine described to be important at first in mice models, was discovered in MS patients (Jakimovski et al., 2018; Rasouli et al., 2015). The close interaction between humans and mouse studies helps in finding new treatments for MS patients and it permits to understand the function of some drugs already in use. This approach allows to identify the patients that could better respond to a specific therapy.

Chapter 5

Termination of the T cell activation

Contents

After its activation, the immune response needs to be shut down. Different processes lead to the termination of the T cell activation and T cell death, in particular, plays a central role in the regulation of the immune system. The mechanisms regulating cell death in naive T cells seem to differ from the ones in activated T cells and in this chapter, I will focus on this second group. In the periphery, upon TCR-CD3 complex activation, T cells become effector Th cells and respond to a specific threat. When the antigen has been cleared away, most of Th cells undergo a programmed cell death process, only a little part of them become T memory cells. In this chapter I will describe the activation-induced cell death (AICD), a programmed cell death, focusing only in activated peripheral T cells. Here I will detail 2 signaling pathways, part of the AICD: apoptosis and necroptosis.

5.1 Apoptosis

Apoptosis is a well regulated process characterized by some morphological changes, such as the cell membrane blebbing, chromatin condensation, DNA fragmentation and cellular degradation. Phosphatidylserine (PS) is exposed on the outer plasma membrane and provides a binding site for the annexin V, used to detect apoptotic cells (D'Arcy, 2019). In some case apoptotic cells, through the exposure of PS, may

send the "eat-me" message and they are able to produce cytokines and chemokines sending the "find me" message to neighbor cells. Some of these cytokines promote the chemotaxis of phagocytes that find the apoptotic cells and phagocytize them (Cullen et al., 2013), but the mechanisms regulating this process are poorly understood. The apoptosis can be activated through two main molecular pathways: the intrinsic and the extrinsic pathways (Figure 5.1). Despite the differences in the initiation of the signaling, these two mechanisms are complementary and they converge on the effector caspase activation.

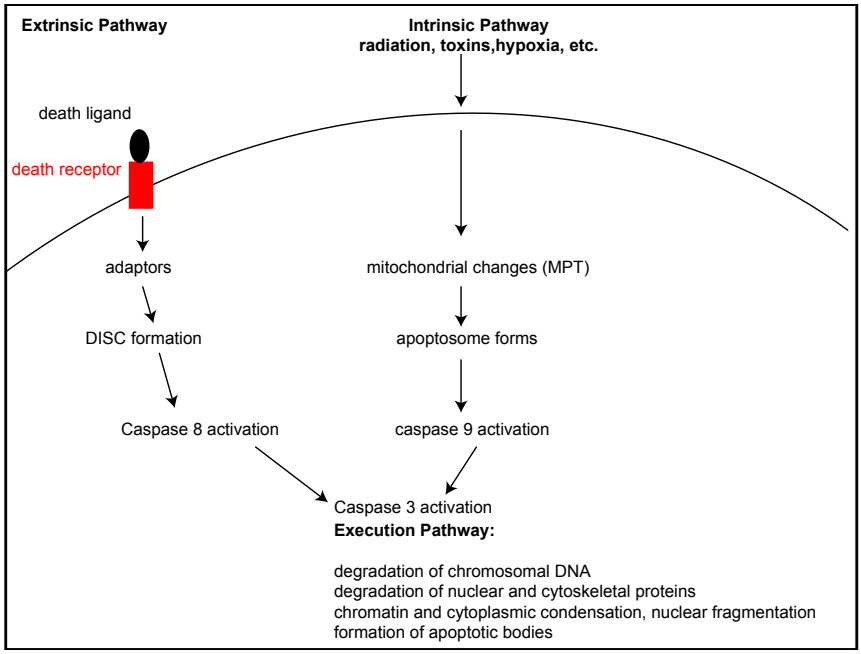

Figure 5.1: **Schematic representation of apoptotic events**. The two main pathways of apoptosis are extrinsic and intrinsic. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate caspase-3. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Adapted from Elmore, 2007).

5.1.1 Intrinsic pathway

Different stimuli trigger the intrinsic pathway, such as genomic toxicity and cytokine withdrawal. Bcl-2 family proteins, crucial elements in the intrinsic pathway, can be divided according to their anti-apoptotic (Bcl-2, Bcl-XL) or pro-apoptotic (Bax, Bid, Bad, Bak) function. Mitochondria is the key player in this type of apoptosis. When the intrinsic pathway is stimulated, Bax and Bak lead to the release of cytochrome c from the mitochondria. Cytochrome c can now bind to Apoptotic

protease activating factor-1 (Apaf-1) and pro-caspase 9 to create the protein complex called the apoptosome (Figure 5.2). This complex activates caspase 9 which in turn activates downstream caspases, including the effector caspase 3 promoting the induction of apoptosis (Green, 2005; D'Arcy, 2019). Caspases are cysteine proteases that cleave their substrates and they are key proteins in apoptosis. However, more recently, an important role of caspases during inflammation processes is also emerging (Cullen et al., 2013; Henry and Martin, 2017). During the apoptosis process, the mitochondrial membrane acquires more permeability and also the Smac proteins (second mitochondria-derived activator of caspases) are released in the cytoplasm. Smacs can bind and inhibit cIAPs permitting to promote the caspase activation (Zhang et al., 2005b).

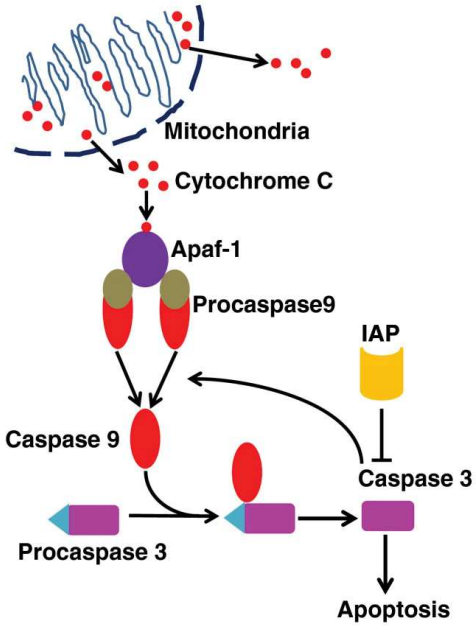

Figure 5.2: **Intrinsic apoptosis signaling pathway**. Schematic diagram of the intrinsic or mitochondria apoptosis signaling pathway. Upon the release of cytochrome C from mitochondria, it binds with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome. The apoptosome activates procaspase-9 into caspase 9, the initiator apoptotic caspase. Caspase 9 then cleaves and activates procaspase-3 to caspase 3, the executioner or effector caspase. In turn, caspase 3 promotes the activation of caspase 9, forming a positive feedback loop. Meanwhile, the inhibitor of apoptosis protein (IAP) inhibits the activity of caspase 3. Elevated activity of caspase 3 induces irreversible fate of apoptosis (Ooi and Ma, 2013).

5.1.2 Extrinsic pathway

The extrinsic pathway is triggered by different extrinsic signals that permit the activation of death receptors (DR), including Fas, TrailR and TNFR1. DRs are composed of an extracellular domain, a transmembrane domain and a cytoplasmic domain that contains the death domain. TNFR1, Fas or TrailR have been shown to have a role in apoptosis but also in inflammation through the induction of the NF-$\varkappa$B pathway (Cullen et al., 2013; Shi and Sun, 2018). Interestingly, the extrinsic pathway is highly interconnected with another type of AICD, the necroptosis.

TNFR1 and Fas signaling

TNFα can signal through two main receptors: TNFR1 and TNFR2. Only the first is considered as a DR and is expressed by many cell types. TNFR1 signaling pathway is one of the more documented in the literature, however, because of its great complexity, it continues to be actively studied. TNFα is produced as a transmembrane protein that can act in this form, but it can also be cleaved and act as a soluble form. Both the membrane and the soluble form of TNFα can act on the TNFR1, but their role may be different and has to be carefully considered when studying the TNF pathway. (Mehta et al., 2018). TNFα-TNFR1 stimulation normally leads to the activation of the NF-$\varkappa$B signaling and the secretion of pro-inflammatory cytokines, as I described in the chapter dedicated to the NF-$\varkappa$B pathway (the "Signal transduction in T cells" chapter). TNFR1 mediates a cytotoxic effect mostly when the NF-$\varkappa$B signaling is blocked (such as in RelA-deficient mice). Briefly, TNFα binds to its receptor and through the recruitment of the adapter proteins TRADD-TRAF1/2-cIAPs-RIPK1 (Complex I) it can lead to the NF-$\varkappa$B activation. However, if this pathway fails to be activated, the complex IIa is formed (TRADD, RIPK1, TRAF2, FADD and Caspase 8) and the cell death through apoptosis is triggered (Figure 5.3) (Shi and Sun, 2018).

I already described the Fas/FasL signaling in the "Signal transduction in T cells" chapter. FasL exists in two forms: soluble or bound to the extracellular membrane (Figure 5.4). Indeed, FasL can be cleaved by matrix-metalloproteinase-7 (Mmp7) permitting the generation of sFasL (soluble FasL) which can be released in the extracellular environment. sFasL seems to be able to bind to Fas but fails to induce apoptosis (Figure 5.4). When Fas is activated by the FasL binding, the FADDosome is assembled and the signaling cascade is activated leading to apoptosis (Cullen et al., 2013; Strasser et al., 2009).

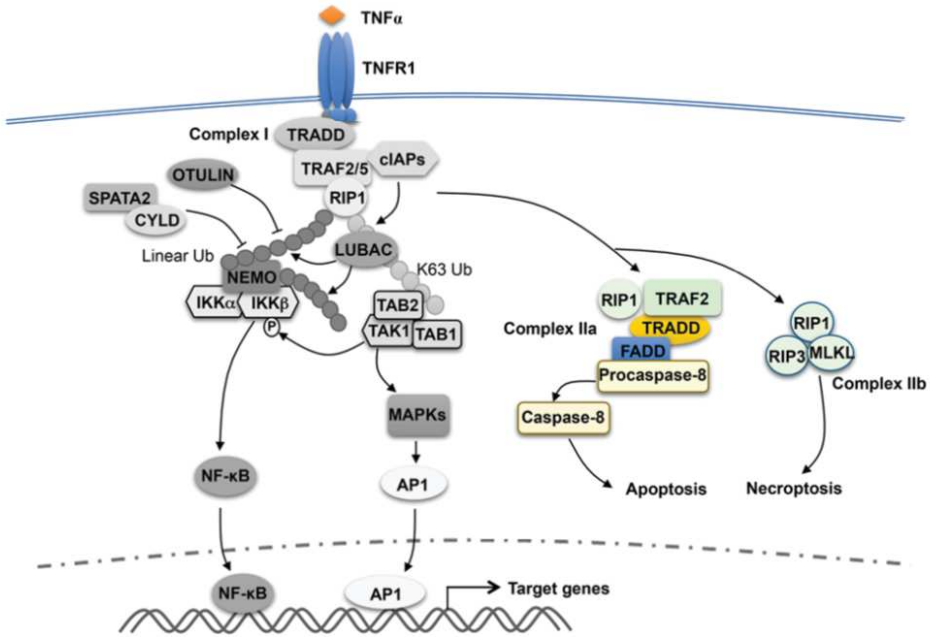

Figure 5.3: **The TNFα signaling: focus on the cell death pathway**. TNFα binding to TNFR1 triggers the assembly of a TNFR1-associated signaling complex, complex I. TRAF2 also participates in the subsequent formation of a cytoplasmic complex, complex IIa, which mediates apoptosis induction. When caspase-8 is inhibited, TNFR1 signaling also leads to formation of complex IIb, leading to necroptosis (Shi and Sun, 2018).

5.2 Necroptosis

The necroptosis is a different mechanism of cell death than apoptosis. How the name itself says, it is a process that stays in the middle between necrosis and apoptosis. It may be triggered by TNFR1 such as apoptosis, but caspase 8 needs to be blocked to bias the cell death process towards the necroptosis (Figure 5.3) (Ch'en et al., 2008; Shi and Sun, 2018), making caspase 8 a crucial factor in the choice between apoptosis and necroptosis (Fritsch et al., 2019). Necroptosis involves three main molecules: RIPK1, RIPK3 and MLKL that seems to be crucial to begin the necroptosis process, however how exactly the cell decides to undergo necroptosis or another AICD or if the cell can redirect the necroptosis towards apoptosis or viceversa are still open questions. RIPK1 inhibition with necrostatine-1 can partially block the necroptosis, while has no effect on apoptosis, on the contrary QVD-OPh, a pan-caspase inhibitor, can block apoptosis and induce necroptosis. Within T cells, the significance of this pathway is not yet fully understood (Ch'en et al., 2008; Henry and Martin, 2017).

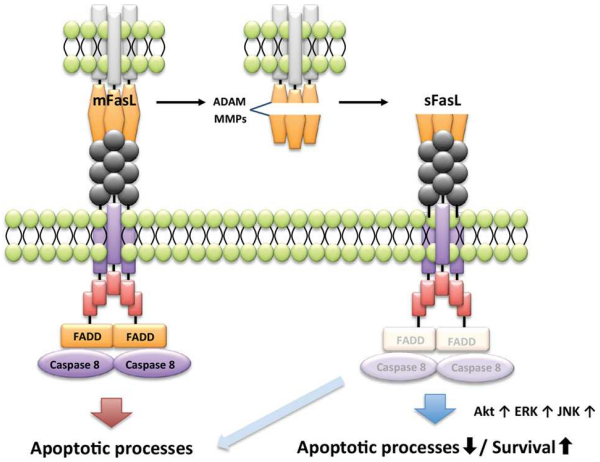

Figure 5.4: **Fas/FasL signaling**. mFasL leads to the recruitment of FADD, the formation of the DISC complex and activates caspase pathways. mFas promotes apoptosis. mFasL is shed by a disintegrin and metalloproteinases (ADAM) or MMPs in the pathological or physiological condition. For instance, sFasL can activates signaling that induce proliferation and inhibit apoptotic activity. Differential signal by the engagement of sFasL is determined by the cell type and the pathological condition (Yamada et al., 2017).

Chapter 6

GM-CSF

Contents

GM-CSF is a cytokine, member of the colony-stimulating factor (CSF) superfamily. This superfamily is named after the ability of the members to stimulate the formation of hematopoietic cell colonies. The three main cytokines that are part of this family are: Macrophage-CSF (M-CSF or CSF-1), Granulocyte-Macrophage-CSF (GM-CSF or CSF-2) and Granulocyte-CSF (G-CSF or CSF-3). The CSFs are solubles factors, however, M-CSF also exists as extracellular-matrix-anchored and cell-associated isoforms (Fixe and Praloran, 1998). They can bind to their receptors and activate intracellular pathways in three main ways as any other cytokine: paracrine (acting on nearby cells), endocrine (acting on faraway cells) or autocrine (acting on the same cell). The function of the three cytokines are often redundant. It seems that M-CSF and G-CSF are important for the myelopoiesis at the steady state, while GM-CSF is more involved during "emergency" conditions such as inflammation (Zhan et al., 1998; Becher et al., 2016). In this chapter I will focus on the structure, regulation and role of GM-CSF.

6.1 GM-CSF structure and function

GM-CSF is a small 22-kDa glycoprotein and its molecular weight can vary depending on its glycosylation state. It is nearly undetectable in the circulation at the steady state, however its levels can quickly increase during inflammation or infection. GM-CSF was isolated for the first time in mouse lung-conditioned medium in 1977 and it was described as a molecule able to generate granulocyte and macrophage colonies in vitro (Burgess et al., 1977). GM-CSF is produced by different cell types such as epithelial cells, endothelial cells, fibroblasts, B cells and activated T cells and its production can be induced by diverse factors, including LPS, TNFα, IL-1, IL-23 and by the TCR-CD3 complex trigger in T cells (Shi et al., 2006). GM-CSF influences cells that express the GM-CSFR, such as DCs and their precursors, granulocytes, monocytes and macrophages. While T lymphocytes are one of the major producers of GM-CSF, they do not express the GM-CSFR, suggesting that an autocrine effect of this cytokine do not exist in this context (Rosas et al., 2007; El-Behi et al., 2011; Becher et al., 2016).

Today the known role of GM-CSF is much more heterogeneous than the one described in the past. Indeed, GM-CSF is involved in diverse processes such as growth and development of granulocyte, macrophage and DCs, stimulation and initiation of myeloblast or monoblast differentiation, chemotaxis of eosinophils and its increased production is detected in some diseases, such as MS and RA (Galli et al., 2019; Hamilton and Tak, 2009). It seems that in a steady state condition, GM-CSF has a redundant role, because mice lacking GM-CSF do not have important perturbations of the myeloid system, they show mostly an absence of alveolar macrophages leading to an impaired ability to clear pulmonary surfactant (Stanley et al., 1994). However, the deregulation of the GM-CSF production leading to its over-expression is associated to drastic changes in the immune system. Mice models where GM-CSF is over-expressed show important abnormalities characterized by enhanced macrophage and DC recruitment/differentiation permitting the installation of a drastic phenotype including blindness due to accumulation of macrophages in the eye (Lang et al., 1987) or autoimmune gastritis through activation of local APCs (Biondo et al., 2001). Mice with the over-expression of the GM-CSF specifically in T cells showed an important extramedullary hematopoiesis associated with histiocyte infiltration. Moreover mutant T cells produced more pro-inflammatory cytokines than WT cells (Nieuwenhuijze et al., 2014). Another mouse model with a specific and inducible over-expression of GM-CSF in peripheral $CD4^+$ T cells demonstrated that the over-expression of the GM-CSF secretion alone caused an important expansion of myeloid cell population that could infiltrate different organs, but mostly the CNS causing neurological deficit (Spath et al., 2017). In conclusion, in our or-

ganism, in the steady state, the GM-CSF seems to have a redundant role, while its increased expression during inflammation has the main role to recruit myeloid cells and to enhance their activation, thus revealing a pro-inflammatory effect.

6.1.1 GM-CSF Receptor

The GM-CSFR is a member of the type I cytokine receptor family. The molecular mechanisms regulating GM-CSFR function and activation has been lately clarified thanks also to the crystallization of the receptor itself in 2008 (Hansen et al., 2008). GM-CSFR is formed by a 60- to 80-kDa ligand-specific α-chain (αc) subunit (*Csfra*) and a 120- to 140-kDa βc subunit (*Csfrb*) that is common with IL-3 and IL-5 receptors. Each αc subunit of the GM-CSFR binds GM-CSF with low affinity and the βc subunit binds cytokines very poorly by itself. However, it is the presence of this βc subunit that transforms the cytokine binding of the αc from low-affinity to high affinity causing the heterodimerization of both chain subunits forming thus a hexameric complex (two βc, two αc and two GM-CSF molecules) (Stomski et al., 1996; Broughton et al., 2016; Hamilton, 2019) (Figure 6.1). Then, two hexamer com-

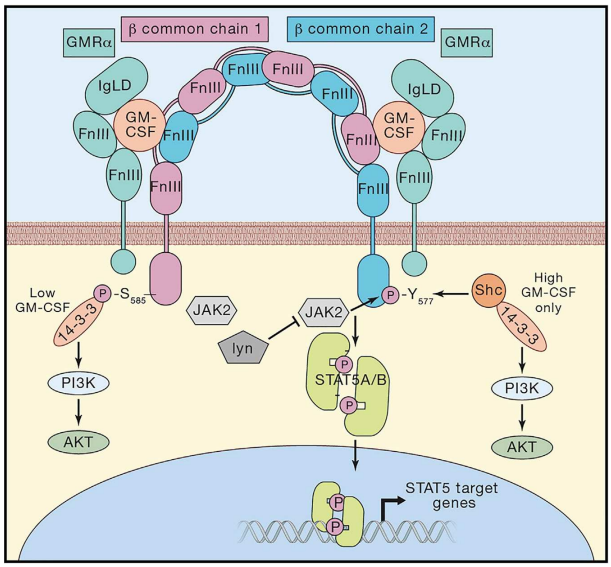

Figure 6.1: **Diagram of the GM-CSF receptor and downstream signaling**. The functional GM-CSFR is a hexameric structure comprising two αc, two βc, and two molecules of GM-CSF. These complexes associated laterally to form a 12- protein complex that is responsible for signaling. Signaling downstream of the GM-CSFR depends on JAK2 and activation of STAT5A/B. Additionally, mutually exclusive phosphorylation at Y577 and S585 (mediated by PI3K) recruit either 14-3-3 at low concentrations of GM-CSF (S585) or Shc in the setting of high GM-CSF concentrations, mediating a molecular switch between survival (low) and survival and growth (high). The Src family kinase Lyn acts as a negative regulator of signaling (Dougan et al., 2019).

plexes aggregate and form a dodecamer complex that can start the signaling cascade. GM-CSFR does not have intrinsic tyrosine kinase activity, but the βc subunit can associate with the tyrosine kinase Jak2 that allows the trans-phosphorylation of several tyrosine residues in the cytoplasmic part of the βc creating binding site for SH2 domains of other proteins such as members of the STAT family. Jak2 can also directly phosphorylate STAT proteins themselves leading to their dimerization and signaling activation (Hansen et al., 2008). Following the GM-CSFR activation, the STAT5 signaling pathway is the best characterized in myeloid cells, that are mostly expressing GM-CSFR (Seydel et al., 2008). Additional signaling pathways can be activated by βc subunits, such as the ERK 1/2 and the PI3K pathways. The NF-κB signaling pathway can also be activated by the GM-CSFR. IKKβ seems to be able to directly interact with the GM-CSFR αc subunit, however, the molecular mechanism for this signal transduction is not clearly understood (Ebner et al., 2003; Bozinovski et al., 2002; Guthridge et al., 2004). The regulation of the GM-CSFR activation can also be modulated by the concentrations of the GM-CSF itself (Figure 6.1). At low cytokine concentration, GM-CSFR is phosphorylated on the Ser585 leading to cell survival, while at high cytokine concentration GM-CSFR is phosphorylated on the Tyr577 leading to both cell survival and proliferation. Thus, only in presence of high concentrations of GM-CSF the STAT5 signaling pathway is activated (Guthridge et al., 2006). In humans, increased levels of *CSFRA*, encoding the αc subunit, and over-activation of the GM-CSF/STAT5 signaling axis have been described in hematologic malignancies such as chronic myelomonocytic leukemia (CMML) causing an important monocytosis (Padron et al., 2013).

6.1.2 *Csf2* gene regulation

GM-CSF is encoded by the *Csf2* gene, composed of 4 exons. The mouse and human genes share the same exon/intron organization. In the human genome, *CSF2* is located on chromosome 5, while in the murine genome, *Csf2* is on chromosome 11. Murine and human GM-CSF share modest structural homology at the level of the nucleotide (70%) and amino acid (56%) sequences. As mentioned in the section above, GM-CSF expression has to be highly regulated in our organism in order to correctly balance the immune response. In this section, I will describe the regulation of the murine *Csf2* gene, focusing mostly on T cells. As specified above, in T cells, GM-CSF expression requires the TCR-CD3 complex activation to be secreted and it can be expressed by many $CD4^+$ T cell subtypes (Mirabella et al., 2010; Wurster et al., 2011). GM-CSF expression can be regulated by extrinsic and intrinsic factors that act on three main regions of the *Csf2* gene: the enhancer, the promoter and the Conserved non-coding sequence a (CNSa) (Figure 6.2). Of note, the

majority of the studies on the regulation of the *Csf2* gene were mostly done at the beginning of the 90's. Then, the interest for the regulation of the GM-CSF expression faded for some years, and a new attention appeared when the new millenium began. More recently, we observe a renewed interest to understand the fine regulation of GM-CSF expression very probably due to its discovered link with diseases such as RA or MS.

Csf2 enhancer

The *Csf2* enhancer is located around 1.5kb upstream of the promoter and it is strongly conserved between humans and mice (Figure 6.2) (Cockerill et al., 1993; Osborne et al., 1995). It contains binding sites for AP-1 and NFAT proteins. While NFAT is critical to initiate chromatin remodeling in this region, the cooperation between AP-1 and NFAT is required for an optimal function of the enhancer (Johnson et al., 2004a). CsA, inhibiting the activation of NFATs, suppresses the expression of GM-CSF by reducing the *Csf2* enhancer activity (Brettingham-Moore et al., 2005). Another way to inhibit the *Csf2* enhancer activity is through the introduction of Glucocorticoids and 1,25-$(OH)_2D_3$ (Vitamin D) which receptors are able to compete with NFAT/AP-1 proteins for the binding sites on the enhancer (Smith et al., 2001; Towers and Freedman, 1998; Sheng, 2015). The Sp-1 like motif located close the NFAT/AP1 sites seems to be required only for the maximal activity of the enhancer. On this same region, GATA binding sites are also described and that is not surprising given that GM-CSF is also expressed by myeloid cells (Johnson et al., 2004a).

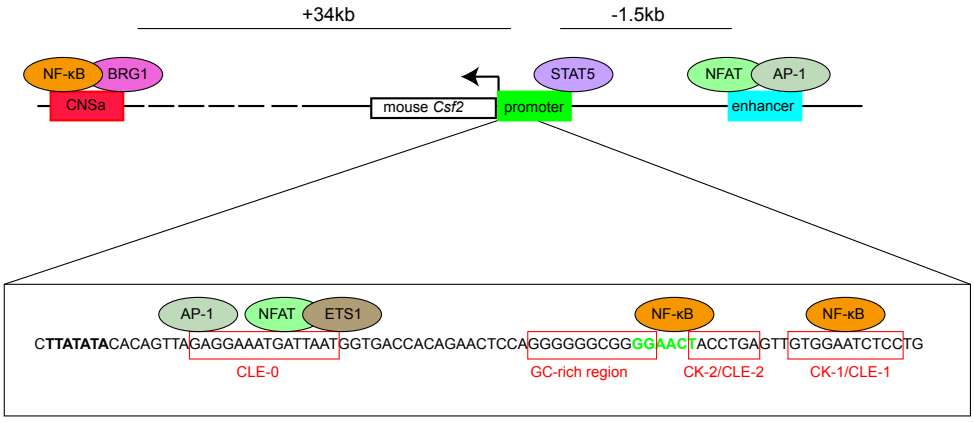

Figure 6.2: **The *Csf2* gene with a focus on the *Csf2* promoter**. The heart of the *Csf2* promoter is here described: the TATA box is detailed in bold; the CLE-0, CLE-1 and CLE-2 and the GC-rich regions in red rectangles; the κB region is depicted in green bold.

Csf2 promoter

The murine *Csf2* promoter has a much higher level of activity than the human promoter and its activation is less dependent to the upstream enhancer than in the human (Osborne et al., 1995). The study of the *Csf2* promoter via accessibility experiments with MNase and restriction enzymes in EL4 cells could suggest the presence of a single nucleosome (<200bp) occupying the promoter site in resting T cells and that was then remodeled upon T cell activation (Holloway et al., 2003). In this region, five different sequences were described as crucial for the correct functioning of the promoter: 1) cytokine consensus 1 (CK-1), also referred to as conserved lymphokine element 1 (CLE-1); 2) CK-2/CLE-2; 3) the $\varkappa$B region or classical NF-$\varkappa$B site; 4) a GC-rich region downstream of the CK-2 sequence and 5) the CLE-0 containing two CATT repetitions (Figure 6.2) (Nimer et al., 1990; Thomas et al., 1997; Holloway et al., 2003). Many TFs influence the GM-CSF expression by directly binding on *Csf2* promoter. In the CLE-0 region binding sites for AP-1 and ETS1/NFAT, present also in the enhancer, were described, however NFAT proteins seem to be dispensable for the chromatin remodeling events at the level of the *Csf2* promoter (Thomas et al., 1997; Brettingham-Moore et al., 2005).

NF-$\varkappa$B is one of the most important factors regulating the *Csf2* promoter activity. In activated T cells, NF-$\varkappa$B can bind on CLE-2 and CLE-1, and on the $\varkappa$B element, highly conserved between species (Figure 6.2). In more detail, RelA, c-Rel and p50 were described to bind the *Csf2* promoter (Himes et al., 1996; Holloway et al., 2003; Poke et al., 2012). More recently, both the canonical and the non-canonical NF-$\varkappa$B signaling pathways were shown to play a key role in the regulation of GM-CSF expression. By using *NF$\varkappa$B2*$^{\text{lym1}}$ mice that express a non-processable p100, and thus characterized by an inactive NF-$\varkappa$B non-canonical pathway, it was demonstrated that mutant mice were refractory to the EAE induction and produced lower levels of GM-CSF. By luciferase experiments, only p52, member of the non-canonical pathway, and c-Rel, component of the canonical pathway, were able to bind to the $\varkappa$B region directly regulating the expression of GM-CSF (Yu et al., 2014). Also the canonical NF-$\varkappa$B pathway has been directly implicated in the GM-CSF regulation. After T cell activation, RelA- or c-Rel- deficient mice reveal an impaired GM-CSF expression and in mice deficient for Malt1, an important protein permitting the activation of the canonical pathway, the expression of GM-CSF was drastically reduced (Gerondakis et al., 1996; Beg and Baltimore, 1996; Liou et al., 1999; Brüstle et al., 2012). A more recent work, using ATAC-seq analysis, shows a more important enrichment in NF-$\varkappa$B binding sites in pTh17 cells cultured *in vitro* and in an *in vivo* EAE model, indicating that NF-$\varkappa$B could be necessary also for the GM-CSF expression (Qiu et al., 2020). More specifically, the canonical and non-canonical pathways

seem to have a critical role in GM-CSF production both acting alone or modulating each others (Sánchez-Valdepeñas et al., 2006). Individual mutations introduced in two NF-κB binding sites in the *Csf2* promoter, CLE-1 and κB regions, block the binding of the NF-κB members to these regions preventing chromatin remodeling events (Schreck and Baeuerle, 1990). Moreover, in T cells, the NF-κB affinity for its binding site on the CLE-1 region was increased by the co-stimulation of CD28, leading to the identification of a region particularly responsive to CD28 (CD28RR) (Fraser and Weiss, 1992; Holloway et al., 2003).

Upstream from the CLE-1 region, on the *Csf2* promoter, in activated T cells, STAT5 can also directly bind and positively regulate the murine GM-CSF expression in activated T cells (Sheng et al., 2014). The Th-GM cells develop through the IL-7/STAT5 pathway activation. STAT5-deficient mice show resistance to EAE induction (Sheng et al., 2014). Also in human $CD4^+$ T cells, it was shown that the GM-CSF production was dependent on the STAT5 signaling. In humans, STAT3 seems to antagonize STAT5 for the regulation of the GM-CSF-expression (Noster et al., 2014). Another STAT family member, STAT4, has been found to be able to bind the murine *Csf2* promoter and positively regulate GM-CSF expression. Indeed, in *STAT4*$^{-/-}$ mice, the secretion of GM-CSF was reduced and the mutant mice were not able to induce EAE (McWilliams et al., 2015).

Csf2 CNSa

The CNSa, a region with *Csf2* enhancer activity, is located 34kb in mouse and 30kb in human downstream of the *Csf2* promoter (Precht et al., 2010; Wurster et al., 2011). The CNSa needs the binding of BRG1 (a multi-subunit ATP-dependent remodeling complexe) and NF-κB to function. NFAT does not act in the CNSa, CsA in fact has no effect on the regulation of the CNSa activity (Wurster et al 2011). More recently, around 10kb upstream the CNSa, two more p50 sites were found and seem to be responsible for the GM-CSF expression by T cells in an *in vivo* EAE mouse model (Peer et al., 2018).

Role of extrinsic factors

Apart from the different TFs that can directly regulate the *Csf2* gene and so are directly responsible for the GM-CSF expression, the secretion of GM-CSF can be controlled by different extrinsic factors. It exists a positive regulation of the IL-7, IL-23 and IL-1β and an inhibitory effect of TGFβ1 and IL-12 on GM-CSF expression (El-Behi et al., 2011; Codarri et al., 2011; Sheng et al., 2014). GM-CSF can be inhibited by IL-10 and IL-4 in human PBMC, probably through the inhibition of the STAT5 signaling, however, IL-10 seems not to have any effect on the GM-CSF

expression in mice (Sagawa et al., 1996; El-Behi et al., 2011). Moreover, in T cells, also IFNγ seems to be able to inhibit GM-CSF expression (Codarri et al., 2011). Very recently, it was shown that the IL-17 cytokine can modulate the GM-CSF expression too. Indeed, *Il17*$^{-/-}$ Th17 cells produce more GM-CSF than WT Th17 cells and the addition of IL-17 in the cell culture inhibits GM-CSF expression (Chong et al., 2020). Given the role of the GM-CSF expression in the pathogenic phenotype of many T cells, understanding the regulation of the *Csf2* gene in T cells could help to actively modulate GM-CSF expression by T cells in *in vivo* mouse models in order to find new treatments for diseases where GM-CSF is important for the pathogenesis.

6.2 Role of GM-CSF expression: from mouse to human diseases

GM-CSF has recently gained more interest in human diseases because of its pro-inflammatory properties. Its expression has been associated to several inflammatory and/or autoimmune diseases, including MS, RA and psoriasis (Hamilton, 2019). However, GM-CSF expression can also be protective against certain types of infection and also seems to be able to stimulate an immune response against tumor cells. Here I will describe the role of GM-CSF expression in some mouse and human diseases.

6.2.1 Multiple Sclerosis

I already introduced the crucial role played by GM-CSF in the pathogenesis of EAE and its importance in shaping the phenotype of pathogenic Th in the "Pathogenic T cells" chapter. In the mouse, the selective deletion of the *Csf2rb* from different immune cells demonstrated that only the deletion in CCR2^{+} Ly6C^{hi} monocytes is necessary to reduce the EAE pathogenesis (Croxford et al., 2015). GM-CSF-deficient mice are highly resistant to EAE induction (McQualter et al., 2001) and if a treatment with GM-CSF-neutralizing or GM-CSFRα-blocking antibody is initiated when the first EAE clinical signs appear, the symptoms developed are less severe (Codarri et al., 2011; Duncker et al., 2018; Ifergan et al., 2017). The over-expression of GM-CSF exacerbates the EAE severity and its specific and induced expression in peripheral CD4^{+} T cells is sufficient to induce CNS lesions (Spath et al., 2017). In *in vivo* mouse models, GM-CSF expression can be regulated by different stimuli: IL-23, important cytokine in pTh17, enhances the GM-CSF expression, while TGFβ1, crucial cytokine in cTh17, inhibits its expression (Komuczki et al., 2019; Codarri et al., 2011; El-Behi et al., 2011); the blockade of IL-12 and IFNγ, typical

Th1 cytokines, can enhance GM-SCF expression (Codarri et al., 2011). IL-1β signaling is essential for the expansion of cells producing GM-CSF *in vivo* (Mufazalov et al., 2017). Among TFs, T-bet is not necessary to regulate the GM-CSF expression (El-Behi et al., 2011). The role of RORγt in GM-CSF expression remains unclear. Indeed, enforced expression of RORγt increases GM-CSF expression (Codarri et al., 2011), while T cells of *Rorc*-mice cultured under cTh17 condition are able to produced GM-CSF at similar levels to WT cells (El-Behi et al., 2011).

The interplay and communication between T cells and myeloid cells, precisely monocytes, is fundamental for the pathogenesis of the MS. GM-CSF mostly produced by the pathogenic T cells facilitates the egression of myeloid cells, mostly monocytes from the BM leading to their infiltration to the CNS. Once in the CNS, the monocytes stimulated by the GM-CSF produce pro-inflammatory cytokines maintaining an inflammatory environment that sustains the inflammatory disease (Monaghan and Wan, 2020).

6.2.2 Psoriasis and Rheumatoid Arthritis

GM-CSF and Psoriasis

Psoriasis is a skin chronic inflammation that affects 2% to 3% of the population worldwilde. As many other chronic diseases, psoriasis is characterized by a following of relapse and remission phases. The cause is not well known and a genetic predisposition has been described, including SNPs in the IL-23 signaling (Nair et al., 2009). Psoriasis seems to the result from a problem of the immune system: normally the skin cell takes 3-4 weeks to turnover and new skin cells gradually move up from deepest layer of the skin until they reach the more external outlier where they die. In psoriasis patients, skin cells regenerate faster than normal taking only 3 to 7 days, resulting in the formation of red patches of skin characterized by thick and silvery scales, called plaques (Lowes et al., 2014). It seems that the IL-23/Th17 axis plays a key role in the psoriasis pathogenesis. In the acute phase of the disease, the first trigger (for instance stress or a pathogen infection) leads to the activation of DCs that produce mainly IL-23 but also other inflammatory cytokines. These cytokines are able to stimulate the production of IL-17 and IL-22 by γδ T cells, still part of the innate immune response, and to amplify the loop of inflammation stimulating the hyperproliferation of the keratynocytes (Becher and Pantelyushin, 2012). It is only the late events of psoriasis that are mediated by the adaptive immune response and by $CD4^+$ T cells. Neutralizing Abs anti-IL-17 and anti-IL-23 treatments are actually effective in psoriasis (Sakkas et al., 2019).

GM-CSF has an unclear role in the initiation or maintenance of this chronic inflammatory process. As described in case-reports, the therapeutic application

of GM-CSF have resulted in re-exacerbated psoriatic disease (Kelly and Marsden, 1994) or in the emergency of a new onset (Cho et al., 1998). In a psoriasis murine model the neutralization of the GM-CSF ameliorated the severity of skin inflammation, however *Csf2*$^{-/-}$ mice still were susceptible to the disease and could not prevent the psoriasis appearance (Scholz et al., 2017). In a recent study, GM-CSF expression seemed to be elevated in inflamed skin only in absence of Treg cells (Hartwig et al., 2018). Thus for some time, GM-CSF was considered to be a potential pharmacological target in psoriasis, until lately, when a monoclonal antibody GM-CSF inhibitor, Namilumab, was studied in phase II clinical trial: GM-CSF blockade didn't affect at all the course of psoriasis, meaning that GM-CSF signaling is not critical for the evolution of psoriasis (Papp et al., 2019). Thus, in psoriasis, the central role is played by the cytokine secretion in the IL-23/Th17 axis that permits the close communication between the innate and adaptive immune response with the dialogue between pTh17, DCs and keratinocytes able to amplify the loop of inflammation (Figure 6.3).

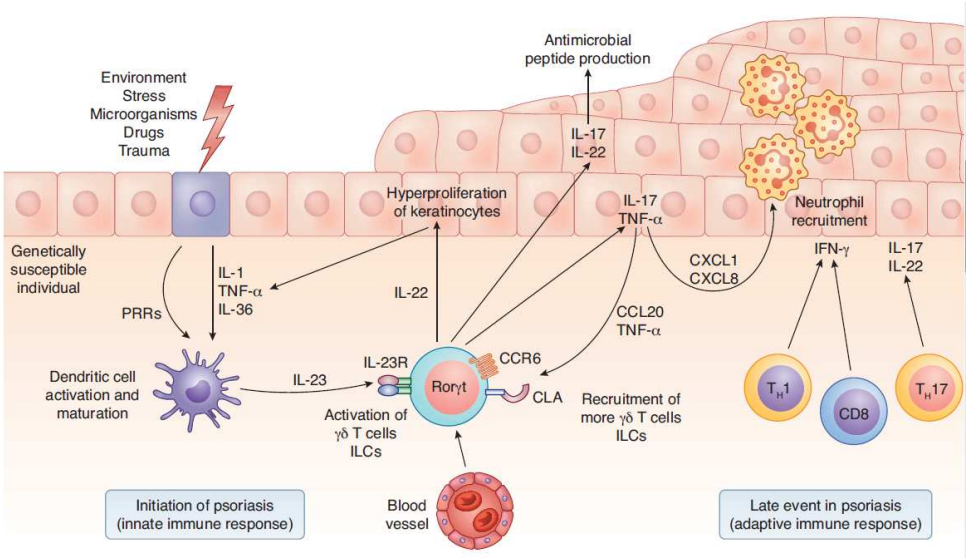

Figure 6.3: **Initiation of psoriasis**. Several insults can directly activate DCs through pattern recognition receptors (PRRs) or indirectly through keratinocyte stress (release of IL-1, TNF-a and IL-36). Stress-sensing DCs can produce IL-23, which activates γδ T cells and ILCs to make TNFa, IL-17 and IL-22, representing an early event in psoriasis that is mediated by the innate immune response. IL-22 induces keratinocyte proliferation, and TNF-α and IL-17 activate DCs and keratinocytes, leading to upregulation of adhesion molecules by the skin epithelium, angiogenesis and chemokine production. IL-17–induced chemokines include CXCL1 and CXCL8 and are responsible for the recruitment of neutrophils. IL-17R engagement by keratinocytes also leads to local production of CCL20, which attracts more circulating CCR6^{+} γδ T cells and ILCs. This escalates into a self-amplifying inflammatory loop that can also be mediated by the adaptive immune system (Becher and Pantelyushin, 2012).

GM-CSF and Rheumatoid Arthritis

RA is a chronic autoimmune and inflammatory disease that affects joints with cartilage and bone damages. The etiology of RA is not completely known. In the literature, there is a genetic association with MHC II HLA-DR4 that is described, however, the genetic component can not totally explain the disease and other factors intervene. Many cell types (fibroblasts, T and B lymphocytes, particularly pTh17 cells, and macrophages) are involved in joint inflammation (Kotake et al., 2017; Bartok and Firestein, 2010). Among many cytokines involved in the RA, the possible role of GM-CSF was suspected because RA patients who were treated with GM-CSF to correct the neutropenia following cancer chemotherapy aggravated RA symptoms (Hamilton and Tak, 2009). Moreover, GM-CSF has been found in RA synovial fluids, plasma and synoviocytes (Bell et al., 1995; Wright et al., 2012). It seems that GM-CSF also participates in the joint inflammation: in a RA mouse model, the disease is much less severe in GM-CSF-deficient mice than in WT mice (Hirota et al., 2018). GM-CSF probably acts through the activation, differentiation and survival of neutrophils and macrophages. Mavrilimumab is a human monoclonal antibody that blocks GM-CSFRα. It has been studied in phase IIb clinical trial revealing that Mavrilimumab was associated with lower disease activity scores after 12 weeks. Phase III studies in RA are ongoing (Burmester et al., 2013; Burmester et al., 2017). Currently, in the clinic, among other treatments, anti-cytokine therapies are used for RA patients, such as inhibitors of TNFα, IL-17 and IL-23 and these treatments lead to reduce cellular infiltration in the joints.

6.2.3 Influenza Infection

GM-CSF can play also a role of protection against some peculiar infections such as pulmonary infections. Alveolar Macrophages are in first line to answer to respiratory infections and their development is mediated by GM-CSF. GM-CSF expression in the lung was shown to reduce the mortality from Influenza by enhancing the alveolar macrophages activity (Huang et al., 2011; Huang et al., 2010; Halstead et al., 2018). Given the important health issue created by the seasonal influenza and the recent COVID-19 pandemia, more investigations could be done in order to better understand the role of the GM-CSF expression in the respiratory tract (Lang et al., 2020).

6.2.4 Cancer

The first to describe the possible role of GM-CSF in cancer was Dranoff and his team in 1993. They observed that vaccination with genetically modified-secreting

GM-CSF/B16 irradiated cells (B16 melanoma cells are a murine tumor cell line) stimulated the antitumor activity permitting mice to survive the tumor when they were challenged with live B16 cells (Dranoff et al., 1993). The hypothesis for the protection activity of the GM-CSF-vaccine against cancer lies on the idea that at first, GM-CSF recruits DCs at the vaccination site and stimulates the maturation of DCs specializing them against a specific antigen expressed by tumor cells and introduced by the vaccination; then, in a second time, DCs enhance antigen presentation to T cells, which finally evokes an antitumor response. Different types of GM-CSF-based cancer immunotherapy were developed in clinical practice and generally they seem to be safe, however, some results are inconsistent not permitting to really understand the complex role of GM-CSF in cancer (Becher et al., 2016).

Recently, GM-CSF got a lot of attention, however it still is a mysterious cytokine that can have a protective or deleterious function depending on the type of threat that challenges our organism and its specific tropism. Currently, the GM-CSF is used in the every-day clinic only because of its ability to stimulate granulocytes development and differentiation. The yeast-derived recombinant human GM-CSF, sargramostin, can be used to reconstitute hematopoietic progenitor cells permitting white blood cells recovery in post-chemotherapeutic patients (approved by FDA in 1991).

Chapter 7

The transcription factor Ikaros

Contents

Ikaros is the founding member of a TF family called Ikaros. The family is composed of 5 members: Ikaros (*Ikzf1*), Helios (*Ikzf2*), Aiolos (*Ikzf3*), Eos (*Ikzf4*) and Pegasus (*Ikzf5*). All these TFs have crucial roles in the hematopoiesis and lymphocyte differentiation. In this chapter I will focus on the role of Ikaros in T cells.

7.1 Ikaros

Ikaros is expressed in the hematopoietic and nervous system and it was discovered in lymphocytes by two independent teams at the beginning of the '90s (Lo et al., 1991; Georgopoulos et al., 1992). At first, Lo et al. described a new DNA-binding protein, Lyf-1, able to bind the *Dntt* promoter (Lo et al., 1991). Then, Georgeopoulos et al. characterized the same protein able to bind to the *Cd3d* regulatory regions in T cells and called it Ikaros, a name from the Greek mythology where Ikaros is

the son of Dedalus, the craftsman that created the labyrinth of King Mynos. Today this protein keeps the name of Ikaros (Georgopoulos et al., 1992).

7.1.1 The gene *Ikzf1*

In humans, Ikaros is encoded by the *IKZF1* gene on the chromosome 7 that is highly conserved in mice (*Ikzf1*) but located on the chromosome 11 (Molnár and Georgopoulos, 1994). *Ikzf1* is composed by 8 exons: exon 1 is non-coding, while exons 2 to 8 encode Ikaros mRNAs. The alternative splicing leads to the generation of different protein isoforms of Ikaros. Ik-1 and Ik-2 are the major functional isoforms observed in murine and human lymphocytes (Payne et al., 2003; Ronni et al., 2007).

7.1.2 Structure of the Ikaros protein and isoforms

Ikaros is a Kruppel-type zinc finger protein (Figure 7.1). The N-terminal domain contains 4 zinc fingers (ZFs) that are important for the DNA-binding ability, with the ZF 2 and 3 being essential for this activity (Cobb et al., 2000; Molnár and Georgopoulos, 1994). Ikaros binds to its target genes in the consensus sequence (G)GGAA (Molnár and Georgopoulos, 1994; Schjerven et al., 2013). The C-terminal domain contains 2ZFs (encoded by exon 8) that are crucial for the homo- and hetero-dimerization ability of Ikaros.

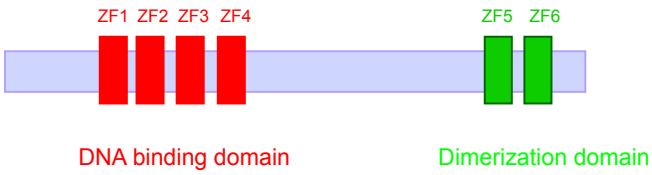

Figure 7.1: **Structure of the Ikaros protein**.

Because of the alternative splicing, *Ikzf1* can generate different isoforms (Figure 7.2). The isoforms of Ikaros described until now have an intact and functional dimerization domain, but they can lack different part of the DNA binding domain and, as described above, when they lack the ZF2-3 in the exon 5 (isoforms 5 to 7 in the figure 7.2) they can not successfully bind to the DNA and form the dominant negative (DN) isoforms (this process is schematized in Figure 7.3) (Sun et al., 1996;

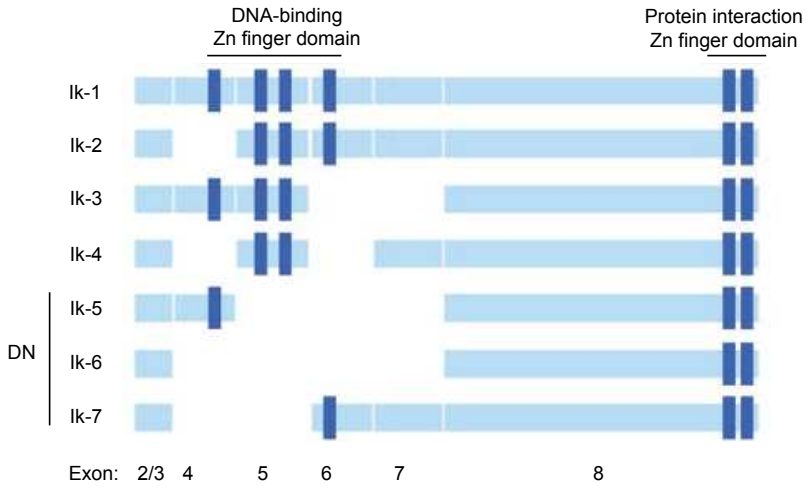

Figure 7.2: **Schematic representation of the Ikaros isoforms**. Exon composition containing Zn finger motifs involved in DNA binding and protein dimerization is shown for Ikaros isoforms. Exons are shown as light blue boxes. Dark blue bars indicate zinc fingers (Yoshida and Georgopoulos, 2014).

Li et al., 2011). High expression of these isoforms has been described in human and murine hematopoietic diseases (Winandy et al., 1995).

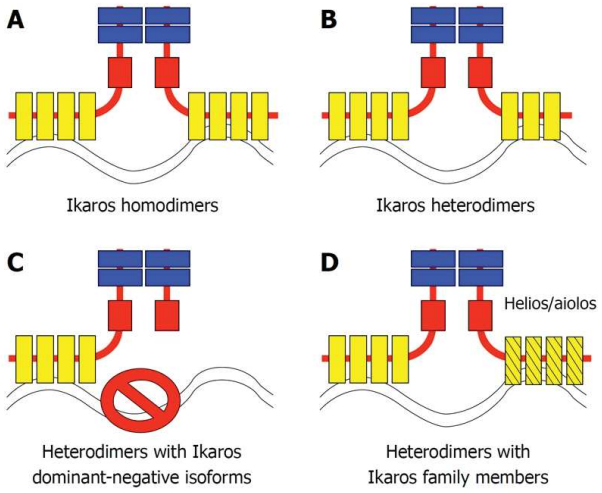

Figure 7.3: **The association of Ikaros proteins with Ikaros isoforms and Ikaros family members controls the activity of the Ikaros protein complex**. The four main types of Ikaros family-containing protein complexes are shown. A: Homodimers of the most abundant Ikaros isoform (IK-1); B: Heterodimers of IK-1 with DNA-binding Ikaros isoforms; C: Heterodimers of IK-1 and DNA-nonbinding (DN) Ikaros isoforms - this complex does not bind DNA; D: Heterodimers of Ikaros with its other family members (e.g. Helios or Aiolos) (Li et al., 2011).

7.1.3 Post-translational regulation of Ikaros

Ikaros is subjected to several post-translationally modifications that modulate its activity. The phosphorylation of Ikaros at multiple serine and threonine residues by the kinase CK2 reduces the affinity of Ikaros to the DNA affecting cell cycle progression (Gómez-del-Arco et al., 2005; Gurel et al., 2008). Ikaros phosphorylation by CK2 occurs in the 2 PEST domains (identified by PEST-finder software) in the N- and C-terminal regions of Ikaros. This leads to Ikaros ubiquitination and proteasome-dependent degradation (Popescu et al., 2009). Indeed, a dephosphorylation of Ikaros by the phosphatase PP1 prevents Ikaros degradation and therefore stabilizes the protein (Popescu et al., 2009). Thus, both CK2 and PP1 pathways are important to modulate the balance between phosphorylation and dephosphorylation and fine tune the levels of Ikaros, ultimately controlling its activity. Ikaros can also be sumoylated on lysine residues in its N-terminal domain, interfering with its transciptional activity. In more detail, the sumoylation does not seem to influence the nuclear localization of Ikaros, but rather influences its activity. Indeed, the unsumoylated Ikaros is more effective in its repressive activity than the sumoylated form (Gómez-del-Arco et al., 2005; Apostolov et al., 2016). Very interestingly, some immunomodulatory drugs, such as lenalidomide, are able to directly bind Cereblon, a protein part of an ubiquitin ligase complex, and promote the recruitment of new targets proteins, such as Ikaros, for degradation (Petzold et al., 2016; Mori et al., 2018), suggesting that other mechanisms could regulate and modulate Ikaros activity.

7.2 Ikaros function

From the first studies, it was described that in B cells Ikaros was preferentially localized in pericentromeric heterochromatin in the nucleus (Brown et al., 1997). Pericentromeric heterochromatin is normally characterized by repressive regions, thus Ikaros was considered mostly as a repressor of the transcription. However, the role of Ikaros is much more heterogeneous and it is now known to be able to act as a repressor or activator of the transcription (Davis, 2011; Georgopoulos, 2017; Heizmann et al., 2018).

7.2.1 Ikaros interacts with chromatin remodeling factors

Ikaros can regulate the transcription of its target genes by remodeling the chromatin via the interaction with nucleosome remodeling complexes. These complexes are formed by many subunits able to directly interact with the DNA and with histone markers modifying the chromatin structure and accessibility. Nucleosome

remodeling ATPase complexes in particular use the ATP hydrolyses to modulate the nucleosome organization in order to open chromatin regions that before where closed (activation) or to close regions that where opened (repression). Two of the most well described ATP-dependent remodeling enzyme complexes are SWI/SNF and Mi-2/NURD. As ATPase subunit, the SWI/SNF (SWItch/ Sucrose Non-Fer mentable) complex contains BRG1, while Mi-2/NURD (Nucleosome Remodeling Dea cetylase) complex contains the Mi-2α or Mi-2β. These two ATP-dependent remodeling complexes can lead to the activation or repression of the transcription. The SWI/SNF complex is principally described to be a transcription activator by its activity to remodel nucleosomes and generate open chromatin regions at the levels of promoters and enhancers. The NURD complex, instead, is mostly associated to transcription repression. However, the NURD complex, because of its remodeling nucleosomes and histone deacetylation activities, can also function as an activator of the transcription (Wurster and Pazin, 2012; Dege and Hagman, 2014; Bornelöv et al., 2018). It seems that these complexes do not have a specific DNA binding site consensus sequence, but they interact with DNA through the TFs that recruit them (Wurster and Pazin, 2012). A variety of TFs can interact with nucleosome remodeling ATPase complexes and target their binding to specific genes. Ikaros is described to have a crucial role in remodeling the chromatin and functions as an activator or repressor of the transcription by interacting with the SWI/SNF complex, via its association with BRG1, and the NURD complex, via its association with Mi-2β (Kim et al., 1999; Koipally and Georgopoulos, 2002). Thus, depending on the context (cell type, stage of development, Th subtype), it would seem that Ikaros is a key factor to open or close the chromatin and influences positively or negatively gene transcription (Figure 7.4) (Heizmann et al., 2018). However, many questions remain unanswered, and one of particular interest is: which mechanism allows Ikaros to prefer the interaction with activator or repressive complexes on specific target genes?

Since the discovery of Ikaros, its activity as a repressor of the transcription is the one that has been the best characterized. Indeed, Ikaros interacts directly with HDAC proteins (Histone deacetylases), that via the lysines deacetylation in H3 and H4 are able to repress chromatin regions. Ikaros can also interact indirectly with HDAC proteins via its association with corepressor proteins, such as Sin3 and CtBP (Koipally, 1999; Koipally and Georgopoulos, 2000; Koipally and Georgopoulos, 2002). Moreover, Ikaros is able to interact with many corepressors that have already a chromatin remodeling activity such as Mi-2β (Figure 7.5) (Kim et al., 1999; Koipally and Georgopoulos, 2002; Dege and Hagman, 2014; Zhang et al., 2012). In more detail, by using an important number of mutations of the N- and/or C-terminal domains of Ikaros, Koipally and colleagues describe that not only the C-terminal, but also the N-terminal domain is able to interact with repressor proteins and that

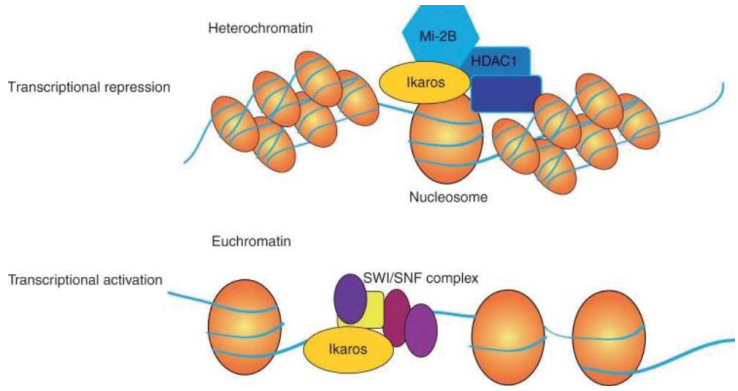

Figure 7.4: **Ikaros can act as a transcriptional repressor or activator based on cell type and developmental context**. By its interactions with different chromatin-remodeling complexes, Ikaros mediates different transcriptional outcomes. HDAC, histone deacetylase (Davis, 2011).

the two Ikaros domains seem to be important for the repression activity of Ikaros (Figure 7.5) (Koipally, 1999; Koipally and Georgopoulos, 2002).

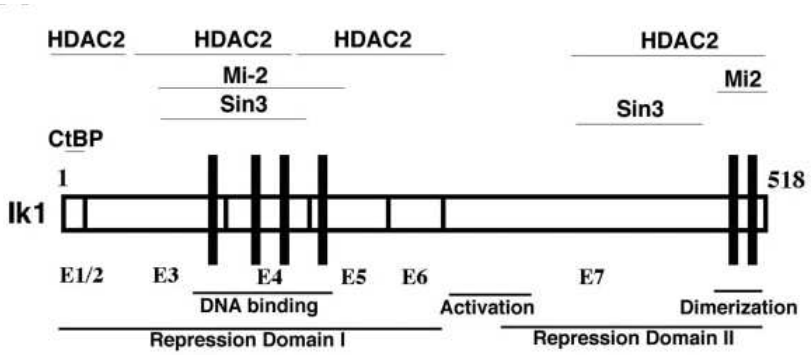

Figure 7.5: **Dissection of the two repression domains of Ikaros**. Schematic representation of Ik1 and its subregions. Exons (E) are indicated as horizontal rectangles, and zinc fingers by vertical rectangles (Koipally and Georgopoulos, 2002).

Interestingly, in primary murine double positive (DP) WT cells, Ikaros has been confirmed to interact with Mi-2β and and ChIP-seq analyses show that Ikaros and Mi-2β can bind to the same target genes. While in DP cells deficient for Ikaros, a surprisingly enrichment of Mi-2β binding was described and the analyses of the Mi-2β enriched peaks suggest the possibility that in WT cells Ikaros itself could target the Mi-2/NURD complex to bind specific regulatory regions (Zhang et al., 2012). Moreover, in double negative (DN) thymocytes, Ikaros is described to be a crucial factor able to epigenetically silence target genes via its direct interaction with the Polycomb repressive complex 2 (PRC2). Consistent with these results, in absence of Ikaros, an important loss of H3K27m3, a repressive mark, is found on these same genes and correlates with their increased mRNA expression (Oravecz et al., 2015), introducing a new mechanism that could be responsible for the repressive activity

of Ikaros on the transcription. Very interestingly, Ikaros has also been described to have a pioneering activity. In a T-ALL cell line derived from Ikaros null mice, ChiP-seq and ATAC-seq analyses in KO cells and in cells where Ikaros was re-introduced by retroviral infection show that Ikaros is able to bind closed chromatin regions and to induce their opening (Ding et al., 2019). How Ikaros works to induce these modifications in the chromatin landscape remains still unknown.

7.2.2 Interaction and/or competition of Ikaros with other TFs

Ikaros functions as an hetero-dimer by interacting with the other members of the Ikaros family but also with other TFs, including GATA family members in myeloid and erythroid cells or Ahr in ILC3 cells (Bottardi et al., 2013; Li et al., 2016a). However, in murine $CD4^+$ T cells, there is no evidence that Ikaros interacts with other TFs, while an other member of the family, Aiolos, was shown to directly interact with STAT3 to positively regulate Bcl-6 expression in follicular Th cell (Read et al., 2017). A demonstrated interaction between Ikaros and other TFs in T cells has not been shown yet.

In B cells, Stat5 and Ikaros can have an antagonizing action on the same target genes by competing for the same binding sites, but no direct interaction between these TFs have been detected so far (Katerndahl et al., 2017, Heizmann et al., unpublished data). A possible competition for the same binding sites between Ikaros and the NF-$\varkappa$B members was already suggested by Molnár in 1994 because of the similarity in their binding sequences (Molnár and Georgopoulos, 1994). More recently, ChiP analysis in a human cell line show that Ikaros and NF-$\varkappa$B are able to bind a specific target sequence, the M4 motif ACTAYRNNNCCCR (Y being C or T, R being A or G, and N as any nucleotide), and by immunoprecipitation experiments Ikaros co-precipitate with NF-$\varkappa$B subunit, p50 (Trung et al., 2016), suggesting that these two TFs may bind the same binding sites and/or be part of the same protein complex.

7.3 The role of Ikaros in the hematopoietic system

Ikaros is a TF crucial for the hematopoiesis and it plays an important role in differentiation and development of different hematopoietic cell lineages. It regulates the expression of the globin genes during erythropoiesis (Bottardi et al., 2009), it can influence the megakaryopoiesis (Malinge et al., 2013), the DCs development

(Mastio et al., 2018) and the B cell differentiation (Heizmann et al., 2018). Given its action in different cell types, its fine regulated function has been studied thanks to the use of different mouse models (Figure 7.6). Within T cells, Ikaros is described to have different roles, here I will focus on the most important ones.

7.3.1 The role of Ikaros in T cells

Ikaros loss induces an impaired T cell development and differentiation. Indeed, in absence of Ikaros some crucial step of T cell development are not correctly regulated in the thymus, such as the β-selection, leading to the generation of impaired T cells. For the purpose to study the role of Ikaros in the β-selection, *Ikzf1*$^{-/-}$ *Rag1*$^{-/-}$ mice were used (Winandy et al., 1999), because in *Rag1*$^{-/-}$ mice the TCRβ chain rearrangement does not happen resulting in immunodeficient mice (Mombaerts et al., 1992). Very surprisingly, *Ikzf1*$^{-/-}$ *Rag1*$^{-/-}$ mice expressed mature T cells (Winandy et al., 1999) suggesting that in absence of Ikaros T cell differentiation happens in absence of the pre-TCR engagement and that Ikaros has a key role in the development of T cells. However the mechanism that leads Ikaros to regulate this process remains still unclear.

Ikaros regulates the expression of some Notch target genes

The Notch pathway plays an important role in the T cell development and it is switched on already in Early T-cell Precursor cells that differentiate towards the T lineage and it is switched off at the DP stage (Rothenberg et al., 2008). The deregulation of the Notch pathway can be dramatic inducing the onset of T-ALL diseases (T acute lymphoblastic leukemia) and mice with a deletion of *Ikzf1* develop Notch-dependent T-cell malignancies (Jeannet et al., 2010; Dumortier et al., 2006). The loss of Ikaros results in the up-regulation of some Notch target genes (Dumortier et al., 2006; Geimer Le Lay et al., 2014) and this may happen because of different mechanisms: 1) EMSA and ChIP-seq analysis showed that Ikaros and RBP-J are able to compete for the same target genes (Kleinmann et al., 2008; Geimer Le Lay et al., 2014); 2) without knowing the exact molecular mechanism, Ikaros loss is associated to the reduced presence of H3K27me3, a silencing mark (Oravecz et al., 2015), suggesting that in absence of Ikaros some Notch target genes could be more expressed because they have a more accessible chromatin; 3) immunoprecipitation experiments indicate that Ikaros interacts with RBP-J in a T cell line (Geimer Le Lay et al., 2014), suggesting that this interaction may be another possible mechanism implicated in the repression of the transcription of some Notch target genes. An interesting result is that *Ikzf1*$^{L/L}$ adult thymectomized mice remain healthy and do not develop any cancer (Dumortier et al., 2006), moreover it would seem that the

Mouse strain	*Ikzf1* $^{null/null}$	*Ikzf1* $^{L/L}$	*Ikzf1* $^{DN/DN}$	*Ikzf1* $^{Plstc/Plstc}$	*Ikzf1* $^{\Delta F1/\Delta F1}$	*Ikzf1* $^{\Delta F4/\Delta F4}$	*Ikzf1* $^{flox/flox}$
Mutation							
	Deletion exon 8	LacZ knock-in Exon2 (Hypomorphic allele)	Deletion Exon 4-5 (ZF1-3)	Mutation Exon 4 (ZF3)	Deletion exon 4 (ZF1)	Deletion exon 6 (ZF4)	Conditional deletion exon 8
B-lymphoid phenotype	*B cells absent	*Mild block B cell development; *Activated B cells; *Disturbed Ig class switch recombination	*B cells absent	***B cells absent**	*Block at pre-B cell stage; *Reduction of large pre-B cells.	*Mild reduction progenitor B cells; *Increase of large pre-BII cells.	*dLck-hCre^{3779} : B cells present
T-lymphoid phenotype	*Fetal T cells absent; *Post-natal T cells activated *Skewing towards CD4^{+} lineage.	*Normal thymic cellularity; *Activated thymocytes and T cells.	*T cells absent	*T cells absent	*Fetal T cells present; *Mild reduction thymic cellularity.	*Fetal T cells absent; *Reduced thymic cellularity.	*dLck-hCre^{3779} : normal T cell maturation in the thymus. CD8^{+} cells normal.
Hematopoietic phenotype	*Mild reduction in HSC activity; *NK cells and pDCs absent.	*NK cells and pDCs absent; *Increase of neutrophil precursors in fetal liver.	*Strong reduction in HSC activity; *Reduction of erythroid progenitors; *NK cells and pDCs absent.	*Loss of LT-HSC pool at E15,5; *Increase of GMPs at E15,5; *Fatal fetal anemia	*NK cells and pDCs present.	*NK cells and pDCs absent.	*dLck-hCre^{3779} : Myeloid cells present.
T-lineage malignancies	*Fraction Ikzf1 $^{null/+}$ mice develops T-cell malignancy.	*All *Ikzf1* $^{L/L}$ mice develop thymic lymphoma in 10 mo.	*All *Ikzf1* $^{DN/+}$ mice develop thymic lymphoma in 4 mo.	*Most *Ikzf1* $^{Plstc/+}$ mice develop T cell malignancy in 4 mo	*None	*All *Ikzf1* $^{\Delta F4/\Delta F4}$ mice develop thymic lymphoma in 10 mo	*CD4-Cre: thymic lymphoma in 7 weeks
References	*Wang et al 1996 *Yoshida et al 1999 *Winandy et al 1999	*Kirstetter et al 2002 *Sellars et al 2009 *Heizmann et al 2016 *Dumortier et al 2006	*Georgopoulos et al 1994 *Winandy et al 1995	*Papathanasiou et al 2003 *Manta et al 2007	*Schjerven et al 2013	*Schjerven et al 2013	*Badea et al 2003 *Geimer Le Lay et al 2014 *Lyon de Ana et al 2019

Figure 7.6: **Summary of the observed phenotypes in the different constitutive *Ikzf1* knockout mouse models**. DN: dominant negative; Plstc: ENU-induced dominant-negative point mutation, called Plastic; Neo: neomycin gene; βGeo: fusion between LacZ and neomycin gene; ZF: zinc-finger; HSC: hematopoietic stem cell; NK: natural killer; pDCs: plasmacytoid dendritic cells; LT-HSC: long-term hematopoietic stem cell; GMPs: granulocyte-macrophage precursors; mo: months. Modified From Marke et al., 2018

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deletion of Ikaros at the late stage of T cell development, such as at the SP T cell stage, is not sufficient to lead the development of a T-ALL disease (Lyon De Ana et al., 2019), suggesting that the cell responsible for the transformation process is probably an immature cell in the thymus.

Ikaros binds the *Dntt* gene

The Terminal deoxynucleotidyl Transferase (TdT), encoded by the *Dntt* gene, is a DNA polymerase that is expressed in immature B and T cells and has a critical function in the VDJ recombination permitting the junctional diversity. The *Dntt* promoter has been described to be a target of Ikaros from its very first discovery (Lo et al., 1991). Competition experiments by gel shift assays indicate that Ikaros competes with Ets TF family to bind on the *Dntt* promoter in a DP thymocyte line suggesting that the binding of Ikaros could determine the inhibition of TdT expression in mature T cells (Trinh et al., 2001). An elegant study demonstrates that also the post-translational modifications of Ikaros by CK2 and PP1 can influence the regulation of the binding activity of Ikaros on the *Dntt* gene (Wang et al., 2014). Currently, the complex mechanism that allows Ikaros to bind the *Dntt* gene and if this binding is responsible for the inhibition of TdT expression remain unclear. It would be interesting to study, in cells deficient for Ikaros, the effect of the failed decreased expression of TdT by analyzing the TCR repertoire in order to determine which TCR clones are more represented in mice deficient for Ikaros and if this could influence their susceptibility to answer to different *in vivo* challenges.

Ikaros may regulate CD4 and CD8 expression

Ikaros is so important for the T cell development that it seems to be able to influence the expression of CD4 and CD8 itself. In the CD4 regulation a close interaction between Mi-2β and Ikaros has been demonstrated. Really interestingly, Mi-2β seems to play a role in the regulation of the CD4 expression in DP cells by two complementary mechanisms: 1) immunoprecipitation and ChIP-qPCR experiments show that in WT cells Mi-2β interacts with the co-activator p300 protein and they bind the *Cd4* enhancer (Williams et al., 2004), suggesting that Mi-2β may be able to induce the CD4 expression. 2) In complement to these results, in another study, the same type of experiments in thymocytes find an interaction between Mi-2β and Ikaros and a binding of Mi-2β at the level of the *Cd4* silencer (Naito et al., 2007), suggesting that Mi-2β may be able to induce the CD4 expression by inhibiting the silencer action in collaboration with Ikaros.

By immunoprecipitation experiments, Ikaros is able to bind also the *Cd8a* regulatory regions in thymocytes (Harker et al., 2002). However, if Ikaros could directly

regulate the expression of CD8 remain unclear.

Ikaros sets the TCR threshold

In an early study, Ikaros was described to be important to set the threshold for the T cell activation. In more detail, splenic T cells from heterozygotes *Ikzf1* $^{null/+}$ and *Ikzf1* $^{DN/+}$ mice stimulated via different concentrations of CD3ε Abs proliferated more than WT cells, suggesting that Ikaros lowered the threshold of T cell activation (Avitahl et al., 1999). However, a more recent study shows the opposite phenomena: in a mouse model where Ikaros is deleted from the single positive (SP) T cell stage, the loss of Ikaros leads to defects in $CD4^+$ T cell proliferation (Lyon De Ana et al., 2019). This opposite result may be explained by the observation that, in the two studies, Ikaros deletion happens in different step of the T cell development and that the two mouse models used have a different genetic background (C57BL/6 x 129SV background mice in the first study and C57BL/6 background mice in the second one). In conclusion, the effect of the Ikaros loss on proliferation ability of $CD4^+$ T cells still remain unclear.

Ikaros binds the *Il2* gene

Ikaros has been described to remodel the chromatin and directly bind the *Il2* gene in $CD4^+$ T cells (Thomas et al., 2007). EMSA and Ikaros ChIP experiments show that Ikaros binds the *Il2* gene and that its DNA binding domain is crucial for this interaction, because in presence of a DN isoform of Ikaros, this binding is not anymore possible (Thomas et al., 2007). In more detail, the ChIP analyses of histone acetylation indicate that in absence of Ikaros, the *Il2* promoter is characterized by an hyperacetylated state (Thomas et al., 2007), suggesting that Ikaros could regulate the acetylation state of the *Il2* promoter. Very interestingly, one of the binding sites of Ikaros identified in the *Il2* gene is at -203 and overlap with the binding site of NF-χB (Paliogianni et al., 1993), suggesting that these two TFs could bind on the same target genes.

Ikaros regulates T cell differentiation

Ikaros has an important and fine regulated role in the T cell differentiation and it can influence the polarization of different subtypes of Th and Treg cells. Ikaros may be able to play a role already in T cells at the naive state (TN), however, this topic has not been further studied and still remains unclear. Naive $CD4^+$ T cells deficient for Ikaros were described to have a reduced survival than WT cells in presence of IL-7, while the survival was similar if the IL-7 was not introduced in the culture cell (Agnihotri et al., 2017). The mechanism that has been proposed by

the authors is that in absence of Ikaros, the reduced expression of Foxo1 induces the impaired expression of IL-7Rα, however the over expression of Foxo1 by retroviral expression does not rescue the IL-7Rα expression (Agnihotri et al., 2017), suggesting that other mechanisms may be implicated. The homing of TN cells is another crucial characteristic of these cells and it was described as impaired in TN cells deficient for Ikaros (Agnihotri et al., 2017). Indeed, by transferring CFSE labeled-$CD4^+$ T cells from WT and *Ikzf1*$^{-/-}$ mice into WT host mice, the absence of Ikaros induced a reduced homing of TN cells to the LN in comparison to WT TN cells (Agnihotri et al., 2017). The reduced expression of CD62L in TN KO cells could be responsible for this altered homing (Agnihotri et al., 2017). However other factors need to be considered in cells deficient for Ikaros such as the viability of the cells *in vivo*, the possibility that TN cells are sequestered in other sites or the expression of other membrane molecule that could influence the journey of TN cells from the bloodstrem to the LN.

Ikaros is known to promote the Th2 cell development and to regulate the Th1 cell differentiation. During Th2 cell differentiation, in an Ikaros null mouse model, the expression of IFNγ, important cytokine of Th1 cells, is increased and ChIP-qPCR analyses in WT cells demonstrate that, in these same culture conditions, Ikaros is able to bind the *Ifng* CNS22 (Quirion et al., 2009). While in Th1 cell condition, in cells deficient for Ikaros, the expression of IFNγ is also increased, but in WT cells no Ikaros peak has been identified by ChIP-qPCR on *Ifng* regulatory regions (Quirion et al., 2009). These results suggest that Ikaros could directly repress the IFNγ expression in Th2 cells, however there is no confirmation of the functional binding of Ikaros, while in Th1 cells other mechanisms regulated by Ikaros seem to be responsible for the increased expression of IFNγ. An important limitation of this study is that total $CD4^+$ T cells were purified indicating that the pool of cells analyzed is composed by naive and activated $CD4^+$ T cells. The same mechanism proposed for IFNγ has been also described for T-bet (Thomas et al., 2010). These results highlight the importance of polarizing cytokines in influencing the binding of Ikaros on its target genes in $CD4^+$ T cells.

Under *in vitro* iTreg culture conditions, the absence of Ikaros does not allow the expression of Foxp3 (Agnihotri et al., 2017) neither if the deletion of Ikaros happen only in more mature $CD4^+$ cells (Lyon De Ana et al., 2019). Moreover, the same phenotype is present in cells missing the ZF4 of Ikaros, suggesting that probably it is the DNA-binding domain of Ikaros that is important for the Foxp3 expression (Heller et al., 2014). It would be interesting to evaluate what is the specific function of Treg cells deficient for Ikaros in the suppression of the immune response.

In Th17 cell differentiation, the role of Ikaros remains uncertain. By using *Ikzf1*$^{\Delta F4/\Delta F4}$ mice (Figure 7.6), an enhanced IL-17 production was detected in *in vitro*

cultures, suggesting that the ZF4, located in the DNA-binding domain of Ikaros, could be important for the regulation of the IL-17 expression (Heller et al., 2011). However, in a germline deletion of Ikaros, an impaired production of IL-17 was described (Wong et al., 2013). This last result was confirmed by our team, using a *Ikzf1*$^{f/fCD4Cre}$ mouse model, where the *Ikzf1* deletion is conditioned to the CD4 expression, and a *Ikzf1*$^{f/fRosaCre26ER}$ mouse model (Figure 7.6) (Maurer thesis, unpublished results). A recent study shows that, in*Ikzf1*$^{f/fdLckCre}$ mice, where the Cre expression is regulated by the distal *Lck* promoter permitting the deletion of Ikaros from the SP cell stage, IL-17 production is not impaired in total CD4$^+$ T cells (Lyon De Ana et al., 2019). These different results could be explained by the different types of mouse models (different time of *Ikzf1* deletion and different background of mice), and the way the experiments were performed (naive CD4$^+$ T cells activated in culture in the study of Wong and colleagues or total CD4$^+$ T cells in the study of Lyon de Ana and colleagues).

Globally, Ikaros has a crucial role in the development and differentiation of T cells (Figure 7.7) and its deregulation has been mostly studied *in vitro*. There are not many *in vivo* mouse models where the effect of the loss of Ikaros is studied in T cells. Only Thomas and his colleagues studied the effect of Ikaros loss *in vivo* by using a *S.mansoni* worm infection in WT and *Ikzf1*$^{DN/+}$ mice. They confirmed that the absence of Ikaros induces an increased expression of IFNγ among cells polarized towards a Th2 response (Thomas et al., 2010). The lack of studies *in vivo* using mice deficient for Ikaros is probably due to the fact that these mice do not live long and die of T-ALL if the *Ikzf1* deletion takes place during the T cell development (Dumortier et al., 2006). However, it would be really amazing to understand and analyze the effect of the loss of Ikaros specifically in T cells *in vivo*.

Loss of Ikaros enhances inflammatory cytokine production

In the absence of Ikaros, total CD4$^+$ T cells in Th17 culture conditions strongly express *Ifng* and *Csf2* mRNA (Lyon De Ana et al., 2019). In this same study, total CD4$^+$ T cells from WT and *Ikzf1*$^{f/f\ dLCKCre}$ mice were analyzed by microarray analysis at the naive state or after 1 day of activation by anti-CD3 and anti-CD28 antibodies (Th0 condition) and the authors describe that, in absence of Ikaros, the gene signature was enriched with genes associated to inflammation and autoimmunity processes (Lyon De Ana et al., 2019), suggesting an interesting role of Ikaros in the regulation of these genes. However, in this article, total CD4$^+$ cells, composed by naive and activated cells, are used for the experiments. Indeed, it has already been described that the loss of Ikaros promotes the Th1 cell differentiation via the expression of

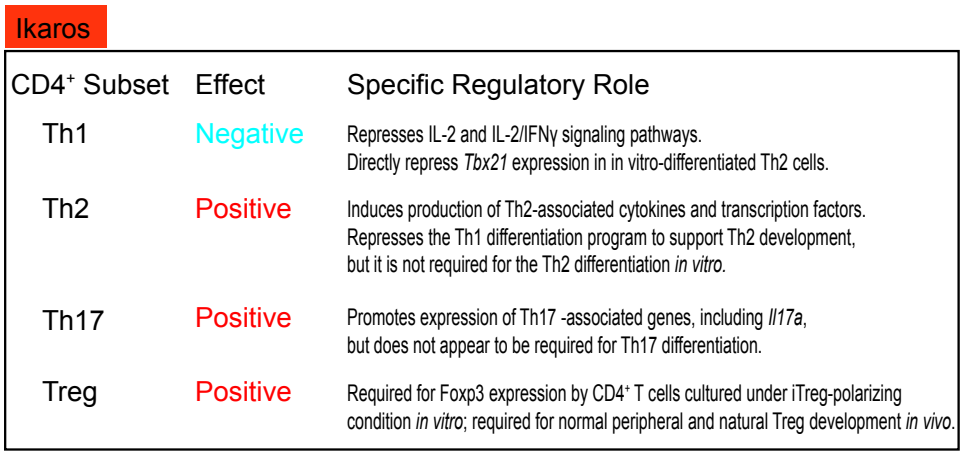

Figure 7.7: **Regulation of CD4⁺ T cell differentiation programs by Ikaros**. Summary of the known effects of Ikaros on expression of Th1, Th2, Th17 and Treg gene programs, including associated cytokines and transcription factors (Adapted from Powell et al., 2019).

IFNγ and T-bet (Quirion et al., 2009; Thomas et al., 2010), thus, with this study, we can not exclude the possibility that the enriched gene signature observed in cells deficient for Ikaros is the consequence of activated cells that were already biased towards a Th1-like polarization by the loss of Ikaros. Moreover, the specific mouse model used by the author, *Ikzf1*$^{f/f\ dLck\text{-}3779Cre}$, allows the deletion of Ikaros in SP cells and it would seem that the deletion of a gene under the distal Lck-promoter-3779 is not 100% complete in peripheral CD4⁺ cells (Zhang et al., 2005a). Indeed, as show by Lyon de Ana and colleagues, peripheral CD4⁺ cells seem to be composed by a pool of cells that in majority do not express Ikaros but in little percentage express Ikaros (Lyon De Ana et al., 2019), suggesting that the results of the microarray analysis could be biased by the presence of little amounts of Ikaros.

7.4 Association between Ikaros mutations and human diseases

As I described above, the lack of Ikaros is mostly associated to T-ALL development in mice. However, in humans, *IKZF1* gene defects are much less frequent in T-ALL and are mostly implicated in B acute lymphoblastic leukemia (B-ALL) where they are considered important for the stratification of the risk and the therapy choice (Marke et al., 2018).

IKZF1 mutations have also been associated to common variable immunodeficiency (CVID). Interestingly, mutations on different residues within the same Ikaros domain can have different outcomes. As an example, an heterozygous mutation in the N159 residue, in the ZF2, leads to the transcription of a DN Ikaros isoform

that was described in 7 unrelated CVID patients and this mutation affects B and T cell function and the myeloid cell development (Boutboul et al., 2018). However, the substitution of an arginine for an histidine at codon 167 (H167R), in the same ZF, is associated to a loss of function activity (Kuehn et al., 2016; Dieudonné et al., 2019). Indeed, it would seem that the structure of the Ikaros protein is much more complex and fine regulated that the one described until now.

GWAS analysis associated the presence of *IKZF1* SNPs to Inflammatory Bowel Disease, type 1 diabetes and systemic lupus erythematosus (SLE), however these associations are not clear yet and are to be confirmed (Franke et al., 2010; Jostins et al., 2012; Bentham et al., 2015).

Chapter 8

Study aim

Ikaros is an important TF that regulates the differentiation and development of T cells (Powell et al., 2019). As an example, Ikaros plays a critical role in the Th1/Th2 balance as it has been shown to promote the generation of Th2 cells, while inhibiting the Th1 cell differentiation (Quirion et al., 2009; Thomas et al., 2010). Its role in Th17 cell polarization has been investigated by several groups, but it still is a matter of debate. Indeed, opposing effects on IL-17 expression have been observed, depending on the mouse model of *Ikzf1* used and the time of its deletion. Using different mice models, before my arrival in the laboratory, my colleagues found that Ikaros is essential to promote IL-17 expression. Strikingly, they also observed that during cTh17 polarization, Ikaros loss leads to an increase of pathogenic gene expression, that include *Csf2*, encoding GM-CSF, *Ifng*, and *Il3*. GM-CSF, in particular, is an old cytokine which expression can be regulated by different TFs, and that over the late years has been considered to have a crucial role in inflammation (Hamilton, 2019). The aim of my thesis is to give more insight in how Ikaros regulates the fate of T lymphocytes and more precisely, three main questions constitute the axis of the research project of my thesis:

1) How Ikaros regulates the IL-17 expression?

2) What are the requirements for Ikaros deficient T cells to acquire the pathogenic profile?

3) How does Ikaros regulate pro-inflammatory genes expression, especially *Csf2*?

4) Are these Ikaros deficient cells having a pathogenic function *in vivo*?

Part III

Manuscript

CD4$^+$ T cells require Ikaros to restrain STAT5 and NFκB-dependent GM-CSF expression and their pathogenic phenotype

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ABSTRACT

Th17 cells are important mediators against extracellular pathogens but they also contribute to the onset of autoimmune and inflammatory diseases depending on their degree of pathogenicity. The factors modulating Th17 cell pathogenicity, however, remain poorly understood. Here we show that, under conventional Th17 polarizing conditions, naive Ikaros null $CD4^+$ T cells do not develop normally into IL-17^+ cells, but instead express genes associated with high pathogenicity (eg. *Csf2*, encoding GM-CSF, *Il3*, *Ifng*), and produce significantly higher levels of GM-CSF compared with WT cells. Interestingly, only T cell receptor and CD28 co-receptor signaling are required for high GM-CSF production in the mutant cells, which drive them towards a pathogenic Th1 phenotype. Moreover, we show that Ikaros antagonizes STAT5 and NFκB-induced GM-CSF expression, upon T cell activation. High throughput analysis in TCR/CD28-activated cells shows that Ikaros binding correlates with less of chromatin accessibility and repression of gene transcription. These data highlight an essential function of Ikaros in repressing pathogenic gene expression and shaping the cellular response to T cell activation and polarization.

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INTRODUCTION

Upon antigen recognition and depending on the cytokine context, naive $CD4^+$ T cells develop into distinct effector populations. In the presence of IL-6 and transforming growth factor beta 1 (TGFβ1), $CD4^+$ T cells are driven by the transcription factor RAR-related orphan receptor γt (RORγt) to become IL-17-secreting Th17 cells *(1, 2)*, which play a key role in clearing fungi and extracellular bacterial infections. $IL\text{-}17^+$ cells are described as conventional (c) or "non-pathogenic" Th17 cells, as they do not induce tissue inflammation and may play a role in gut homeostasis *(3)*, and because they possess immunoregulatory functions through IL-10 secretion *(4)*. However, with the inclusion of IL-23, or in the presence of IL-23, IL-1β and IL-6, $CD4^+$ T cells differentiate into "pathogenic" (p)Th17 cells that can promote autoimmunity *(5)*. pTh17 cells have been reported to induce murine experimental autoimmune encephalomyelitis (EAE) via their production of pro-inflammatory cytokines like granulocyte-macrophage colony stimulating factor (GM-CSF) *(6-9)*. Thus, Th17 cells appear to be heterogenous and possess different functional characteristics. In addition, Th17 cells can also be converted to other types of effector cells (eg. Th1, Th2, Tr1, Tfh and Treg) when exposed to different environmental cues, suggesting cellular plasticity *(10-13)*. Nonetheless, the factors and pathways that shape the identity of Th17 cells are only partially understood.

The transcription factor Ikaros, encoded by the *Ikzf1* gene, is a zinc finger DNA binding protein that exerts broad functions in T cells. In the thymus, it affects dynamic gene expression changes and establishes epigenetic patterns in developing T cells *(14-17)*. In the periphery, Ikaros negatively regulates T cell responsiveness to TCR stimulation *(18)*, and promotes Th2 cell polarization at the expense of Th1 cell development *(19-21)*. Its role in Th17 cell differentiation is less clear. IL-17 expression by cTh17-polarized cells is severely blunted in germline Ikaros null mice *(22)*, but inconsistently affected in animals where Ikaros is deleted in peripheral T cells *(23)*. In contrast, IL-17 production is enhanced in Th17 cells from mice with a targeted deletion of the fourth DNA-binding zinc finger (ZnF4) of Ikaros *(24)*. Thus, Ikaros may promote or repress cTh17 cell development depending on the *Ikzf1* mutation and/or time of deletion. In addition, Ikaros deficient Th17 cells exhibit increased mRNA levels of *Csf2*

(encoding GM-CSF) or higher production of IFNγ *(23)*, suggesting that Ikaros might also repress some aspects of the pathogenic Th17 cell phenotype.

Here, we studied the role of Ikaros in T cell activation and T cell polarization towards Th17 cells using multiple mouse models and cellular approaches. Our results indicate that Ikaros is essential to repress pathogenic gene expression and shape the fate of $CD4^+$ T cells during T cell activation and Th17 polarization.

RESULTS

Ikaros represses a pathogenic gene expression program in CD4$^+$ T cells

To address the role of Ikaros in Th17 polarization, we studied Ik$^{f/f}$ CD4-Cre$^+$ (TKO) mice where *Ikzf1* is selectively deleted in T cells (**Fig. S1A**) *(14)*. Purified naive CD4$^+$CD25$^-$CD44$^-$TCR$\gamma\delta^-$NK1.1$^-$ T (TN) cells from TKO and WT (Ik$^{f/f}$ CD4-Cre$^-$) lymph nodes were stimulated with anti-CD3 and anti-CD28 antibodies (Ab) alone ("Th0 conditions"), or in the presence of IL-6 and TGFβ1 ("Th17 conditions"). Cytoplasmic IL-17 production was analyzed after 3 days by flow cytometry (**Fig. 1A**). In these cultures, the majority of WT cells were induced to make IL-17 under Th17 compared with Th0 conditions (67% vs. 1.2%, respectively), as expected, while TKO cells made little to no IL-17 in either condition. Therefore, naive CD4$^+$ T cells cannot produce IL-17 in response to Th17 cell polarization without Ikaros.

To begin to understand the Th17 response in the absence of Ikaros, we evaluated the gene expression profiles of WT and TKO naive CD4$^+$ T cells, cultured in Th17 conditions (and in the presence of anti-IFNγ and anti-IL-4 neutralizing Abs to block indirect effects), over time (days 0, 1 and 2), by microarray analysis. These experiments revealed that WT and TKO cells responded similarly in general to Th17 conditions, as the majority of the up- (>3000) and downregulated (>1500) genes overlapped between genotypes at days 1 and 2 (FC>1.42) (**Fig. S1, B and C**). Still, there were clear differences. Principal component analysis (PCA) revealed that WT and TKO cells were distinct at every timepoint (**Fig. 1B**). For example, among the upregulated genes, Th17-associated genes were less upregulated in TKO cells with time, like *Il17a* and *-f*, *Il21* and *Rorc* (clusters 11, 12) (**Fig. S1B**). *Il17a* and *Rorc* mRNA levels were confirmed by qRT-PCR (**Fig. S2D**). Cell cycle-related genes were also less induced in TKO cells (cluster 18) (**Fig. S1, B and E**). Among the downregulated genes, a high number were ectopically expressed in TKO cells at day 0 but they were downregulated in Th17 conditions, like WT (clusters 4, 5), including the gene encoding Aiolos (*Ikzf3*) (**Fig. S1C**).

Interestingly, three clusters of genes were ectopically expressed in Th17 conditions in the absence of Ikaros (**Fig. 1C**). Cluster 1 contained genes that were constitutively expressed in naive TKO CD4$^+$ T cells and remained expressed over time; it included genes encoding Helios (*Ikzf2*) and NFATc1 (*Nfatc1*). Clusters 2 and 3 contained genes that were similarly regulated between WT and TKO cells at day 0, but

were strongly induced in the mutant cells at day 1 or 2; they included genes encoding pro-inflammatory cytokines (eg. *Ifng*, *Csf2*, *Il1a*, *Il3*, *Il13*, *Il22*, *Ccl3*, *Ccl4*) or their receptors (eg. *Il23r*, *Cxcr3*) (**Fig. 1, C-E**). On the other hand, the anti-inflammatory cytokine gene *Il10* was induced but less upregulated in TKO cells (cluster 12) (**Figs. S1B and 1D**).

Since pro-inflammatory cytokines are characteristic of pathogenic Th17 cells, we performed a gene set enrichment analysis (GSEA) with the most differentially expressed genes between TKO and WT samples, and compared them with genes highly expressed in pTh17 or cTh17 cells *(7)*. This showed a strong and direct correlation between TKO T cells and pTh17, but not cTh17, cells (**Figs. 1F and S2A**), suggesting that TKO $CD4^+$ T cells resemble pTh17 cells after stimulation. We also compared TKO cells with the recently described Th-GM cells *(25)*, and found a strong correlation between the genes upregulated in TKO and those expressed in Th-GM cells (**Fig. S2B**).

Collectively, these results indicated that activated $CD4^+$ T cells cannot produce IL-17 and enter a pro-inflammatory gene program in the absence of Ikaros.

Ikaros is required in $CD4^+$ T cells to coordinate cytokine responses

To determine if Ikaros is required early during thymocyte differentiation, or late during the polarization process, we deleted the *Ikzf1* alleles in $Ik^{f/f}$ R26-$CreERT2^+$ (IKO) and $Ik^{f/f}$ R26-$CreERT2^-$ (WT) mice, injected with tamoxifen for 3 days, followed by 3 days of rest (**Fig. S3A**). This protocol did not alter the naive $CD4^+$ T cell compartment in numbers or phenotype (**Fig. S3B**). Naive $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 Abs, in the absence or presence of IL-6 and increasing concentrations of TGFβ1, and IL-17 production was measured 3 days later (**Fig. 2A**). WT IL-17^+ cells were easily detected in wells with low concentrations of TGFβ1 (0.0017-0.03 ng/ml), and their numbers decreased as TGFβ1 concentrations went up, as expected *(26)*. In contrast, IKO IL-17^+ cells were barely detected in all conditions, similar to TKO cells. Thus, naive peripheral $CD4^+$ T cells need Ikaros to become Th17 cells.

High TGFβ1 concentrations induce the differentiation of $Foxp3^+$ regulatory T (Treg) cells *(27)*, and naive $CD4^+$ T cells may preferentially differentiate into Treg cells in the absence of Ikaros. We therefore measured the appearance of $Foxp3^+$ T cells in Th17 conditions, as described above. This showed that $Foxp3^+$ cells were produced in

the WT samples at the higher TGFβ1 concentrations (0.5-8 ng/ml), but they were not observed in the IKO wells (**Fig. S3C**). Thus, Ikaros is required for the appearance of both Th17 and Treg cells following IL-6 and TGFβ1 stimulation.

As the mRNA of pro-inflammatory cytokines were upregulated in the Ikaros KO T cells, we evaluated GM-CSF production in naive IKO CD4$^+$ T cells stimulated in Th0 or Th17 conditions. Strikingly, more IKO cells made GM-CSF, and more GM-CSF was made per cell, regardless of stimulation, and at all concentrations of TGFβ1 tested, compared with WT (**Fig. 2, B and C**). IKO CD4$^+$ T cells also produced more IFNγ (**Fig. S3D**), confirming a general deregulation of pro-inflammatory cytokine production in the absence of Ikaros. To determine if these results were due to Ikaros, or the indirect effects of IFNγ and IL-4 produced in these cultures *(28)*, we performed the experiments in the presence of neutralizing Abs against these cytokines. This treatment inhibited endogenous IFNγ production (**Fig. S3E**), but there was no detectable effect on the generation of IKO IL-17$^+$, Foxp3$^+$, or GM-CSF$^+$ cells (**Fig. S3, F-H**). Thus, Ikaros promotes IL-17 and inhibits pro-inflammatory cytokine production during Th17 polarization.

In the course of these experiments, we noticed more cell death in the Ikaros null cultures, a process that was reduced by the addition of the pan-caspase inhibitor QVD-OPh (**Fig. S4A**), suggesting death by apoptosis. Since *Fasl* mRNA was upregulated in stimulated TKO T cells (**Fig. 1C**), we asked if FasL signaling promoted Ikaros KO cell death, and perhaps the overproduction of GM-CSF$^+$ cells, since this pathway is associated with the production of pro-inflammatory cytokines *(29)*. To address these questions, we first measured *Fasl* mRNA in naive WT and IKO CD4$^+$ T cells after 0-2 days of culture in Th0 or Th17 conditions; *Fasl* mRNA was easily detected in the IKO cells within 1 day of stimulation in either condition compared with WT (**Fig. S4B**). Surface FasL was also increased on IKO cells (**Fig. S4C**). We then cultured naive WT and IKO CD4$^+$ T cells in Th0 conditions in the absence or presence of anti-FasL neutralizing Abs, and analyzed the cells for annexin V and propidium iodide positivity after 2 days. IKO cells had significantly less annexin V$^+$ cells when FasL signaling was blocked (**Fig. S4D**). To determine if FasL deregulation promotes GM-CSF expression in Ikaros null cells, we cultured naive WT and IKO T cells in Th0 or Th17 conditions, in the presence of anti-FasL Abs, and measured GM-CSF expression. Interestingly, while the anti-FasL Ab blocked the appearance of WT GM-CSF$^+$ cells, as expected, it had

little effect on IKO cells (**Fig. S4E**). Indeed, the number of IKO GM-CSF$^+$ cells seemed to increase when FasL signaling was blocked. Thus, Ikaros promotes cell survival by preventing *Fasl* upregulation, but blocks pro-inflammatory cytokine production via a different mechanism. In addition, QVD-OPh was included in the experiments below, unless specified, to maintain cell viability.

To determine if Ikaros is directly required during T cell polarization, we deleted *Ikzf1* in vitro, during the culture period, CD4$^+$ T cells of untreated WT and IKO mice. Cells were cultured in Th0 or Th17 conditions, and simultaneously treated with 4-hydroxytamoxifen (4-OHT) or ethanol (EtOH). Ikaros, GM-CSF and IL-17 expression were evaluated after 1-3 days of culture (**Fig. S5**). In these cultures, loss of Ikaros was observed in the large majority of cells after 2 days of 4-OHT. GM-CSF was easily detected in the Ikaros$^{lo/-}$ IKO cells after 3 days of Th0 or Th17 stimulation compared with Ikaros sufficient cells (**Fig. S5A**), though it was less robust in the Th17 cultures, due probably to the inhibition of GM-CSF production by exogenous TGFβ1 *(9)*. In contrast, IL-17 production decreased with Ikaros loss in the IKO cells, starting at day 2 in Th17 conditions (**Fig. S5B**). These results indicated that Ikaros loss immediately results in the disappearance of cTh17 and the appearance of pTh17 cells. In a complementary experiment, we retrovirally expressed Ikaros (and GFP) or GFP only (pMIG) in naive WT and TKO CD4$^+$ T cells during Th0- or Th17-inducing conditions (**Fig. 2D**). In WT cells cultured in Th17 conditions, ectopic Ikaros slightly enhanced IL-17 production but had no effect on GM-CSF expression. In TKO cells, however, Ikaros re-expression rescued IL-17 production in Th17 conditions, and reduced GM-CSF in both Th0 and Th17 conditions. These results demonstrated that Ikaros promotes cTh17 polarization even in cells where Ikaros was deleted in the thymus.

To determine if T cell homeostasis in vivo is affected by Ikaros, we analyzed the T cell populations in TKO mice. Mutant mice showed decreases in total LN cellularity, and CD4$^+$ and CD8$^+$ T cell numbers, compared with WT (**Fig. S6, A and B**), though splenic T cells seemed unaffected. The frequencies of CD4$^+$ T cells were also lower. In contrast, B cell numbers were unchanged, but their frequencies were higher in the TKO LNs (**Fig. S6C**). In addition, naive TKO CD4$^+$ T cells were significantly decreased in numbers and frequencies compared with WT (**Fig. S6, D and E**), while TKO effector CD4$^+$ T cells were increased in frequencies (**Fig. S6F**). Interestingly, IL-7Rα levels were reduced on naive TKO CD4$^+$ T cells (**Fig. S4G**), suggesting a possible defect in cell survival. To determine if Ikaros null effector T cells produced more pro-

inflammatory cytokines in vivo, we evaluated the production of IL-17 and GM-CSF in freshly isolated WT and TKO cells. These experiments indicated that more LN and splenic TKO CD4$^+$T cells, and not CD8$^+$, were consistently and selectively positive for GM-CSF (**Figs. 2E and S7A**). On the other hand, IL-17 production was increased only in frequency in TKO LN CD4$^+$ T cells (**Fig. S7B**). These results suggested that Ikaros deficiency is closely associated with an increase in GM-CSF production in vivo.

Altogether, our results showed that naive CD4$^+$ T cells can become IL-17- or GM-CSF-producing cells depending on the stimulation and their intrinsic levels of Ikaros -- IL-17 expression requires high Ikaros levels, while GM-CSF is induced in cells that have lost Ikaros.

GM-CSF is produced by a specific CD4$^+$ T cell population in Ikaros null cells

Because GM-CSF is induced in Ikaros null cells cultured in Th0 conditions, we evaluated its activation requirements. Naive WT and IKO CD4$^+$ T cells were isolated from tamoxifen-treated mice, and cultured in the absence of TCR stimulation (but maintained in IL-7 to help survival), with anti-CD3 Abs alone, or with anti-CD3 + anti-CD28 Abs. GM-CSF and IL-17 levels were measured over 3 days (**Figs. 3A and S8A**). In the absence of TCR stimulation, WT and IKO cells did not make GM-CSF nor IL-17. Indeed, cells from either genotype did not make IL-17 in any of these conditions. However, some WT cells were GM-CSF$^+$ after 3 days of anti-CD3 + anti-CD28 stimulation. Strikingly, the great number of IKO cells made GM-CSF in response to either anti-CD3, or anti-CD3 + anti-CD28, at day 3. These results indicated that Ikaros null T cells needed only TCR stimulation to make GM-CSF, while WT cells required co-stimulatory CD28 signals, suggesting that Ikaros is required to set the activation threshold for pro-inflammatory cytokine production in CD4$^+$ T cells.

To determine why Ikaros null T cells respond so differently to TCR and CD28 stimulation, we evaluated the transcriptomes of naive WT and TKO CD4$^+$ T cells activated for 0-2 days in Th0 conditions, by RNA-sequencing. Three samples were analyzed per condition. Principal component analysis (PCA) of the expressed genes indicated that, globally, freshly isolated WT and TKO cells resembled each other, while activated cells of both genotypes clustered together (PC1; **Fig. 3B**), indicating that cell activation causes the biggest differences in gene expression. Among the upregulated genes upon T cell activation (**Fig. S8B**), some clusters (ie. 14, 18, 19) were less induced in the mutant cells. Similar results were observed among the downregulated

genes (**Fig. S8C**), where genes from a few clusters were less downregulated (ie. 7, 9-11) in TKO cells. WT cells were also distinct from TKO cells (PC2; **Fig. 3B**). Differentially expressed genes that were upregulated only in the TKO cells included those that were also upregulated in Th17 conditions, such as genes encoding pro-inflammatory cytokines or their receptors (eg. *Csf2*, *Csf1*, *Ifng*, *Il23a*, *Il3*, *Il1a*, *Il15ra*, *Il2rb*), *Nlrp3*, associated with the inflammasome pathway, and *Foxo3* (**Fig. 3, C and D**). *Tbx21*, encoding Tbet, an activator of Th1 polarization, was also upregulated. In direct correlation, metascape analysis revealed the top pathways to be associated with cytokine signaling, actin cytoskeleton organization, and migration (**Figs. 3E and S9**).

We also observed that cell cycle-related genes responded slower to activation, and were less upregulated, in Ikaros null cells compared with WT (cluster 18; **Fig. S8B**). This suggested that the proliferative response is impaired without Ikaros. To investigate this issue, we stimulated naive $CD4^+$ T cells from tamoxifen-treated WT and IKO mice in Th0 and Th17 conditions, and measured their division rates and kinetics of cytokine production. These results showed that IKO cells divided less than WT cells in both conditions, a phenotype that was most striking in Th0 conditions, where the majority of Ikaros null cells divided 2 cycles less than WT cells (**Fig. S10A**). To correlate cytokine production with cell cycle, we evaluated the percentage of GM-CSF^+ and IL-17^+ cells at every division after a 3 day culture. This showed that GM-CSF was detected in low-proliferating, while IL-17 was observed in high-proliferating, IKO cells (**Fig. S10, B and C**), and reveal that IL-17 and GM-CSF are produced by different cell populations.

Together, these results indicate that Ikaros represses the appearance of a specific and distinct pro-inflammatory cytokine-producing $CD4^+$ T cell population in response to activation.

Loss of Ikaros alters the T cell epigenome

To determine how Ikaros influences chromatin accessibility, we studied the epigenome of WT and TKO $CD4^+$ T cells after Th0 activation over a 2-day period, by ATAC-sequencing. Three samples were analyzed for each condition. A total of 158,327 ATAC-seq peaks was identified for all conditions combined. The chromatin accessibility signatures of the different populations were analyzed by PCA (**Fig. 4A**), which separated the samples along lines similar to their RNA-seq profiles (**Fig. 3B**). Most of the ATAC-seq peaks were similar between WT and TKO chromatin (**Fig. 4B**),

suggesting that they were Ikaros-independent. A small percentage showed increased or decreased accessibility in the TKO samples compared with WT, but the ratios of up vs. down were similar between different days of culture (**Figs. S11A and 4C**). The genes with increased ATAC-seq peaks over time included many encoding pro-inflammatory cytokines, and *Fasl* (**Fig. S11B**). Most of the peaks were found in gene introns or intergenic regions, regardless of condition, and this was not changed with the loss of Ikaros (**Fig. S11C**). The majority of the regions with increased accessibility was correlated with genes whose expression levels were increased in the TKO cells (**Figs. S11D and 4D**), and included, among others, pro-inflammatory genes. Interestingly, the up and down changes in ATAC-seq peak signals were frequently observed within the same locus, as was observed for the *Csf2*, *Fasl*, *Il3* and *Ifng* genes (**Figs. 4E and S11E**), highlighting the complexity of gene regulation. Some of these changes were seen at day 0 in freshly isolated cells, while others were induced upon Th0 activation. These results indicated that increased chromatin accessibility correlates with increased gene expression in Ikaros null cells.

To determine if specific biological pathways are affected by Ikaros loss, we evaluated the ATAC-seq peaks that were greatly increased or decreased (Log_2FC TKO/WT >+2 or <-2; padj$\leq$0.01) in these samples. Gene ontology analysis revealed that few pathways were significantly altered at d0 and after one day of activation (**Fig. S12A**). However, the genes associated with the STAT5 and NFκB pathways displayed some of the highest increases in ATAC-seq peak signals after 2 days of activation (**Fig. 4F**). We then searched for Ikaros, STAT5 and NFκB binding motifs under the peaks that were either increased (TKO), or decreased (WT), in TKO chromatin compared with WT. This showed that, under the peaks that were increased in TKO chromatin, the Ikaros and STAT5 motifs were selectively over-represented at all timepoints, while RelA (p65) motifs were over-represented only upon TCR + CD28 stimulation (**Figs. S12B and 4G**). To determine the amount of overlap between Ikaros and STAT5, and Ikaros and RelA, motifs under the increased (TKO) vs. decreased (WT) ATAC-seq peaks, we quantified the peaks with Ikaros, STAT5, or the combination of Ikaros and STAT5 (**Fig. S12C**), as well as RelA, or the combination of Ikaros and RelA (**Fig. S12D**), at day 0 or day 2 samples. These analyses showed strong enrichment of peaks with both Ikaros and STAT5, or Ikaros and RelA under the peaks that were increased in TKO chromatin, suggesting a molecular significance for overlapping motifs.

Ikaros binding correlates with chromatin closing

To determine if the changes in chromatin accessibility were due to Ikaros, we evaluated Ikaros binding on the chromatin of naive WT cells, freshly isolated or activated in Th0 conditions for 1 day, by ChIP-sequencing. These experiments showed that Ikaros bound largely similar regions in both conditions (**Fig. 5A**), which were concentrated at promoters, introns and intergenic regions (**Fig. 5B**). Interestingly, Ikaros binding was globally reduced in WT cells upon activation (**Fig. 5C**). Further, at regions where Ikaros bound to WT chromatin, we observed a significant increase in chromatin accessibility in TKO cells (**Fig. 5, C and D**), along with increases in gene expression (**Fig. 5E**). These results suggested that Ikaros binding correlates with chromatin compaction and gene repression. Nonetheless, our analysis of the Ikaros-bound regions at loci containing *Csf2*, *Ifng*, *Il7r, Fasl* and *Cish* (positive control for Ikaros binding *(30)*) as well as those containing key regulatory proteins (ie. *Foxo3*, *Tbx21*, *Nfat5*, *Rorc*), did not reveal obvious differences in the levels of ATAC-seq peaks between WT and TKO chromatin (**Figs. 5F and S13**). Thus, we concluded that Ikaros does not directly limit chromatin accessibility to repress a pro-inflammatory phenotype in activated T cells.

Ikaros antagonizes STAT5 and NFκB function in activated T cells

Because Ikaros motifs overlapped with STAT5 and RelA motifs at regions of increased accessibility in Ikaros KO chromatin, we investigated the relationship between Ikaros and these pathways in regulating pro-inflammatory cytokine production. To assess the importance of STAT5, we evaluated the levels of STAT5 and activated STAT5 (p-STAT5) in naive $CD4^+$ T cells from tamoxifen-treated WT and IKO mice, cultured for 2 days in Th0 conditions, by Western blot, which showed that p-STAT5 levels were increased approximately 3-fold in IKO cells (**Fig. 6A**). To determine if increased STAT5 activity influences GM-CSF expression, we cultured these cells in Th0 conditions, and low concentrations of a STAT5 inhibitor (STAT5i). These results showed that both *Csf2* mRNA and GM-CSF levels were reduced with increasing STAT5i concentrations (**Fig. 6, B and C**), suggesting that STAT5 activation plays a role in promoting GM-CSF production in Ikaros null cells.

To evaluate the importance of the NFκB pathway, we first treated WT and IKO cells with an inhibitor of IKK2 (IKK2i), a subunit of the IKK kinase that is required for

the phosphorylation and degradation of the inhibitor of NFκB (IκBα), thereby inducing NFκB activation. Strikingly, IKK2i treatment almost completely inhibited *Csf2* and GM-CSF expression in the activated IKO cells (**Fig. 6, D and E**). Similar results were obtained when we expressed a mutant IκBα protein (IκBαSR), that cannot be phosphorylated, to block NFκB activation in WT and IKO cells (**Fig. 6F**). This showed that GM-CSF production was prevented >7-fold in activated IKO cells expressing IκBαSR (and GFP), suggesting a pivotal role for NFκB activation in boosting GM-CSF expression in the absence of Ikaros.

Together, these results indicated that the STAT5 and NFκB pathways are ectopically activated upon TCR and CD28 activation in Ikaros null T cells.

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DISCUSSION

In this study we reveal a central role for Ikaros in modulating the cellular response to T cell pathogenicity. Ikaros directly promotes cTh17 development from naive $CD4^+$ T cells, and is needed only during the polarization process. Equally important, we show that Ikaros is also required to suppress the development of pro-inflammatory T cells regardless of polarizing cytokines. In the absence of Ikaros, activated T cells in Th17 culture, express a group of pro-inflammatory genes that resemble those expressed by pTh17 and Th-GM cells, but with the sole activation via TCR/CD28, these cells turn towards a pathogenic Th1 profile. Strikingly, the upregulation of *Csf2* mRNA is a prominent feature of Ikaros null cells. Using three high throughput analysis, we show that, upon T cell activation, Ikaros is an essential repressor of gene transcription acting via both chromatin remodeling and binding on target genes, including *Csf2*. With these results, we highlight an essential function of Ikaros in controlling the balance between conventional versus pro-inflammatory/pathogenic T cells in response to antigen.

Our results clarify the role of Ikaros in promoting IL-17 production. Our conclusion is similar to that of a previous study using mice with a germline *Ikzf1* deletion *(22)*, but different from the one obtained with mice missing ZnF4 of Ikaros *(24)*, which might reflect a distinct role of ZnF4 in modulating the IL-17 production. Other Ikaros family proteins have also been implicated in cTh17 cell differentiation. For example, Aiolos has been reported to promote cTh17 development by silencing *Il2* expression *(31)*, while Eos, on the other hand, has been implicated in repressing IL-17 and promoting IL-2 expression in activated T cells *(32)*. Ikaros has been described as a repressor of *Il2* gene transcription via direct binding on the IL2 promoter and chromatin remodeling in anergic $CD4^+$ T cells *(33, 34)*. While we also observe a binding of Ikaros to the *Il2* promoter in TCR + CD28-activated $CD4^+$ T cells (data not shown), our transcriptome data show that *Il2* RNA is similar in WT and KO Th17 cultures. Further investigations will be required to clarify the involvement of IL-2 in the regulation of IL-17 expression by Ikaros.

Our results show that TCR stimulation increases both the number of $GM\text{-}CSF^+$ Ikaros null cells, as well as the level of GM-CSF per cell. Ikaros is probably not required during the early phase of TCR and coreceptor engagement, as *Ikzf1* deletion in vitro results in protein loss only after one day in culture. The exogenous addition of Th17-

polarizing cytokines reduced GM-CSF production in WT cells, a similar result was previously described *(9)*. However, in the absence of Ikaros, TGFβ1 is not able to reduce GM-CSF expression, unless used at very high concentrations. Interestingly, Ikaros loss also leads to an impairment of IL-17 and Foxp3 expression, both depending on the TGFβ1 pathway. Together, these data suggest that Ikaros could modulate the threshold of TGFβ1 signaling. Ikaros has been shown to antagonize the TGFβ1 signals in common dendritic progenitors *(35)*. However, in $CD4^+$ T cells, the role of Ikaros on TGFβ1 signaling remains unclear and it will be interesting to further determine if it can impact the TGFβ1 molecular pathway.

In an attempt to understand the role of Ikaros in T cell responses, without Th17 polarizing cytokines, we found that upon TCR and CD28 activation, the loss of Ikaros also leads to a high expression of *Ifng*, *Foxo3, Tbx21*, and *Eomes* (cluster 7 RNA-seq) along with *Csf2*. Interestingly, these genes that have been described to drive Th1 pathogenicity in an EAE model *(36)*, suggesting that in the absence of IL-6 and TGFβ1, activated $CD4^+$ KO T cells still reach a pathogenic state that is different from pTh17 cells. The analysis of genome-wide binding profile shows that Ikaros binds described enhancers of *Ifng (19, 37)* and *Csf2 (38)* and the promoters of *Foxo3*, *Tbx21 and Eomes* (data not shown). Ikaros has been described as a silencer of *Tbx21* and *Ifng* expression during Th2 polarization *(20)*. However, its role as a repressor for *Foxo3* and *Eomes* expression and the interplay between Ikaros and these transcription factors for T cell pathogenicity remains to be investigated.

One important question is whether cTh17 and pro-inflammatory T cells derive from common precursors. The present results do not exclude either possibility. *Ikzf1* deletion in vitro leads to a rapid disappearance of $IL\text{-}17^+$ cells which occurs one day before the appearance of $GM\text{-}CSF^+$ cells. This difference in timing is compatible with our interpretation that IL-17 production directly depends on Ikaros, while GM-CSF expression happens only after Ikaros is lost. The fact that IL-17 and GM-CSF are detected following different types of stimulation (Th17 vs. Th0 conditions) suggests that the mechanisms by which Ikaros regulates their gene expression are distinct. Moreover, our data show that, while IL-17 production is concomitant with T cell proliferation, GM-CSF production in KO cells coincide mainly with cells with a low proliferative index, suggesting again different requirements for IL-17 and GM-CSF production. Finally, Ikaros re-expression in KO cells is able to restore the conventional

program of Th17 cell differentiation, indicating that loss of Ikaros does not lead to irreversible CD4$^+$ T cell pathogenicity.

The strong expression of GM-CSF is a recurrent characteristic in Ikaros null CD4$^+$ T cells, but how does Ikaros inhibit GM-CSF production? Our results suggest that it might act at least at three different levels. First, Ikaros binds on the CNSa sequence (+34 kb from the TSS), a distal regulatory sequence enhancing the *Csf2* promoter activity *(38)*. NFκB and BRG1, a SWI/SNF remodeling ATPase, have also been shown to bind on the CNSa sequence and enhance *Il3* and *Csf2* transcription *(38)*. Ikaros is already present in this region in naive CD4$^+$ T cells and remains present upon TCR/CD28 activation. It is therefore tempting to speculate that the binding of Ikaros to the CNSa region might disturb the recruitment of NFκB and BRG1, occurring upon activation *(38)*, leading to a tight control of GM-CSF expression. Second, we clearly demonstrate that STAT5 and NFκB activities are required for the strong expression of GM-CSF expression in KO cells. While the role of Ikaros on NFκB activation remains to be determined, Ikaros seems to repress, directly or indirectly, STAT5 phosphorylation, suggesting that it is able to exert a control on signaling cascades leading to STAT5 activation. As Ikaros binds to the CNSa, close to sites binding STAT5 *(39)* and NFκB *(38)*, it could potentially disloge these TFs from their binding sites. However, we do not detect any binding of Ikaros on the *Csf2* promoter or the enhancer located -1.5kb from the promoter, ruling out a potential competition of Ikaros, STAT5 and NFκB in these regions. Third, we found that the loss of Ikaros results in an increased chromatin accessibility at the enhancer (-1.5 kb from the murine *Csf2* promoter), where NFAT, and AP1 can bind *(40)*. Ikaros is not present on this enhancer. However, by binding to distal regulatory regions, it could recruit the chromatin remodeler Mi2β, the NuRD remodeling ATPase *(41)* and Polycomb Repressive Complex (PRC2) *(15)*, and bring them to the enhancer to impede the chromatin accessibility. Thus, Ikaros could block the access of critical transcription factors on the enhancer of *Csf2* by closing the chromatin in this critical region.

How do the GM-CSF-producing T cells observed here fit into the current descriptions of pathogenic T cells? Our transcriptome and RNA-seq results suggest that Ikaros negatively regulates the pathogenic gene expression programs of pTh17, Th-GM and pTh1 cells. T cells expressing high levels of GM-CSF have been found in the cerebrospinal fluid of multiple sclerosis patients, and they have been shown to be

pathogenic in murine EAE models *(25, 42, 43)*. Moreover, recent studies highlighted GM-CSF as an essential cytokine defining a Th subset playing a key role in neuroinflammation in patients with MS *(44, 45)*. It will, thus, be important to evaluate the functional consequences of Ikaros deficiency in models of autoimmunity. Finally, single-nucleotide polymorphisms in the *IKZF1* gene have been associated with autoimmune diseases like systemic lupus erythematosus, inflammatory bowel disease and primary Sjögren's syndrome *(46-49)*. It is thus possible that genetic variations leading to changes in Ikaros levels may contribute to the pathogenic program in these patients.

MATERIALS AND METHODS

Mice and ethics statement

Ikaros$^{f/f}$ Cre^{-}, Ikaros$^{f/f}$ CD4Cre^{+} mice and Ikaros$^{f/f}$ Rosa26-CreERT2^{+} mice were previously described and designated here as WT, TKO and IKO, respectively *(14)*. Although TKO mice die from T-cell acute lymphoblastic lymphomas/leukemias (T-ALL) at 4-6 months of age, the animals used here (5-8 weeks-old) showed no signs of transformation in the peripheral organs, as defined by CD4 and CD8 profiling, T cell receptor Vα and Vβ chain usage, and the absence of a deregulated Notch pathway *(14)*. Inducible deletion of the floxed alleles of *Ikzf1* was performed by injecting adult IKO mice intra-peritoneally with tamoxifen (75 mg/kg; T5648, Sigma-Aldrich) dissolved in sunflower oil every day for 3 days followed by 3 days of rest. TKO mice were 5-8 weeks old and IKO mice were 5-12 weeks old, backcrossed 5-12 generations on C57BL/6; all gave similar results. Mice were bred and maintained in SPF conditions until use. Animal procedures were approved by the IGBMC ethics committee and the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (2012-096 and APAFIS#21777-2019082318358785v3).

Antibodies, cytokines and reagents

Anti-CD4-AF700 (RM 4-5), anti-CD44-FITC (IM7), anti-CD62L-PECF594 (MEL-14), anti-GM-CSF-BV421 (MP1-22E9), anti-IFNγ-PECF594 (XMG 1.2), anti-NK1.1-PE (PK136), anti-TCRγδ-PE (GL3), anti-FasL (MFL3), anti-TCRβ-PerCPCy5.5 (H57-597), anti-CD16/CD32 (2.4G2), purified neutralizing anti-IL-4 (11B11) or anti-IFNγ (XMG1.2) Abs and GolgiPlug^{TM} were from BD Biosciences. Anti-CD8α-PerCPCy5.5 or APC-Fire 750 (53-6.7), anti-CD25-PE (PC61.5), anti-IL7Rα (A7-R34), purified anti-CD3 (2C11), anti-CD28 (37.51), neutralizing anti-FasL (MFL4) Abs, the control isotype for anti-FasL Ab (clone HTK888), and Annexin V-FITC were from Biolegend. Anti-IL-17-eF660 (eBio17B7), anti-CD19-AF700 (1D3), and anti-Foxp3-FITC (FJK-16S) Abs were from eBiosciences, anti-Ikaros (A3; rabbit polyclonal Ab against the Ikaros C-terminus) was produced in-house. Goat anti-Rabbit IgG (H+L) ($F(ab')_2$ fragment)-AF488 was from Jackson ImmunoResearch. Murine rIL-6 (IL-6) (216-16) and human rTGFβ1 (hTGFβ1) (100-21C) were from Peprotech. DAPI (D1306) was from Invitrogen. Phorbol 12-myristate 13-acetate (PMA; P8139), 4-hydroxytamoxifen (H-7904) and propidium

iodide (81845) were from Sigma-Aldrich. Ionomycin was from Life Technologies and QVD-OPh (A1901) was from ApexBio. IKK2 inhibitor (TPCA-1; T1452) and STAT5 inhibitor (573108) were from Sigma-Aldrich and Calbiochem, respectively.

DNA constructs

The murine IκBα super repressor (IκBαSR) contains mutations of Serines into Alanines, preventing its phosphorylation and thereby its degradation *(50)*. IκBαSR was subcloned prom pCDNA3.1 *(51)* into the pMIG vector, upstream of the IRES-GFP sequence, using the restriction enzyme EcoRI. The murine Ikaros was subcloned into the pMIG vector, upstream of the IRES-GFP sequence using the restriction enzymes XhoI and EcoRI.

Th0 and Th17 cultures

Cell suspensions were prepared from total (mesenteric and peripheral) lymph nodes and cells were stained with anti-CD16/CD32 blocking Abs, anti-CD4, anti-CD8, anti-CD44, anti-CD25, anti-NK1.1 and anti-TCRγδ Abs in 1X PBS 10% FCS for 15 min. Naive $CD4^+$ T cells ($CD4^+CD8^-CD44^{hi}CD25^-NK1.1^-TCR\gamma\delta^-$) were sorted on either a FACSAria™ II SORP or a FACS ARIA™ FUSION (BD Biosciences) cell sorter. Sort purity was >98%. Alternatively, $CD4^+$ T cells were enriched using the "Dynabeads untouched mouse CD4 cell" kit (Invitrogen), stained with anti-CD44, anti-CD25, anti-NK1.1 and anti-TCRγδ Abs in 1X PBS 10% FCS for 15 min and naive $CD4^+$ T cells were sorted as previously described *(26)*. Both protocols gave similar results for Th17 differentiation. For Th17 differentiation, naive $CD4^+$ T cells ($4x10^4$ cells/well) were activated with anti-CD3 and anti-CD28 (2 µg/ml each) Abs, both pre-coated overnight on a Nunc-immuno 96-well plate (Thermo Scientific), in the absence or presence of IL-6 (10 ng/ml) and various TGFβ1 concentrations (0.0017-8 ng/ml), in 200 µl of IMDM medium supplemented with 10% inactivated FCS, GlutaMAX™-I, Penicillin/Streptomycin, non-essential amino acids, sodium pyruvate, Hepes and β-mercaptoethanol (IMDM 10% FCS) as previously described *(26)*. Similar results were obtained between human and mouse TGFβ1. When mentioned, naive $CD4^+$ T cells were activated with plate-bound anti-CD3 and anti-CD28 (2 µg/ml each), with or without IL-6 (10 ng/ml) and TGFβ1 (0.03 ng/ml) corresponding to Th17 and Th0 culture conditions, respectively. Where indicated, neutralizing anti-FasL Ab (20 µg/ml),

neutralizing anti-IL-4 and anti-IFNγ Abs (10 µg/ml each), the pan-caspase inhibitor (QVD-OPh, 20 µM) and IL-7 (20 ng/ml) were added on naive $CD4^+$ T cells at d0. At day 3, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h. When mentioned, cells were first stained with a Zombie Aqua™ fixable viability dye (Biolegend) according to the manufacturer's protocol and then stained with anti-CD4, anti-CD8 Abs, fixed and permeabilized using the Foxp3 Fixation/Permeabilization kit (eBiosciences), stained with anti-IL-17, anti-GM-CSF, anti-IFNγ and anti-Foxp3 Abs (where indicated) and analyzed on the cytometer LSRII (BD Biosciences).

T cell division

$CD4^+$ naive T cells (2-3 $x10^6$) from LN were sorted, resuspended in 900 µl of IMDM 10% FCS and labelled with CFSE (10 µM) by adding 100 µl of a 100 µM stock with gentle vortex for 10 min at 37°C. CFSE-labelled cells were then washed 3 times with IMDM 10 % FCS and cultured ($4x10^4$ cells / well) in Th0 or Th17 conditions as described above, with QVD-Oph (20 µM). When indicated, at day 3, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h for cytokine expression. Cells were then first stained with a Zombie Aqua™ fixable viability dye (Biolegend) according to the manufacturer's protocol and with an anti-CD4 Ab, fixed and permeabilized using the Foxp3 Fixation/Permeabilization kit (eBiosciences), and stained with anti-IL-17, anti-GM-CSF. CFSE intensity and intracellular cytokines expression were analyzed in live cells, using the cytometer LSRII (BD Biosciences)

Flow cytometry analyses of freshly-isolated cells

Cells were isolated from mesenteric and peripheral LN or spleen and stained with the indicated fluorochrome-coupled Abs in PBS 10% FCS for 15 min on ice, in the presence of an anti-CD16/CD32 blocking Ab. To detect cytokine expression, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h at 37°C, stained with anti-CD4, anti-CD8 Abs for 15 min on ice, fixed and permeabilized using the Foxp3 kit and stained with anti-GM-CSF, anti-IL-17 Abs in the permeabilization buffer for 30 min on ice. Cells were then analyzed on the cytometer LSRII (BD Biosciences).

Retroviral infections

Primary $CD4^+$ T cell infection was performed as described with minor changes *(26)*. For virus production, Platinum-E packaging cells (plat-E) were transfected either with pMIG, Ikaros (Ik-1)/pMIG or IκBαSR/pMIG using lipofectamine 2000 (Invitrogen) in a 10 cm culture dish. One day later, the medium was replaced by 6 ml of IMDM 10% FCS and the retroviral supernatant was harvested after 24 h and filtered in a 0.45 µm filter. This step was repeated for a 2^{nd} harvest and both harvests were frozen at -80°C. For retroviral infection, naive $CD4^+$ T cells were sorted and directly cultured in Th0 or Th17 conditions in a 96-well NUNC plate at 4 x 10^4 cells/well in 200 µl of IMDM 10% FCS. One day later, the medium was replaced by the retroviral supernatant containing polybrene (8 µg/ml), QVD-Oph (20 µM), with or without IL-6 (10 ng/ml) and TGFβ1 (0.03 ng/ml). Cells were centrifuged 1h at 2000 rpm RT and incubated at 37°C for 3h. This step was then repeated once the same day, the medium was then exchanged with IMDM 10% FCS and QVD-OPh (20 µM) with or without plus IL-6 (10 ng/ml) and TGFβ1 (0.03 ng/ml) and cells were cultured at 37°C. Two days after the infection, cells were stimulated with PMA, ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h, stained with Zombie Aqua dye, anti-CD4 Ab, fixed and permeabilized using the BD Cytofix/Cytoperm kit, and stained intracellularly with GM-CSF and IL-17. Cells were then analyzed on the cytometer LSRII (BD Biosciences).

RT-qPCR

Total RNA was extracted using the RNeasy Plus microkit (QIAGEN) according to the manufacturer's protocol. cDNAs were obtained by using the Superscript IV (Invitrogen) and quantitative PCR was performed using the FastStart SybrGreen system (Roche) with the primers listed in the supplemental Table 1. mRNA expression of the genes of interest was normalized to *L32* mRNA and -fold induction of each condition was calculated.

Western blot

Cells were lysed in 30 µl of lysis buffer *(52)* supplemented with complete protease inhibitor cocktail (11873580001, Roche), phosphatase inhibitor cocktail 3 (P0044, Sigma), SDS 0.1% and Bitnuclease (4 U/µl; B16002, Benzonase® alternative, Biotool)

for 15 min on ice. After centrifugation at 15K rpm at 4°C, the supernatant was harvested and the proteins were separated on a 10% SDS-PAGE and transferred on a nitrocellulose membrane. For STAT5, cells were lysed as above without the addition of SDS and Bitnuclease. Ikaros expression was analyzed using the anti-Ikaros (A3) Ab. Anti-phosphoY694-STAT5 (D47E7) and anti-STAT5 (D206Y) Rabbit mAbs were from Cell Signaling Technologies. Anti-tubulin α (YL1/2) and Anti-β-actin (AC-15) Abs were from Abcam and Sigma-Aldrich, respectively.

Microarray analysis

Purified naive $CD4^+$ T cells (0.5×10^6 / well) from spleen were activated on a 48-well plate, pretreated with Goat anti-Hamster IgG and coated with anti-CD3 (2C11; 1 µg/ml) and anti-CD28 (37.51; 0.25µg/ml) Abs, in the presence of mIL-6 (20 ng/ml), hTGFβ1 (1 ng/ml) and neutralizing anti-IL-4 and anti-IFNγ Abs (2 µg/ml each). A transcriptome analysis was performed using GeneChip Mouse Gene 2.0ST arrays (Affymetrix) with cells cultured for 1 and 2 days as well as with naive $CD4^+$ T cells (day 0). Raw data were analyzed using the software Affymetrix version 1.4.1 and the Robust Multiarray Average (RMA) method. K-means clustering was performed using Cluster 3.0, using genes that were differentially expressed (Log_2 FC > 0.5, corresponding to a FC > 1.42) between WT and TKO cells at any of the timepoints analyzed, or between two timepoints in either WT or TKO cells. Principal component analysis (PCA) were performed using the prcomp function in R software and the figure was performed with the ggplot 2 package. Genes with several probes on the microarray were selected and only probes with the highest FC TKO/WT value was retained. GSEA was then performed using the GSEA 2.0 software after selecting differentially regulated genes in TKO vs. WT T cells by the FCROS method with an error set at 10% *(53)*.

Chromatin ImmunoPrecipitation (ChIP)

The ChIP protocol was performed according to different protocols with some changes *(15, 54)*. Briefly, WT naive $CD4^+$ T cells (10×10^6) and activated for 1 day (9×10^6) were washed in PBS and cross-linked in PBS/0.5%BSA/1% ultrapure formaldehyde (Electron Microscopy Sciences) for 10 min at 37°C. Glycine (125 mM) was adding as a quenching agent and cells were then washed 2 times with 3 ml of cold PBS and permeabilized in 3 ml of cell lysis buffer (0.25%, Triton-X100, 10 mM EDTA, 0.5 mM

EGTA, 10 mM Hepes pH 6.5 and complete proteases inhibitors (Roche)) on ice for 10 min. After centrifugation at 2000 rpm for 10 min, the nuclei pellets were resuspended in 300 µl of SDS lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris pH 8.0 and complete proteases inhibitors. 700 µl of ChIP dilution buffer (0.01%SDS, 1.1%Triton X-100, 1.2 mM EDTA, 16.7mM Tris-HCl pH8.1, 167mM NaCl containing complete proteases inhibitors were then added and samples were sonicated in a 1 ml round-bottom glass tubes (Covaris®) using the Covaris E220 sonicator (Peak incident power 100, Duty factor 50, 200 cycles/burst) for 10 min to generate DNA fragment between 150-300 bp. After sonication, the samples were centrifuged at 14, 000 rpm for 10 min. The supernatant containing the chromatin was pre-cleared with 25 µl of dynabeads magnetic prot A for 1h at 4°C. 2.5 x 10^5 cell equivalent of the sample was kept as the input and the pre-cleared chromatin was then incubated overnight with anti-Ikaros (1/100, homemade). The next day, 25 µl of dynabeads magnetic prot A were added for 5h. The prot-DNA complexes bound to the Ab and the beads were then washed 1x with low-salt buffer (0,1% SDS, 1% TTX, 2mM EDTA, 20mM Tris-Hcl pH8.1, 150mM NaCl + complete protease inhibitors), 1x with high-salt buffer (0,1% SDS, 1% TTX, 2mM EDTA, 20mM Tris-Hcl pH8.1, 500mM NaCl and complete proteases inhibitors), 2x with LiCl buffer (250mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1mM EDTA, 10mM Tris pH8.1 and complete proteases inhibitors) and 1x with TE **(**50 mM Tris pH 8.0, 10 mM EDTA). Samples were then eluted by adding 150 µl of Elution buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS) and incubate at 67°C for 10 min with shaking. This step was repeated twice. The reverse crosslink was performed by the ChIP and input samples with NaCl (200 mM) at 65°C ON. Next morning, proteinase K (80 µg) was added for 1 h at 45°C and DNA was purified using phenol/chloroform and phase lock tubes™ (Invitrogen).

ChIP-sequencing and analysis

Libraries were performed using the MicroPlex Library Preparation kit (#C05010014, Diagenode), following Manufacturer's instructions. The library was sequenced on Illumina 4000 sequencer as Single-Read 50 base reads following Illumina's instructions. Image analysis and base calling were performed using RTA 2.7.7 and bcl2fastq 2.17.1.14. Adapter dimer reads were removed using Dimer Remover (https://sourceforge.net/projects/dimerremover/). Alignment was performed to mm10 mouse genome using Bowtie 1.0.0 *(55)* with the following arguments: -m 1 --strata --

best -y -S -l 40. Peak calling was performed using MACS2 v2.1.1.20160309 with default parameters. The Th0 d1 input was used as the control for peak-calling for the d0 and Th0 d1 conditions. Peaks were annotated with Homer software using Ensembl release 94 gene annotation. In order to find the common peaks bound by Ikaros in the d0 and Th0 d1 conditions, the Ikaros peaks at d0 were intersected with the Th0 d1 Ikaros peaks with the Bedtools program. This comparison was represented with a Venn Diagram. Data were visualized using USCS Genome Browser and seqMINER_1.3.3g. Identification of the ATAC-seq peaks bound by Ikaros at d1 was performed by intersecting all ATAC-seq regions at d1 (present in WT or/and in TKO) with all Ikaros peaks at d1 using the Bedtools program.

Assay for Transposase-Accessible Chromatin (ATAC)

Naive WT and TKO $CD4^+$ T cells were sorted on a FACS ARIA^{TM} FUSION (BD Biosciences) cell sorter. Naive $CD4^+$ WT and TKO T cells (5×10^4) were directly used for the ATAC-seq protocol for the day 0 condition, while the remaining naive WT and TKO $CD4^+$ T cells were activated with coated anti-CD3, anti-CD28 Abs (2 µg/ml) in the presence of neutralizing anti-IL-4 and anti-IFNγ Abs (10 µg/ml). At day 1 and 2, live WT and TKO cells (5×10^4 $DAPI^-$ cells) were sorted on a FACS ARIA^{TM} FUSION (BD Biosciences) and used for the ATAC-seq protocol. Three independent experiments were performed. The ATAC-seq protocol has been performed as previously described (Buenrostro et al., 2013). Briefly, cells were lysed with cold lysis buffer (10 mM Tris-HCl, pH7.4, 10 mM NaCl, 3 mM MgCl2, 0.1 % (v/v) Igepal CA-630) and nuclei were pelleted 10 min at 500g. Nuclei were incubated with the transpose reaction mix for 30 min at 37 °C. Immediately following transposition, the fragmented DNA was purified using Qiagen MinElute PCR Purification Kit and eluted in 10 µL. The transposed DNA fragments was amplified by PCR for 5 cycles. To determine the appropriate number of PCR cycles to add for each condition, a qPCR was performed. The appropriate number of cycles (6-9 cycles) was then added to each sample. PCR fragments were purified two times with SPRIselect (Beckman Coulter, #B23317) and the quality of the purified libraries was analyzed using a Bioanalyzer High-Sensitivity DNA Analysis kit (Agilent).

ATAC-sequencing and analysis.

The libraries were sequenced on Illumina Hiseq 4000 sequencer as Paired-End 100 base reads following Illumina's instructions. Image analysis and base calling were

performed using RTA 2.7.7 and bcl2fastq 2.17.1.14. Data analysis was performed using the Encode ATAC-seq pipeline v1.4.2. Adapter sequences were removed and low-quality ends were trimmed. Sequence alignment was performed into the mm10 assembly of Mus musculus genome using Bowtie2 (version 2.2.6) choosing the zero multi-mapping option. Mitochondrial reads were removed. The Peak calling was performed using MACS2 v2.1.1.20160309. Finally, the conserved overlap peaks were used for the analysis. Peaks from different conditions were merged to form a consensus peak set. The peaks were annotated using annotatePeaks.pl script in Homer program and with Ensembl 94 database. The read coverage for each sample was calculated with multicov function from bedtools program (v2.26.0). Comparisons of interest were performed using the test for differential expression, proposed by Love et al. and implemented in the Bioconductor package DESeq2 version 1.16.1 *(56)*. Motif research for selected peaks specifically significant in the TKO or WT conditions (log_2FC TKO/WT > 2 or < -2 with a padj <0.01) was performed at the meme website using the AME software (http://meme-suite.org) and the ones of interest were represented by bar graph using Illustrator. The same peaks selected for the AME analysis, were also scanned for IKAROS, RelA and STAT5 motifs using FIMO software (http://meme-suite.org). To represent the results, we used Venn Diagrams ((http://meta.chart.com). For the PCA analysis, read counts have been normalized across samples with the median-of-ratios method proposed in DESeq2 package. Principal Component Analysis was computed on variance stabilizing transformed data. ATAC-seq data were represented by Volcano plots using ggplot2 package (R). Data were visualized using USCS Genome Browser, seqMINER_1.3.3g. Pathway analysis were performed at the GSEA website using Hallmark gene set collection in the MSig Datatabase and represented by heatmap using the plotly package (R v3.6.2) *(57-59)*.

RNA-sequencing

At day 0, naive $CD4^+$ WT and TKO T cells (4-6 x 10^6) were sorted on a FACS ARIA™ FUSION (BD Biosciences). Naive $CD4^+$ WT and TKO T cells (1 x 10^6) were used directly and the remaining cells were activated with coated anti-CD3, anti-CD28, and anti-IL-4 and anti-IFNγ antibodies. At day 1 and 2, live WT and TKO cells (1-2 x 10^5 $DAPI^-$ cells) were sorted on a FACS ARIA™ FUSION (BD Biosciences). Total RNA was extracted from using the RNeasy Plus Micro kit (Qiagen) according to the manufacturer's instructions. Libraries were prepared with the Clontech SMART-seq v4

Ultra Low Input RNA Kit for Sequencing and sequenced with HiSeq 4000 (Illumina) with single-end 50 bp read length.

RNA-sequencing analysis

The RNA analysis was done using the GenomEast RNA-seq pipeline version 1.2.2. Reads were preprocessed in order to remove adapters, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using Cutadapt version 1.10 *(60)*. Reads were mapped into the mm10 assembly of Mus musculus genome using STAR version 2.5.3a *(61)*. The reads were aligned across exonic, intronic and intergenic genomic region using annotations from Ensembl 94. Gene expression quantification was performed from uniquely aligned reads using Htseq-count version 0.6.1p1, with annotations from Ensembl version 94 and “union" mode *(62)*. Only non-ambiguously assigned reads have been retained for further analyses. In order to make these counts comparable between samples, read counts have been normalized across samples with the median-of-ratios method proposed by Anders and Huber *(63)*. Comparisons of interest were performed using the test for differential expression, proposed by Love et al. and implemented in the Bioconductor package DESeq2 version 1.16.1 *(56)*. Heatmaps of differentially expressed genes (K-means) were created using Cluster 3 and Java Treeview softwares. Selected genes that were differentially expressed (data normalized and divided by median of transcripts length in kb with a value ≥ 50 and FC ≥ 3.5) between WT and TKO cells at any of the timepoints analyzed, or between two timepoints in either WT or TKO cells. For the GSEA, up- and down-regulated genes in the TKO conditions were selected (pvalue ≤ 0.05 and $\log_2$FC ≥ 2 or ≤ -2). Pathways analysis were performed using the Metascape website *(64)*. For the PCA analysis, read counts have been normalized across samples with the median-of-ratios method proposed in DESeq2 package. Principal Component Analysis was computed on regularized logarithm transformed data. The RNAseq result was integrated into the ATAC-seq data with the same Ensembl gene ID and the data were visualized using ggplot2 (R).

Statistical analysis

Statistical significance was analyzed by a Mann-Whitney test to compare two experimental conditions unless indicated otherwise. Only statistical significances

comparing the two conditions of interest are mentioned, unless indicated otherwise. Graphs and statistics were performed using Prism 7 (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

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FIGURE LEGENDS

Figure 1. Lack of Ikaros induces a pathogenic gene expression program.
(A) Left: representative contour plots of IL-17 and CD4 expression in WT and TKO $CD4^+$ cells stimulated for 3d in Th17 or Th0 conditions. Right: proportion of WT or TKO $IL\text{-}17^+$ cells among $CD4^+$ cells from 3d in Th17 cultures (n=5; mean ± SEM). **(B)** Principal component analysis using expression data (transcriptome) from WT and TKO $CD4^+$ cells at d0 and d1-2 in Th17 cultures. The graph shows the first two principal components. **(C)** Heat maps of 3 clusters of genes that were specifically increased in TKO cells compared to WT (K-means clustering). **(D)** RT-qPCR analysis of *Csf2*, *Ifng*, *Il3* and *Il10* mRNA expression in either naive $CD4^+$ T cells (d0) or naive $CD4^+$ T cells cultured in Th17 polarizing condition with QVD-OPh for 1 or 2 days (n=5; mean ± SEM). **(E)** Metascape analysis of GO term enrichment of genes in cluster 1-3 described in C. **(F)** GSEA enrichment plots of genes enriched in pathogenic Th17 cells (pTh17; top) or conventional Th17 cells (cTh17; bottom) among genes up- or down-regulated by Ikaros at d1. Gene sets correspond to 106 genes enriched in pTh17 cells and 143 genes enriched in cTh17 cells (Lee et al. 2012). NES, normalized enrichment score; FDR, false discovery rate. Statistical significance was analyzed by a Mann-Whitney test (*p ≤0.05 and **p ≤0.01).

Figure 2. Ikaros limits the expression of GM-CSF in Th17 polarizing conditions.
(A) Left: representative graph of the proportions of $IL\text{-}17^+$ cells in activated WT and IKO naive LN $CD4^+$ T cells cultured with anti-CD3 and anti-CD28 antibodies and different amounts of TGFβ1 and constant IL-6 for 3 days, as indicated. Right: graph showing the proportions of WT and IKO $IL\text{-}17^+$ cells among $CD4^+$ T cells in 3d cultures, as indicated (n=5; mean ± SEM). **(B)** Representative contour plot showing IL-17 and GM-CSF expression in WT and IKO $CD4^+$ cells after 3 days of culture in Th0 or Th17 conditions. **(C)** Left: representative graph showing the percentage of $GM\text{-}CSF^+$ cells in WT and IKO LN $CD4^+$ cells cultured as in A. Right: graph showing the proportions of $GM\text{-}CSF^+$ $CD4^+$ T cells (n=5; mean ± SEM). **(D)** Naive LN $CD4^+$ T cells from WT and TKO mice, cultured in Th0 or Th17 conditions for 24h and subjected to 2 rounds of infection with pMIG or Ikaros/pMIG in the presence of QVD-OPh. Representative panel of GFP expression at d3, and expression of IL-17 and GM-CSF in live GFP^+ cells in

Th0 (left) or Th17 (right) condition (n=3). **(E)** Left panels: Representative contour plot of ex vivo GM-CSF-expressing cells in WT and TKO $CD4^+$ T cells in LN and spleen. Middle panel: percentage of $GM\text{-}CSF^+$ cells among $CD4^+$ T cells from LN (n=10) or spleen (n=9) from WT and TKO mice. Right panel: cell counts of $GM\text{-}CSF^+$ cells among WT and TKO $CD4^+$ T cells from LN (n=10) or spleen (n=9) of WT and TKO mice (mean ± SEM). Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01, ***p≤ 0.001).

Figure 3. Ikaros is required to limit GM-CSF expression upon T cell activation by TCR and CD28 signals.

(A) Naive WT and IKO LN $CD4^+$ cells from tamoxifen-induced mice were cultured with IL-7, or activated with anti-CD3 ± anti-CD28 Abs for 1-3 days in the presence of QVD-OPh. Representative contour plot showing GM-CSF and IL-17 expression in live $CD4^+$ cells (n=4). **(B)** Principal component analysis using expression data (RNA-seq) from WT and TKO T $CD4^+$ cells at d0 and d1-2 in Th0 cultures. The graph shows the first two principal components. **(C)** Heat maps of 3 clusters of genes that were specifically increased in TKO cells compared to WT in Th0 cultures (K-means clustering). **(D)** Heat map of a cluster of genes more increased in TKO cells compared to WT in Th0 cultures at d1 and 2 (K-means clustering). **(E)** Metascape analysis of GO term enrichment of genes from cluster 4 in panel D.

Figure 4. Ikaros loss changes the landscape of chromatin accessibility in $CD4^+$ T cells.

(A) Principal component analysis using ATAC-seq data from WT and TKO $CD4^+$ cells at d0 and d1-2 in Th0 cultures. The graph shows the first two principal components. Samples were obtained from three independent experiments. **(B)** Pie chart showing the distribution of ATAC-seq peaks in TKO cells compared to WT cells at d0 and d1-2 in Th0 cultures. Peaks increased in TKO cells (black; Log_2FC TKO/WT>1 and padj≤0.01) or decreased in TKO cells (white; Log_2FC TKO/WT<-1 and padj≤0.01). The peaks that are not significantly changed between WT and TKO samples are shown in grey. **(C)** SeqMINER heat map of all the 87221 ATAC-seq peaks in WT and TKO cells at d2 of Th0 cultures. **(D)** Integration of RNA-seq data with ATAC-seq data from TKO cells compared to WT cells at d2 of Th0 cultures. Only the 8572 peaks with a padj ≤ 0.05 from the ATAC-seq and RNA-seq analysis are shown. Among them, 3576 peaks

have a Log_2FC (TKO/WT) ≥ 1 or ≤ -1 and a Pearson correlation coefficient of 0.58 with a p value< 2.2^{-16}. Genes with a positive (purple) or a negative (black) correlation between ATAC-seq and RNA-seq data are highlighted. **(E)** Genome track view of ATAC-seq profiles of *Csf2* and *Fasl*. The University of California Santa Cruz Genome browser (UCSC) depicts the pooled profile of the three independent experiments in WT and TKO $CD4^+$ T cells at d0 and d1-2 in Th0 cultures. **(F)** Heat map showing GO of the ATAC-seq 4486 peaks significantly increased and 2774 peaks significantly decreased in TKO $CD4^+$ T cells in comparison to WT $CD4^+$ T cells at d2 in Th0 cultures. Peaks significantly increased in the TKO (Log_2FC(TKO/WT) > 2 with a padj ≤ 0.01), and significantly decreased in TKO (Log_2FC(TKO/WT) < -2 with a padj ≤ 0.01). **(G)** Bar graph showing the significant enriched transcription factor motifs identified from AME-MEME motif search in ATAC-seq peaks found in WT and TKO $CD4^+$ T cells at d2 in Th0 cultures. Significant peaks were selected as in G. Only some of the significantly enriched motifs are depicted.

Figure 5. Genome-wide ChIP-seq analysis of Ikaros binding in $CD4^+$ T cells

(A) Venn diagram showing the numbers of all Ikaros ChIP-seq peaks, found with MACS 2, in WT naive $CD4^+$ T cells at d0 and at d1 of Th0 cultures. The input from the condition Th0 d1 was used as a reference for peak calling in naive (d0) and activated $CD4^+$ T cells (Th0 d1). **(B)** Pie chart of the genomic location distribution of all Ikaros peaks in WT naive $CD4^+$ T cells at d0 and at d1 in Th0 cultures. **(C)** SeqMINER heat map showing the Ikaros peaks at d1 distributed in WT naive $CD4^+$ T cells at d0 and d1 of Th0 cultures and in the ATAC-seq signals in both WT and TKO cells (d0 and Th0 d1 conditions). **(D)** Boxplot showing the Log_2FC (TKO/WT) of all ATAC-seq peaks (78378) in TKO versus WT cells bound (26054) or not (52324) by Ikaros in the Th0 d1 condition (Mann-Whitney test, p<2.2^{-16}). **(E)** Integration of ATAC-seq data (Log_2FC TKO/WT) and RNA-seq data (Log_2FC TKO/WT) among the regions directly bound by Ikaros (26054 peaks) at d1 of Th0 culture. Gray dots: ATAC-seq regions with a more significant difference in accessibility between TKO and WT (padj ≤ 0.05) associated with a difference in the RNA-seq expression (padj ≤ 0.05). Red dots: ATAC-seq Log_2FC (TKO/WT) ≥ 1 and an RNA-seq Log_2FC (TKO/WT) ≥1. Blue dots: ATAC-seq Log_2FC (TKO/WT) ≥ 1 and an RNA-seq Log_2FC (TKO/WT) ≤ -1. Black dots: ATAC-seq Log_2FC (TKO/WT) ≤ -1 and RNA-seq Log_2FC (TKO/WT) ≤ -1 or ≥ 1. **(F)** UCSC Genome track view of ChIP-seq, using anti-Ikaros Ab, showing the *Csf2* promoter and

regulatory sequences in WT CD4$^+$ T cells at d0 and d1 of Th0 cultures and ATAC-seq of WT and TKO CD4$^+$ T cells at d0 and d1-2 in Th0 cultures. CNSa and b are shown on the left and a focus on *Csf2* promoter and enhancer is shown on the right.

Figure 6. GM-CSF production in Ikaros-deficient CD4$^+$ T cells depends on STAT5 and NFκB.

(A) Left: Western blot of Ikaros and the phosphorylation of STAT5-Y694 in WT and IKO CD4$^+$ cells cultured 2 days in Th0 conditions with QVD-OPh. Tubulin and STAT5 are shown as loading controls. Right: Ratio of STAT5-pY694/STAT5 in WT and IKO CD4$^+$ T cells at d2 of Th0 cultures with QVD-Oph (n=4; mean ± SEM). **(B)** Naive LN WT and IKO CD4$^+$ cells from tamoxifen-induced mice were cultured in Th0 conditions for 3 days with QVD-OPh in the presence of the STAT5 inhibitor (STAT5i) at indicated concentrations or DMSO as a control. Left: Representative contour plot showing GM-CSF and IL-17 expression in WT and IKO CD4$^+$ cells. Right: Proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 3 days of Th0 culture (n=4-5; mean ± SEM). **(C)** RT-qPCR analysis of *Csf2* mRNA expression in WT and IKO LN naive CD4$^+$ cells cultured in Th0 conditions with QVD-OPh (d2) in the presence of DMSO or STAT5i at indicated concentrations. *Csf2* mRNA expression is represented as -fold induction with respect to the DMSO WT sample. Statistical significance for WT and IKO DMSO condition was analyzed by a Wilcoxon test. **(D)** Naive LN CD4$^+$ cells from tamoxifen-induced WT and IKO mice were cultured in Th0 conditions for 3 days with QVD-OPh in the presence of the IKK2 inhibitor (IKK2i; 2.5 µM) or DMSO as a control. Left: Representative contour plot showing GM-CSF and IL-17 expression in WT and IKO CD4$^+$ cells. Right: Proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 3 days of Th0 culture with DMSO or IKK2i (n=6; mean ± SEM)**. (E)** RT-qPCR analysis of *Csf2* mRNA expression in naive LN WT and IKO CD4$^+$ cells harvested at d0 or cultured in Th0 conditions with QVD-OPh (d2) in the presence of DMSO or IKK2i (2.5 µM) and neutralizing anti-IFNγ and anti-IL-4 Abs (10 µg/ml). *Csf2* mRNA expression is represented as -fold induction with respect to the DMSO WT sample (n=4; mean ± SEM)**. (F)** Naive LN CD4$^+$ T cells from tamoxifen-induced WT and IKO mice were activated with anti-CD3 + anti-CD28 Abs for 24h and subjected to 2 rounds of infection with pMIG or IκBα super repressor (SR)/pMIG in the presence of QVD-OPh. At d3, cells were stimulated with PMA, ionomycin and Golgiplug for 2h, and IL-17 and GM-CSF expression was analyzed.

Top: Representative panels of GFP expression and of IL-17 and GM-CSF expression in GFP^- and GFP^+ cells are shown. Bottom: Proportion of WT and IKO $GM\text{-}CSF^+$ $CD4^+$ T cells infected with pMIG or $I\kappa B\alpha$ super repressor after 3 days of Th0 culture (n=3; mean ± SEM). Statistical significance was analyzed by parametric unpaired t-test. Unless otherwise specified, statistical significance was analyzed by a Mann-Whitney test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

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Figure 1

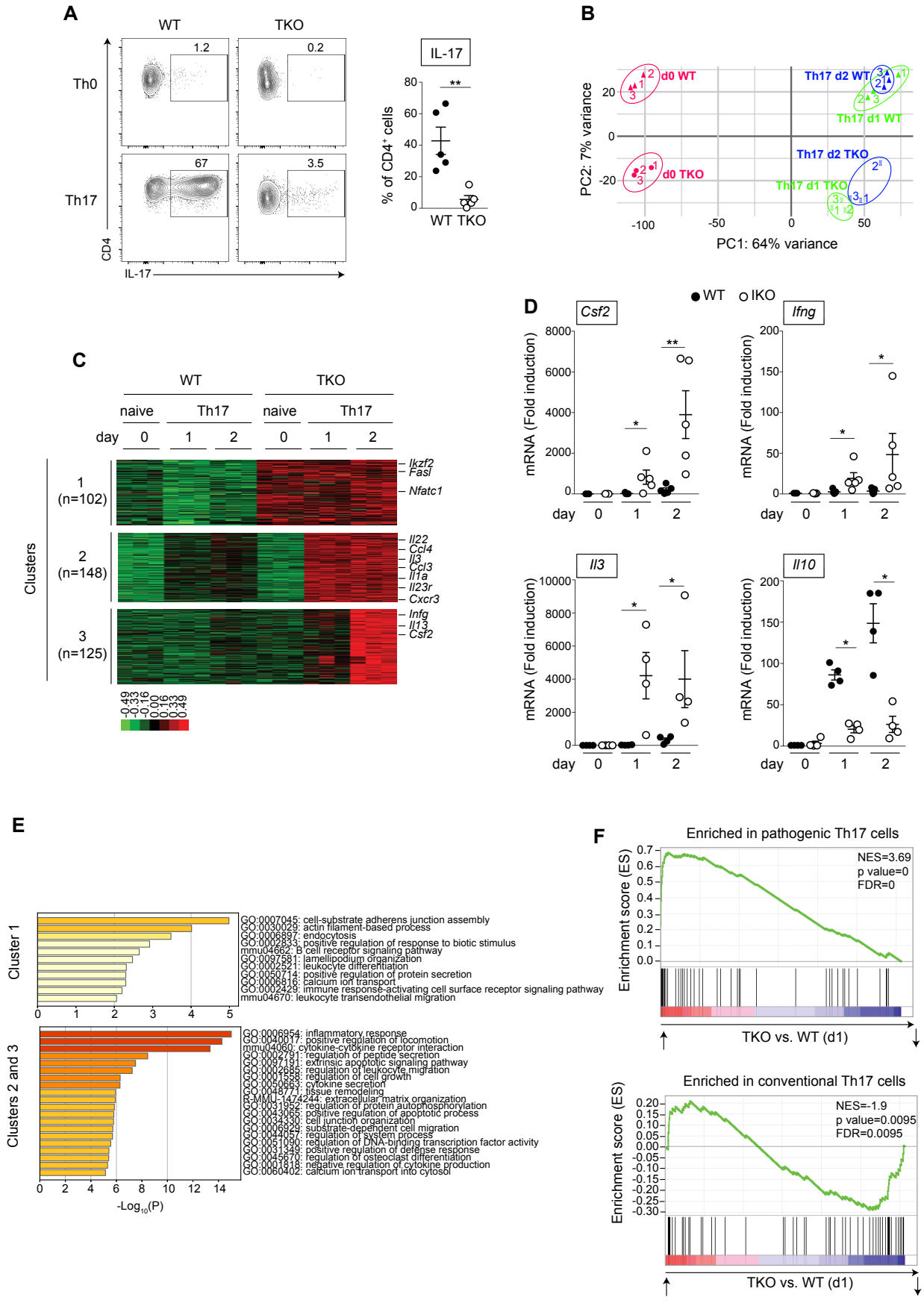

A
WT
TKO
Th0
Th17
1.2
0.2
67
3.5
CD4
IL-17
IL-17
% of CD4⁺ cells
WT TKO
B
d0 WT
Th17 d2 WT
Th17 d1 WT
d0 TKO
Th17 d2 TKO
Th17 d1 TKO
PC2: 7% variance
PC1: 64% variance
C
WT
TKO
naive
Th17
day
Clusters
1 (n=102)
2 (n=148)
3 (n=125)
Ikzf2
Fasl
Nfatc1
Il22
Ccl4
Il3
Ccl3
Il1a
Il23r
Cxcr3
Ifng
Il13
Csf2
D
WT
IKO
Csf2
Ifng
Il3
Il10
mRNA (Fold induction)
day
E
Cluster 1
Clusters 2 and 3
GO:0007045: cell-substrate adherens junction assembly
GO:0030029: actin filament-based process
GO:0006897: endocytosis
GO:0002833: positive regulation of response to biotic stimulus
mmu04662: B cell receptor signaling pathway
GO:0097581: lamellipodium organization
GO:0002521: leukocyte differentiation
GO:0050714: positive regulation of protein secretion
GO:0006816: calcium ion transport
GO:0002429: immune response-activating cell surface receptor signaling pathway
mmu04670: leukocyte transendothelial migration
GO:0006954: inflammatory response
GO:0040017: positive regulation of locomotion
mmu04060: cytokine-cytokine receptor interaction
GO:0002791: regulation of peptide secretion
GO:0097191: extrinsic apoptotic signaling pathway
GO:0002685: regulation of leukocyte migration
GO:0001558: regulation of cell growth
GO:0050663: cytokine secretion
GO:0048771: tissue remodeling
R-MMU-1474244: extracellular matrix organization
GO:0031952: regulation of protein autophosphorylation
GO:0043065: positive regulation of apoptotic process
GO:0034330: cell junction organization
GO:0006929: substrate-dependent cell migration
GO:0044057: regulation of system process
GO:0051090: regulation of DNA-binding transcription factor activity
GO:0031349: positive regulation of defense response
GO:0045670: regulation of osteoclast differentiation
GO:0001818: negative regulation of cytokine production
GO:0060402: calcium ion transport into cytosol
-Log10(P)
F
Enriched in pathogenic Th17 cells
NES=3.69
p value=0
FDR=0
Enrichment score (ES)
TKO vs. WT (d1)
Enriched in conventional Th17 cells
NES=-1.9
p value=0.0095
FDR=0.0095
Enrichment score (ES)
TKO vs. WT (d1)

Figure 2

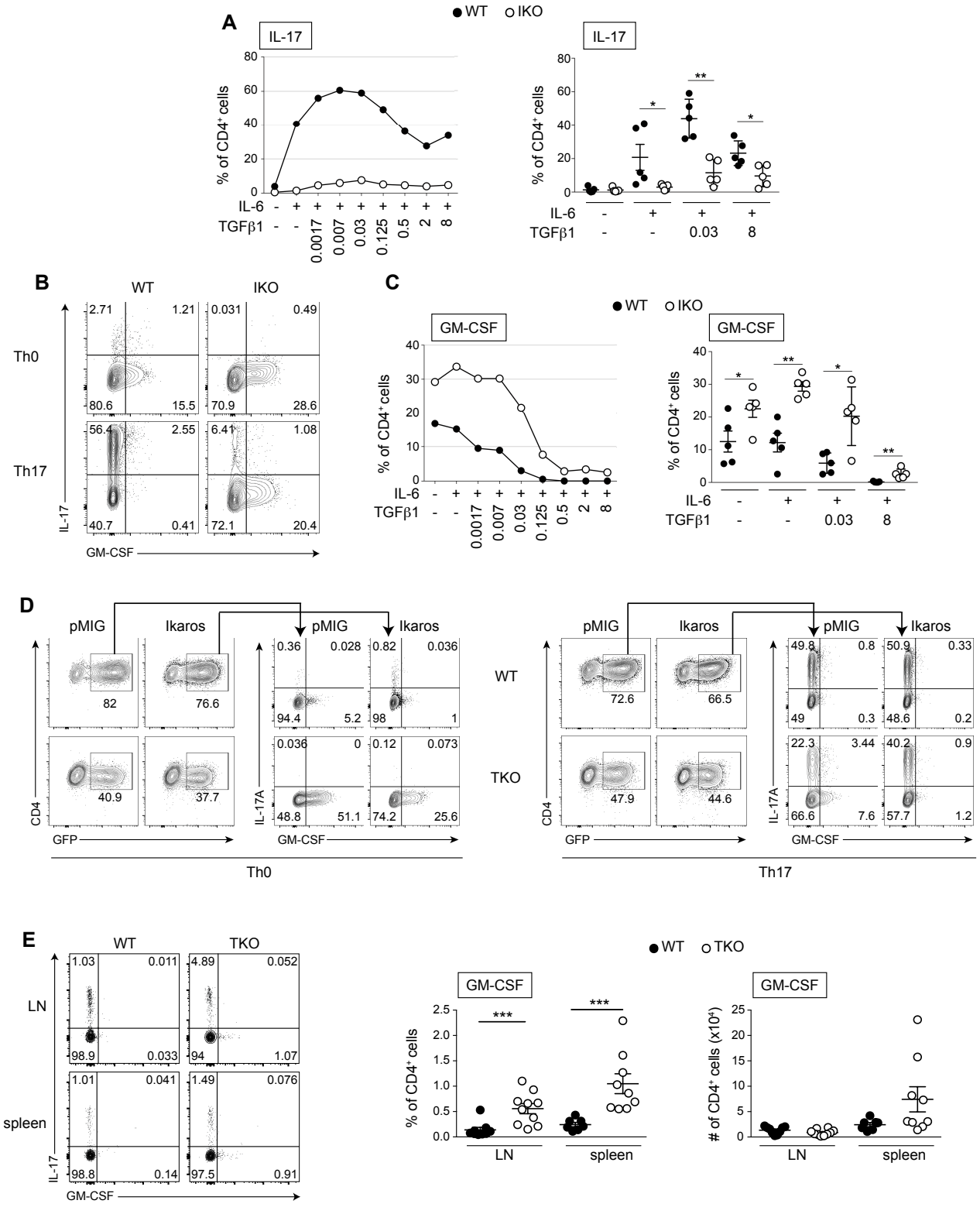

A
WT
IKO
IL-17
% of CD4+ cells
IL-6
TGFβ1
0.0017
0.007
0.03
0.125
0.5
2
8
B
WT
IKO
Th0
Th17
IL-17
GM-CSF
C
GM-CSF
D
pMIG
Ikaros
CD4
GFP
IL-17A
Th0
Th17
WT
TKO
E
LN
spleen
# of CD4+ cells (x10⁴)

Figure 3

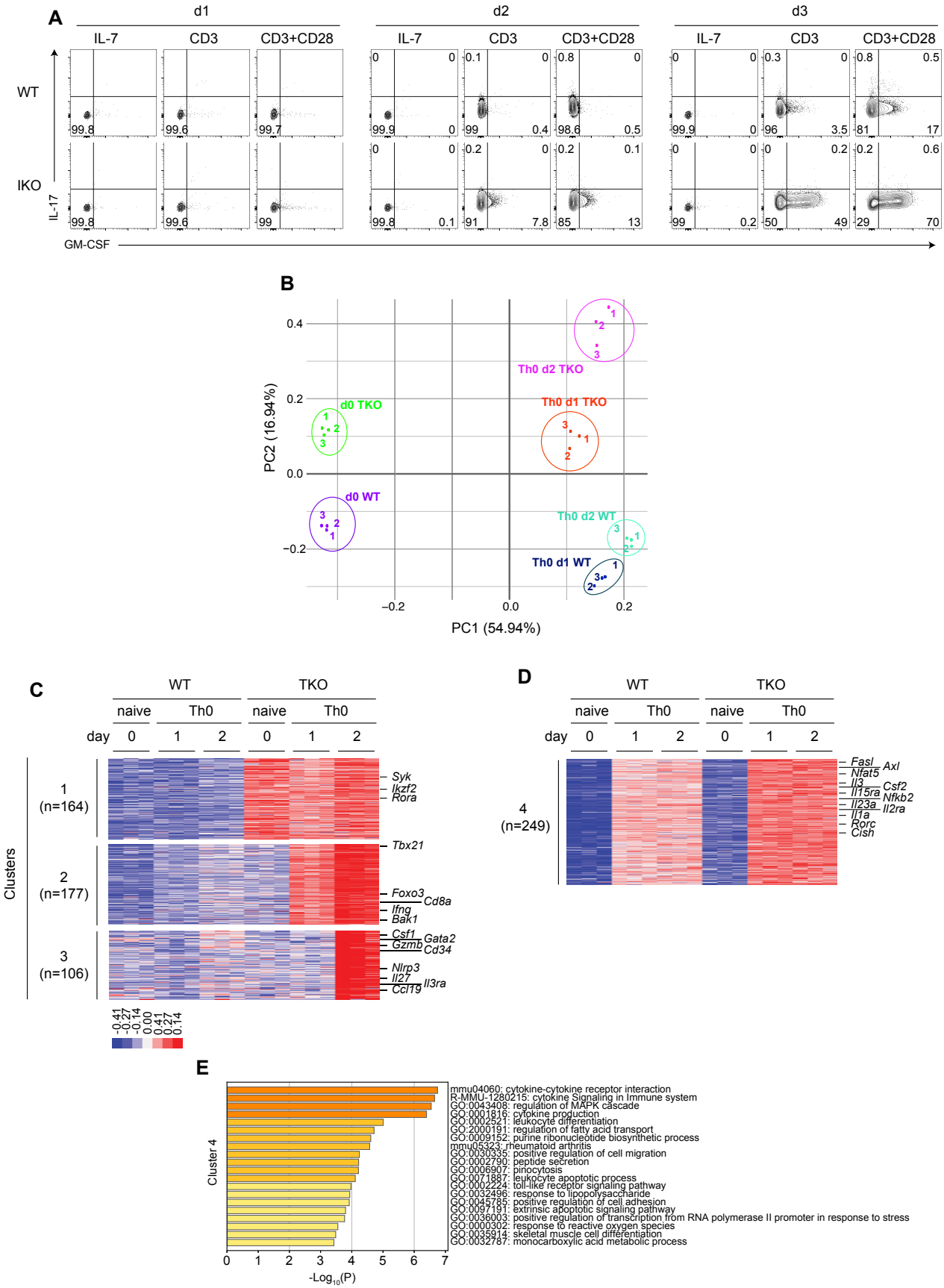

A
d1
d2
d3
IL-7
CD3
CD3+CD28
WT
IKO
IL-17
GM-CSF
B
PC2 (16.94%)
PC1 (54.94%)
Th0 d2 TKO
Th0 d1 TKO
d0 TKO
d0 WT
Th0 d2 WT
Th0 d1 WT
C
WT
TKO
naive
Th0
day
Clusters
1 (n=164)
2 (n=177)
3 (n=106)
Syk
Ikzf2
Rora
Tbx21
Foxo3
Cd8a
Ifng
Bak1
Csf1
Gata2
Gzmb
Cd34
Nlrp3
Il27
Il3ra
Ccl19
D
4 (n=249)
Fasl
Axl
Nfat5
Il3
Csf2
Il15ra
Nfkb2
Il23a
Il2ra
Il1a
Rorc
Cish
E
Cluster 4
-Log10(P)
mmu04060: cytokine-cytokine receptor interaction
R-MMU-1280215: cytokine Signaling in Immune system
GO:0043408: regulation of MAPK cascade
GO:0001816: cytokine production
GO:0002521: leukocyte differentiation
GO:2000191: regulation of fatty acid transport
GO:0009152: purine ribonucleotide biosynthetic process
mmu05323: rheumatoid arthritis
GO:0030335: positive regulation of cell migration
GO:0002790: peptide secretion
GO:0006907: pinocytosis
GO:0071887: leukocyte apoptotic process
GO:0002224: toll-like receptor signaling pathway
GO:0032496: response to lipopolysaccharide
GO:0045785: positive regulation of cell adhesion
GO:0097191: extrinsic apoptotic signaling pathway
GO:0036003: positive regulation of transcription from RNA polymerase II promoter in response to stress
GO:0000302: response to reactive oxygen species
GO:0035914: skeletal muscle cell differentiation
GO:0032787: monocarboxylic acid metabolic process

Figure 4

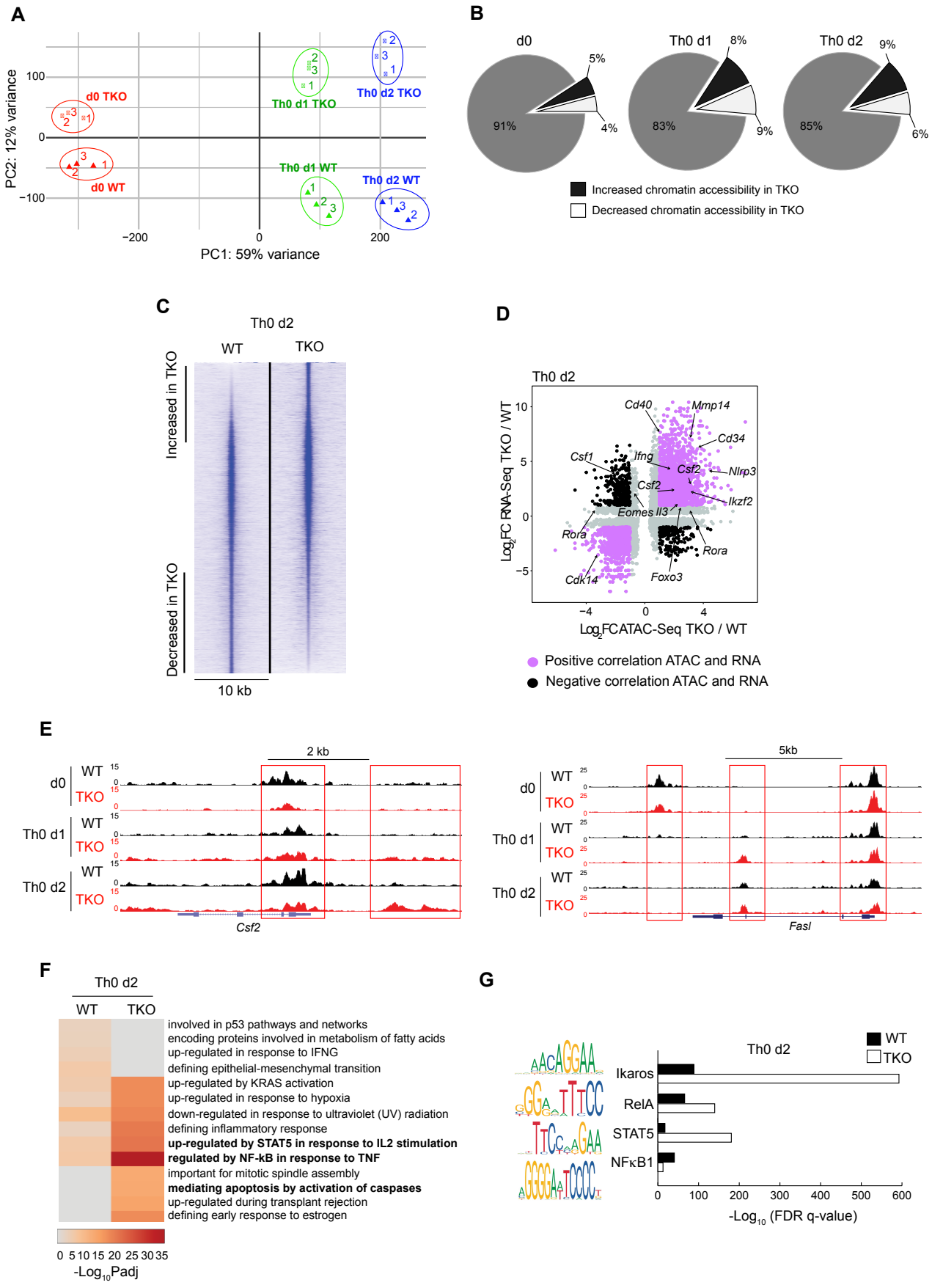

A
PC2: 12% variance
PC1: 59% variance
d0 TKO
Th0 d1 TKO
Th0 d2 TKO
d0 WT
Th0 d1 WT
Th0 d2 WT
B
d0
Th0 d1
Th0 d2
5%
8%
9%
91%
83%
85%
4%
9%
6%
Increased chromatin accessibility in TKO
Decreased chromatin accessibility in TKO
C
Th0 d2
WT
TKO
Increased in TKO
Decreased in TKO
10 kb
D
Th0 d2
Log2FC RNA-Seq TKO / WT
Log2FCATAC-Seq TKO / WT
Cd40
Mmp14
Cd34
Csf1
Ifng
Csf2
Nlrp3
Csf2
Ikzf2
Eomes
Il3
Rora
Rora
Cdk14
Foxo3
Positive correlation ATAC and RNA
Negative correlation ATAC and RNA
E
2 kb
5kb
d0
Th0 d1
Th0 d2
WT
TKO
Csf2
Fasl
F
Th0 d2
WT
TKO
involved in p53 pathways and networks
encoding proteins involved in metabolism of fatty acids
up-regulated in response to IFNG
defining epithelial-mesenchymal transition
up-regulated by KRAS activation
up-regulated in response to hypoxia
down-regulated in response to ultraviolet (UV) radiation
defining inflammatory response
up-regulated by STAT5 in response to IL2 stimulation
regulated by NF-kB in response to TNF
important for mitotic spindle assembly
mediating apoptosis by activation of caspases
up-regulated during transplant rejection
defining early response to estrogen
-Log10Padj
G
Th0 d2
WT
TKO
Ikaros
RelA
STAT5
NFκB1
-Log10 (FDR q-value)

Figure 5

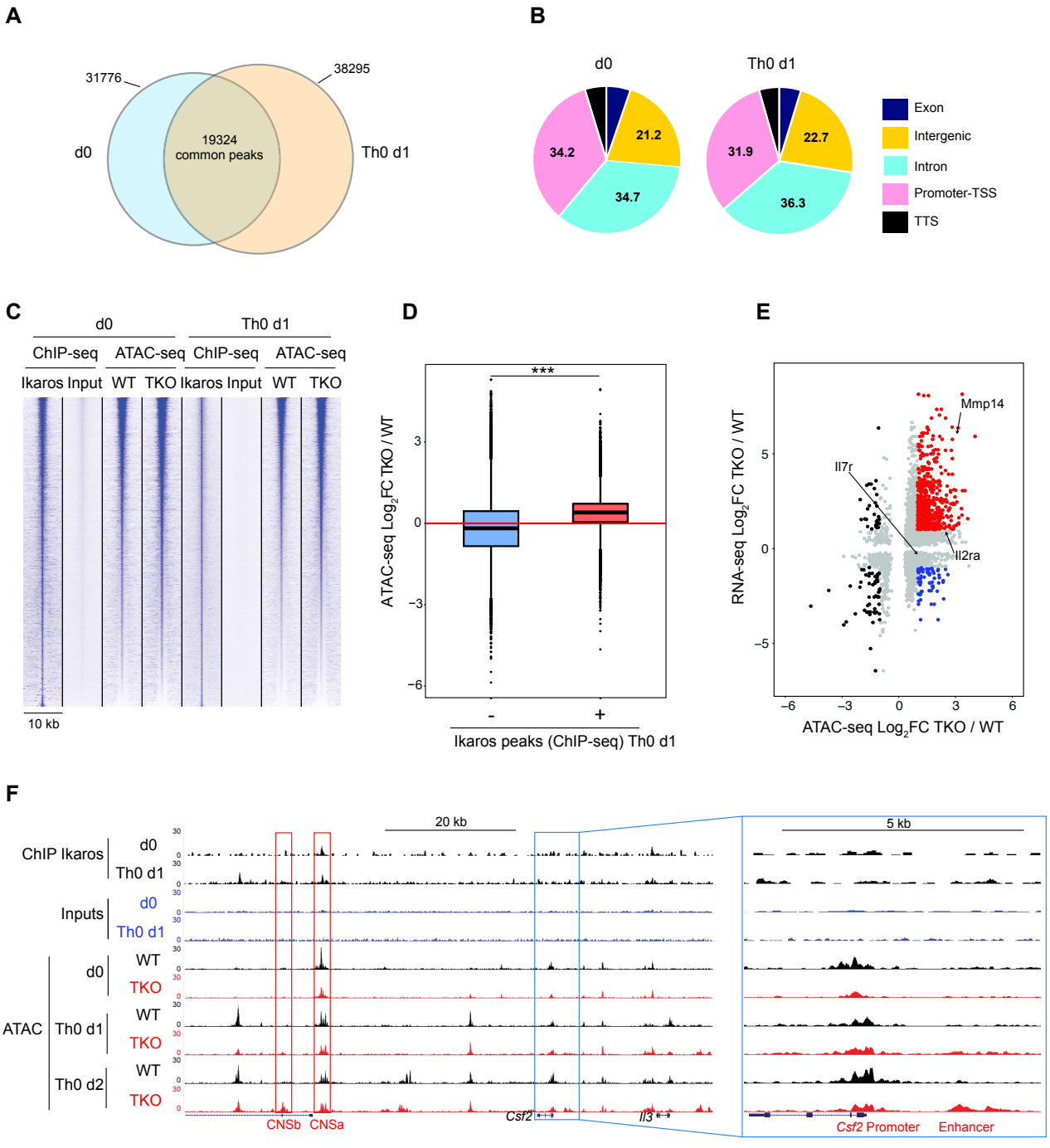
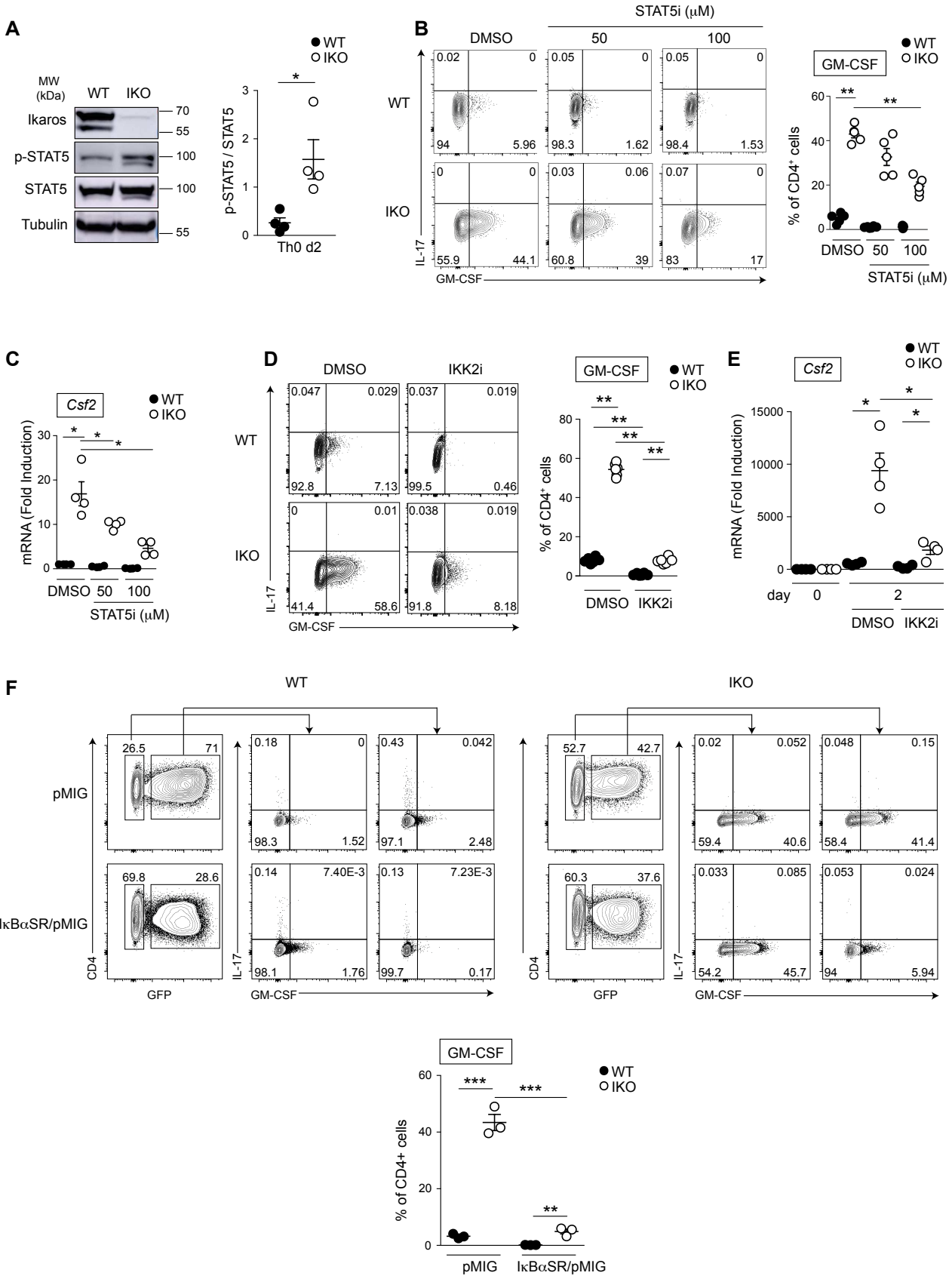

A
31776
38295
19324
common peaks
d0
Th0 d1
B
d0
Th0 d1
34.2
21.2
34.7
31.9
22.7
36.3
Exon
Intergenic
Intron
Promoter-TSS
TTS
C
d0
Th0 d1
ChIP-seq
ATAC-seq
ChIP-seq
ATAC-seq
Ikaros Input
WT
TKO
Ikaros Input
WT
TKO
10 kb
D
***
ATAC-seq Log2FC TKO / WT
3
0
−3
−6
-
+
Ikaros peaks (ChIP-seq) Th0 d1
E
Mmp14
Il7r
Il2ra
RNA-seq Log2FC TKO / WT
5
0
−5
−6
−3
0
3
6
ATAC-seq Log2FC TKO / WT
F
20 kb
5 kb
ChIP Ikaros
d0
Th0 d1
Inputs
d0
Th0 d1
ATAC
d0
WT
TKO
Th0 d1
WT
TKO
Th0 d2
WT
TKO
CNSb
CNSa
Csf2
Il3
Csf2 Promoter
Enhancer

Figure 6


A
MW (kDa)
WT
IKO
Ikaros
p-STAT5
STAT5
Tubulin
70
55
100
100
55
p-STAT5 / STAT5
Th0 d2
B
STAT5i (μM)
DMSO
50
100
WT
IKO
IL-17
GM-CSF
GM-CSF
% of CD4+ cells
C
Csf2
mRNA (Fold Induction)
STAT5i (μM)
D
DMSO
IKK2i
E
day
F
pMIG
IκBαSR/pMIG
CD4
GFP
IL-17
GM-CSF

CD4$^+$ T cells require Ikaros to restrain STAT5 and NFκB-dependent GM-CSF expression and their pathogenic phenotype

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List of supplemental figures

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SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Differentially expressed genes in WT and TKO T cells in Th17 conditions.
(A) Western blot showing the T-cell specific deletion of Ikaros in splenic $CD4^+$ and $CD8^+$ cells vs. $B220^+$ cells in TKO mice. **(B)** Clusters of upregulated genes in WT and TKO $CD4^+$ T cells cultured in Th17 conditions (K-means clustering) **(C)** Clusters of upregulated genes in WT and TKO $CD4^+$ T cells cultured in Th17 conditions (K-means clustering). **(D)** RT-qPCR analysis of *Il17 and Rorc* mRNA expression in freshly isolated naive $CD4^+$ T cells (d0) or after culture in Th17 conditions with QVD-OPh for 1 and 2 days (n=4; mean ± SEM). **(E)** Heat maps of GO term enrichment of genes in cluster 18 shown in B.

Fig. S2. Gene set enrichment analysis of pathogenic Th17 and Th-GM genes in TKO cells.
(A) GSEA enrichment plots of genes enriched in pTh17 (left) or cTh17 cells (right) among genes up- or down-regulated by Ikaros at d2 in Th17 conditions. Gene sets correspond to 106 genes enriched in pTh17 cells and 143 genes enriched in cTh17 cells *(1)*. **(B)** GSEA enrichment plots of genes enriched in Th-GM cells among genes up- or down-regulated by Ikaros at d1 (left) and d2 (right) in Th17 conditions. Gene sets correspond to 210 genes enriched in Th-GM cells *(2)*. NES, normalized enrichment score; FDR, false discovery rate.

Fig. S3. Ikaros loss leads to decreased IL-17 and Foxp3 and increased IFNγ and GM-CSF levels.
(A) Western blot of Ikaros expression in WT or IKO splenic $CD19^+$ or $CD4^+$ cells of tamoxifen-treated mice. β-Actin was used as a loading control. **(B)** Contour plots showing naive $CD4^+$ T cells ($CD4^+CD8^-CD44^{lo}CD25^-TCR^{\gamma\delta-}NK1.1^-$) from peripheral and mesenteric LN of tamoxifen-treated WT and IKO mice. **(C and D)** Proportions of $Foxp3^+CD4^+$ (C; left) or $IFN\gamma^+CD4^+$ cells (D; left) and statistical analysis of the proportion of WT and IKO $Foxp3^+CD4^+$ cells (C; right) or $IFN\gamma^+CD4^+$ (D; right) cells from 3d cultures of WT and IKO cells (from tamoxifen-treated mice), cultured in Th17 conditions with different amounts of TGFβ1 plus constant IL-6. (n=5; mean ± SEM). Statistical significance was analyzed by a Mann-Whitney test (*p ≤0.05, **p ≤ 0.01).

(E-H) Representative graphs showing the intracellular expression of IFNγ (E), IL-17 (F), Foxp3 (G), and GM-CSF (H) in WT and IKO CD4$^+$ T cells cultured for 3 days with different amounts of TGFβ1 plus constant IL-6 with QVD-Oph, and with or without neutralizing anti-IFNγ and anti-IL-4 Abs (n=4).

Fig. S4. Ikaros loss leads to an increased expression of FasL that is responsible for apoptosis but not GM-CSF production.

(A) Left: representative contour plots of Annexin V and PI staining on WT and IKO CD4$^+$ cells cultured for 2d in Th17 conditions with or without QVD-OPh. Right: proportions of WT and IKO Annexin V$^+$ cells. Each dot indicates one mouse (n=4-5; mean ± SEM). **(B)** RT-qPCR analysis of *Fasl* mRNA expression in WT and IKO LN naive CD4$^+$ cells after harvest (d0), or cultured in Th0 or Th17 conditions with QVD-OPh (d1 and 2). *Fasl* mRNA expression is represented as -fold induction with respect to the d0 WT sample. **(C)** Left: representative contour plots showing membrane FasL expression at d3 in WT and IKO cells cultured in Th0 conditions. Right: proportions of WT and IKO FasL$^+$ CD4$^+$ T cells (n=4; mean ± SEM). **(D)** Left: representative contour plot showing Annexin V and PI staining of WT and IKO CD4$^+$ T cells cultured for 2 days in Th0 condition with a neutralizing anti-FasL Ab or an isotype control. Right: proportions of WT and IKO annexin V$^+$ CD4$^+$ T cells at d2 (n=4; mean ± SEM). **(E)** Left: percentage of GM-CSF$^+$ and IL-17$^+$ cells among live WT or IKO CD4$^+$ cells cultured for 3 days in Th0 conditions with a neutralizing anti-FasL Ab or an isotype control. Right: proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells (n=4; mean ± SEM). N indicates the number of independent experiments. Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01).

Fig. S5. GM-CSF expression and IL-17 loss of expression directly correlate with *Ikzf1* deletion.

(A) Left: representative contour plots showing GM-CSF and Ikaros expression in WT and IKO cells treated with ethanol (EtOH)- or 4-hydroxytamoxifen (4-OHT) *in vitro* for 1-3 days in the presence of QVD-OPh, and analyzed at the indicated time point. Right: proportion at d3 of Ikaros$^+$ GM-CSF$^+$ cells from 4-OHT-treated WT cultures, or Ikaros$^-$ GM-CSF$^+$ cells from 4-OHT-treated IKO cultures, performed as in A (n=4; mean ± SEM). **(B)** Left: representative contour plot showing IL-17 and Ikaros expression in

EtOH- and 4-OHT-treated live WT and IKO cells cultured as in A. Right: proportions at d2 and d3 of Ikaros$^+$ IL-17$^+$ cells from 4-OHT-treated WT cultures, and Ikaros$^-$ IL-17$^+$ cells from 4-OHT-treated IKO cultures, performed under in Th17 conditions (n=4; mean ± SEM). Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05).

Fig. S6. Analysis of mature T cells in TKO mice.

All mice were 5-8 weeks old. **(A)** Total cell number in WT and TKO LN and spleen (n=7). **(B)** Left: CD4$^+$ and CD8$^+$ T cell numbers in LN and spleen from WT and TKO mice. Right: proportion of CD4$^+$ and CD8$^+$ T cells in WT and TKO LN and spleen (n=6). **(C)** Left: CD19$^+$ cell count in WT and TKO LN and spleen. Right: corresponding percentage of CD19$^+$ cells (n=5). **(D)** Representative contour plots of naive and effector CD4$^+$ T cells in LN from WT and TKO mice. **(E)** Left: naive CD4$^+$ T cell counts in LN and spleen. Right: percentage of naive cells among CD4$^+$TCR$\alpha\beta^+$ T cells (n=4). **(F)** Left: effector CD4$^+$ T cell counts in LN and spleens. Right: percentage of effector cells among CD4$^+$TCR$\alpha\beta^+$ cells (n=4). **(G)** Relative expression levels of IL-7Rα in WT and TKO naive CD4$^+$ T cells from LN and spleen (n=4). For a given organ (LN or spleen), the values for each mouse analyzed in a given experiment were normalized to the mean expression value for the WT samples analyzed in that experiment. All graphs are represented as mean ± SEM. Each circle indicates one mouse. n indicates the number of independent experiments. Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001).

Fig. S7. Ikaros loss leads to an increase of GM-CSF expression in CD4$^+$ T cells, but not CD8$^+$ T cells, ex vivo.

(A) Left panel: Representative analysis of GM-CSF- and CD8-expressing cells in WT and TKO T cells in LN and spleen. Middle panel: percentage of GM-CSF$^+$ cells among CD8$^+$ T cells from peripheral and/or mesenteric LN (n=9-10) or spleen (n=7-9) from WT and TKO mice. Right panel: cell counts of GM-CSF$^+$ cells among WT and TKO CD8$^+$ T cells from peripheral and/or mesenteric LN (n=9-10) or spleen (n=7-9) of WT and TKO mice (mean ± SEM). **(B)** Left panel: percentage of IL-17$^+$ cells among CD4$^+$ T cells from peripheral and/or mesenteric LN (n=9-10) or spleen (n=7-9) from WT and TKO mice. Right panel: cell counts of IL-17$^+$ cells among WT and TKO CD4$^+$ T cells from peripheral and/or mesenteric LN (n=9-10) or spleen (n=7-9) of WT and TKO mice

(mean ± SEM). Statistical significance was analyzed by a Mann-Whitney test (**p ≤ 0.01).

Fig. S8. Differentially expressed genes in WT and TKO T cells in Th0 conditions. **(A)** Proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 2 and 3 days of culture with anti-CD3 ± anti-CD28 Abs, related to Fig. 3A (n=4; mean ± SEM). Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05)**. (B)** Clusters of upregulated genes in WT and TKO CD4$^+$ T cells cultured in Th0 conditions (K-means clustering). **(C)** Clusters of downregulated genes in WT and TKO CD4$^+$ T cells cultured in Th0 conditions (K-means clustering).

Fig. S9. Metascape analysis of GO term enrichment of genes in clusters 1-3 from RNA-seq.

Fig. S10. CD4$^+$ T cells require Ikaros to proliferate.
(A) Left: Representative histogram of CFSE dilution and proliferative index of WT and IKO CD4$^+$ T cells at d3 in Th0 conditions (n=6; mean ± SEM). Right: Representative histogram of CFSE dilution and proliferative index of WT and IKO CD4$^+$ T cells at d3 in Th17 conditions (n=4; mean ± SEM). **(B)** Left: representative dot plot showing CFSE dilution and GM-CSF expression in WT and IKO CD4$^+$ T cells in Th0 (upper panel) or Th17 conditions (lower panel) for 3d. Right: percentage of GM-CSF$^+$ cells among WT and IKO CD4$^+$ T cells, in Th0 (upper panels; n=5; mean ± SEM) or Th17 conditions (lower panel; (n=4; mean ± SEM), in each division peak. N=number of independent experiments. Statistical significance was analyzed by a Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01). **(C)** Left: representative dot plot showing CFSE dilution and IL-17 expression in WT and IKO CD4$^+$ T cells in Th17 conditions for 3d. Right: percentage of IL-17$^+$ cells among WT and IKO CD4$^+$ T cells, in Th17 conditions, in each division peak (n=4; mean ± SEM).

Fig. S11. Ikaros loss changes the landscape of chromatin accessibility in CD4$^+$ T cells.
(A) Seqminer heat map of all ATAC-seq peaks in WT and TKO CD4$^+$ T cells at d0 (67165 peaks) and at d1 of Th0 cultures (78378 peaks). **(C)** Pie chart of the genomic location distribution of all ATAC-seq peaks in WT and TKO CD4$^+$ T cells at d0 and d2

in Th0 cultures. **(B)** Volcano plot showing log_2FC TKO/WT identified by ATAC-seq in naïve T cells (d0) and in-vitro-differentiated T cells at day 2 (Th0 d2). Up- and down-regulated genes with changes significantly greater than Log_2FC ≥ 1 or ≤ -1 are highlighted in red and blue, respectively (padj ≤ 0.01). At d0, red dots: 3969 peaks; blue dots: 2740 peaks; in Th0 d2, red dots: 8950, blue dots: 6380. **(D)** Integration of RNA-seq data and ATAC-Seq data from TKO cells compared to WT cells at d0 and d1 of Th0 cultures. Left: In naive T cells at d0, only the 3677 peaks with a padj ≤ 0.05 in the ATAC-seq AND RNA-seq analysis are depicted. Among them, 1491 peaks have a log_2FC (TKO/WT) ≥ 1 or ≤ -1 with a Pearson correlation equal to 0.63 with a p value< 2.2^{-16}. Right: In the Th0 d1 condition, only the 9954 peaks with a padj ≤ 0.05 in the ATAC-seq and RNA-seq analysis are depicted. Among them, 2995 peaks have a log2FC (TKO/WT) ≥ 1 or ≤ -1 in both analysis and have a Pearson correlation coefficient equal to 0.59 with a p value< 2.2^{-16}. Genes with a positive (purple) or a negative (black) correlation between ATAC-seq and RNA-seq data are highlighted. **(E)** Genome track view of ATAC-seq profiles of *Il3* (left) and *Ifng (right)* genes. The University of California Santa Cruz Genome browser (UCSC) depicts the pooled profile of the three independent experiments in in WT and TKO $CD4^+$ T cells at d0 and d1-2 in Th0 cultures.

Fig. S12. Ikaros loss leads to an enrichment of NFκB and Stat5 motifs on open chromatin regions.

(A) Heat map showing GO of the ATAC-seq peaks significantly increased (1219 peaks at d0 and 2687 peaks at Th0d1) and decreased (900 at d0 and 3040 at Th0 d1) in TKO compared to WT at d0 and d1 in Th0 cultures. Peaks are considered significantly increased in the TKO when the log_2FC (TKO/WT) > 2 with a padj ≤ 0.01, while they are considered significantly decreased in TKO when the log_2FC (TKO/WT) <- 2 with a padj ≤ 0.01. **(B)** Bar graph showing the indicated transcription factor motifs significantly enriched in WT and TKO ATAC-seq peaks at d0 (upper panel) and d1 in Th0 cultures (lower panel). The motifs were identified from AME-MEME motif search. Significant peaks were selected as in A. **(C and D)** Venn diagram showing the peaks enriched for Ikaros or/and Stat5 (C) or Ikaros and/or RelA (D) from a FIMO-MEME analysis in WT and TKO cells at d0 (TN) and d2 in Th0 conditions, Significant peaks were selected as in A.

Fig. S13. ChIP-seq analysis of Ikaros binding profile on differentially expressed genes.

UCSC Genome track views of ChIP-seq showing genes with a binding Ikaros on promoters or enhancers and genes not bound by Ikaros.

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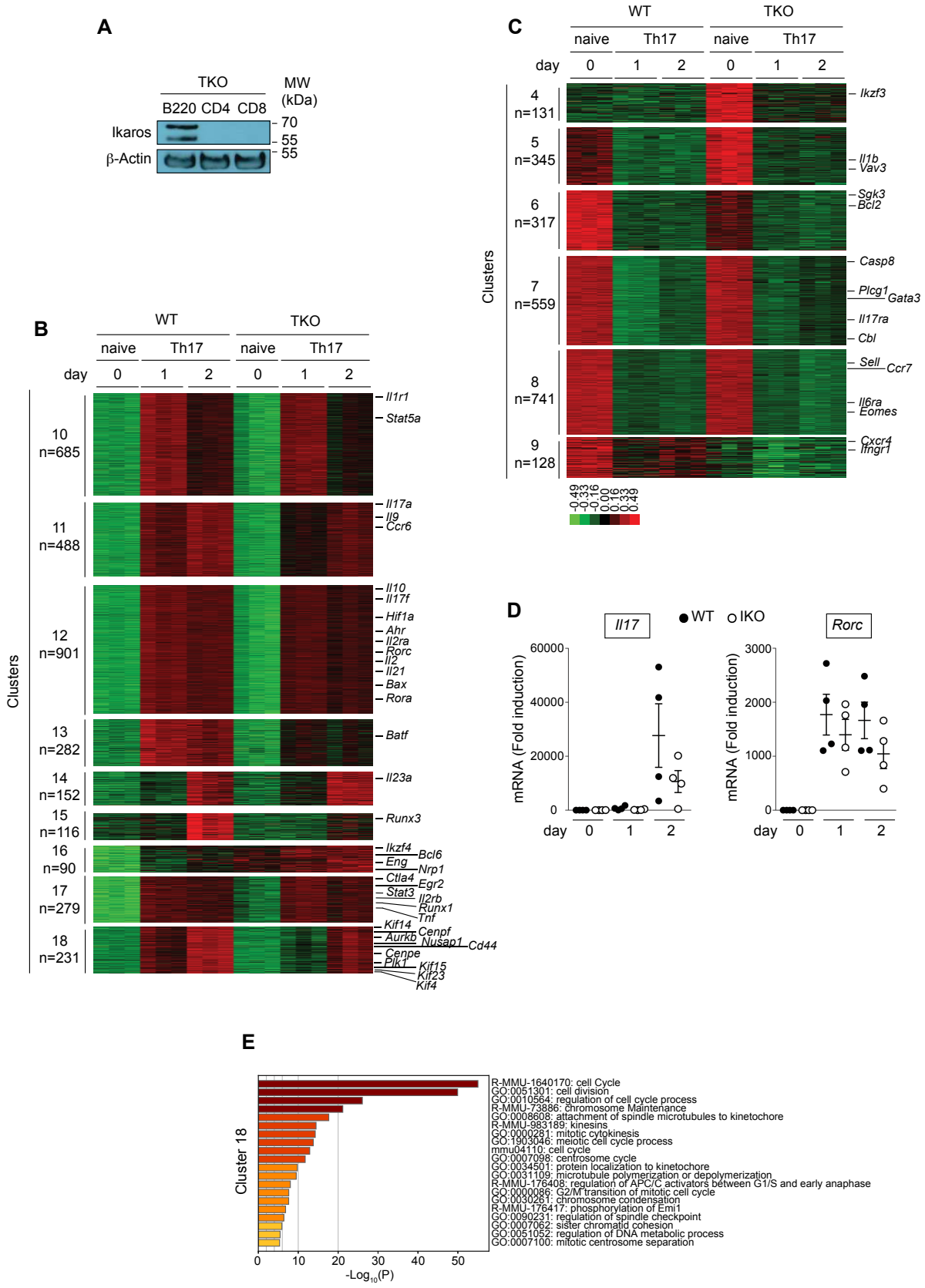
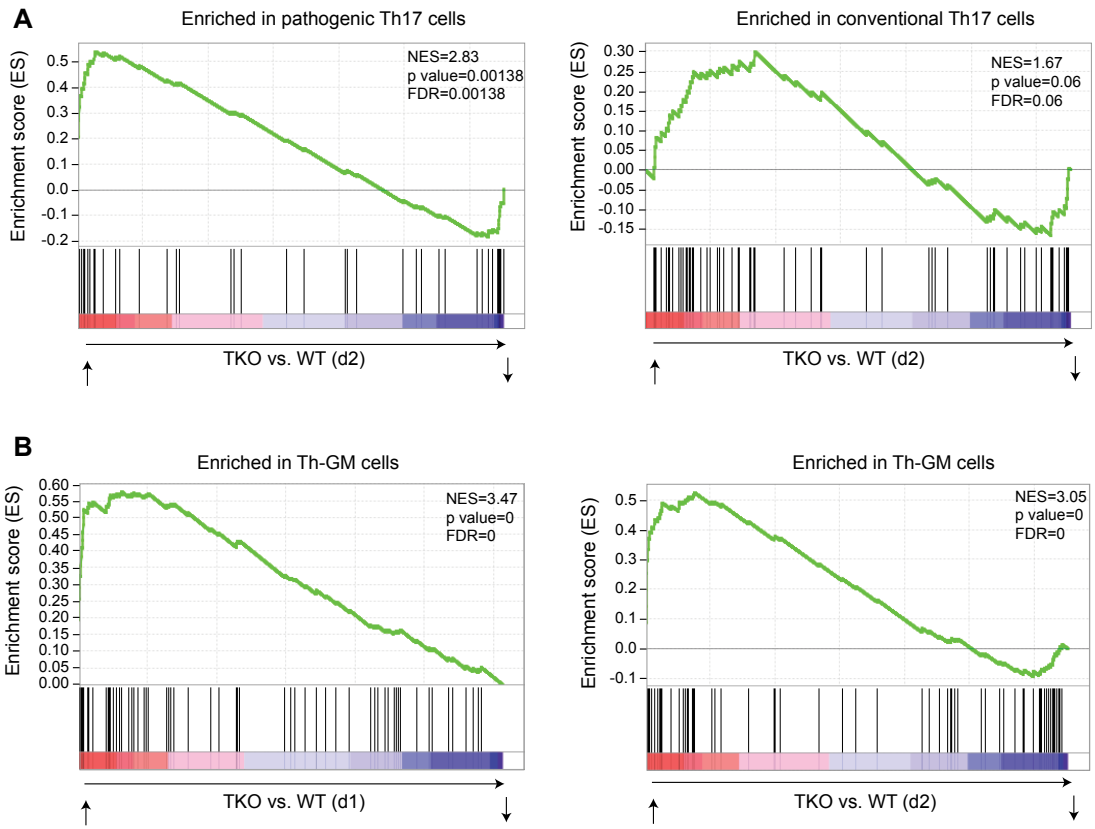
Supplemental Figure 1



Supplemental Figure 2



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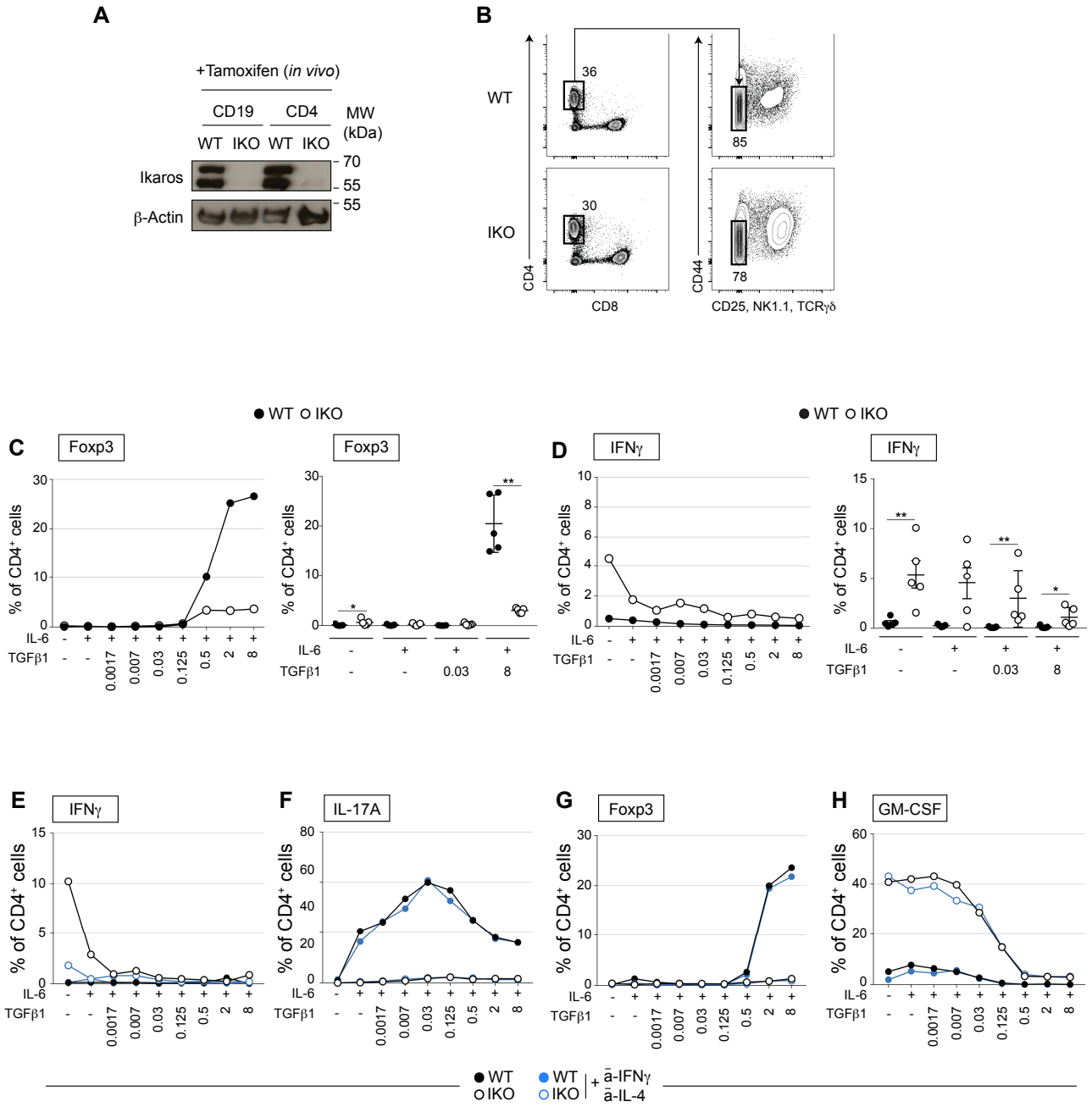

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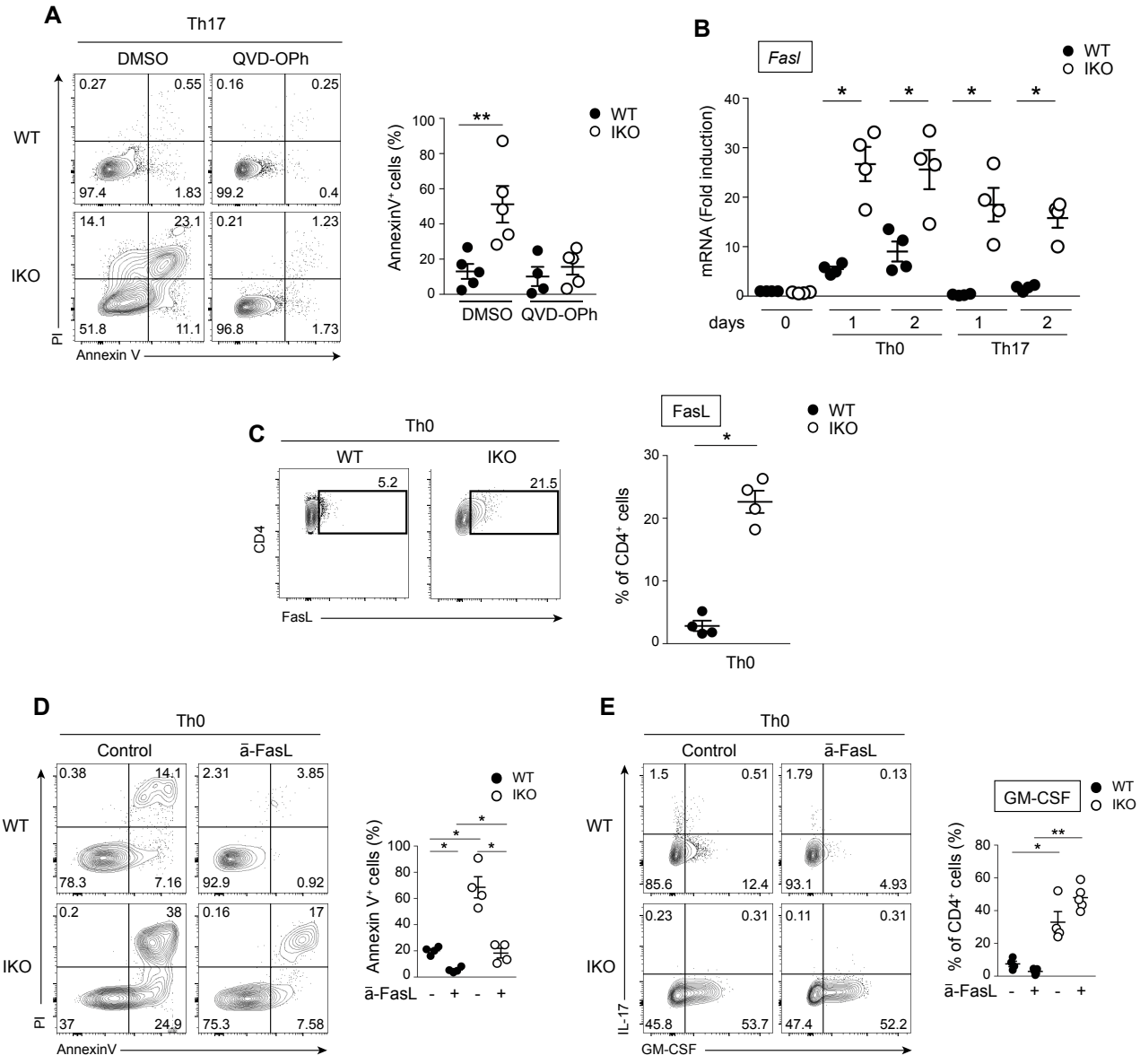

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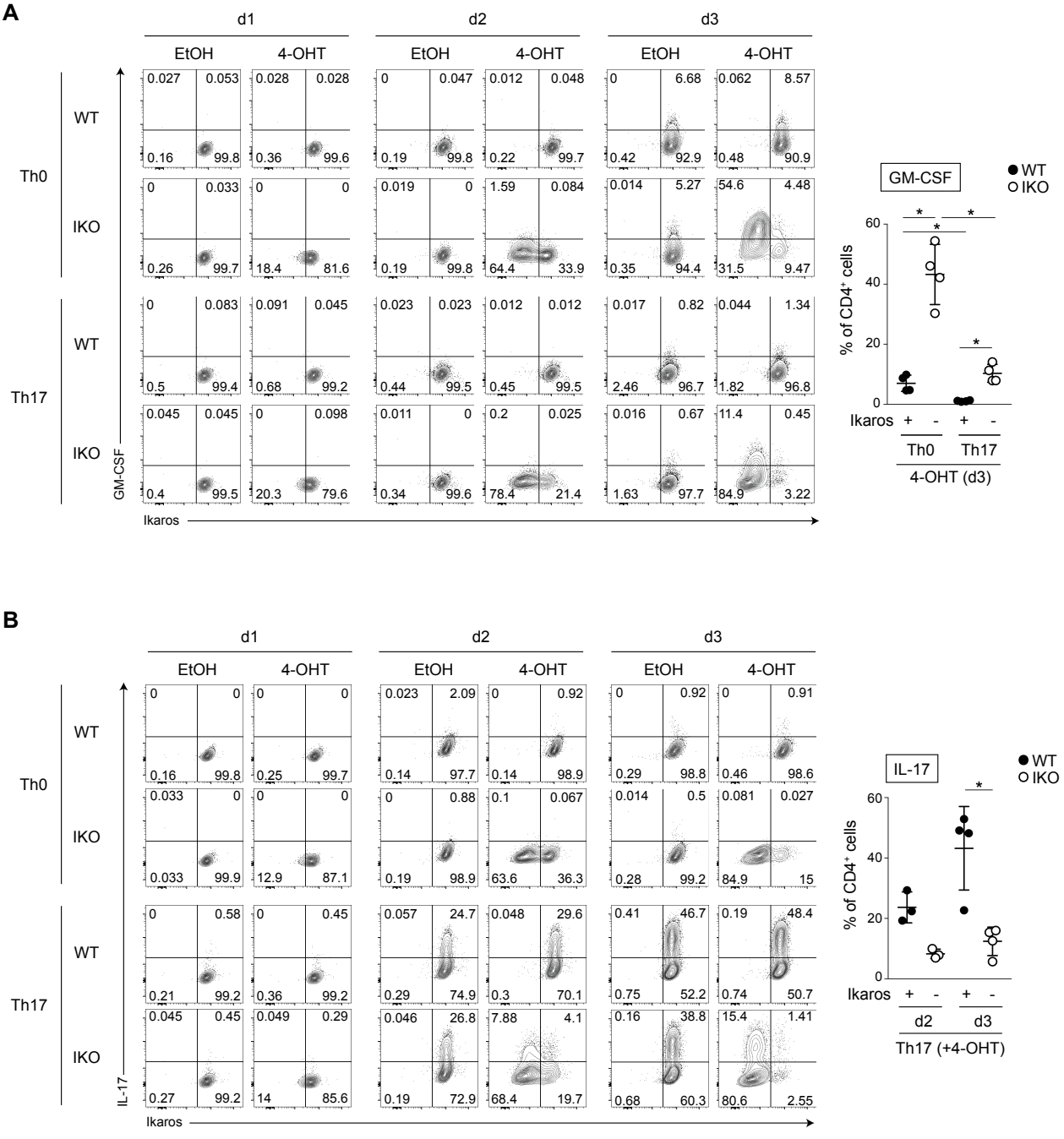

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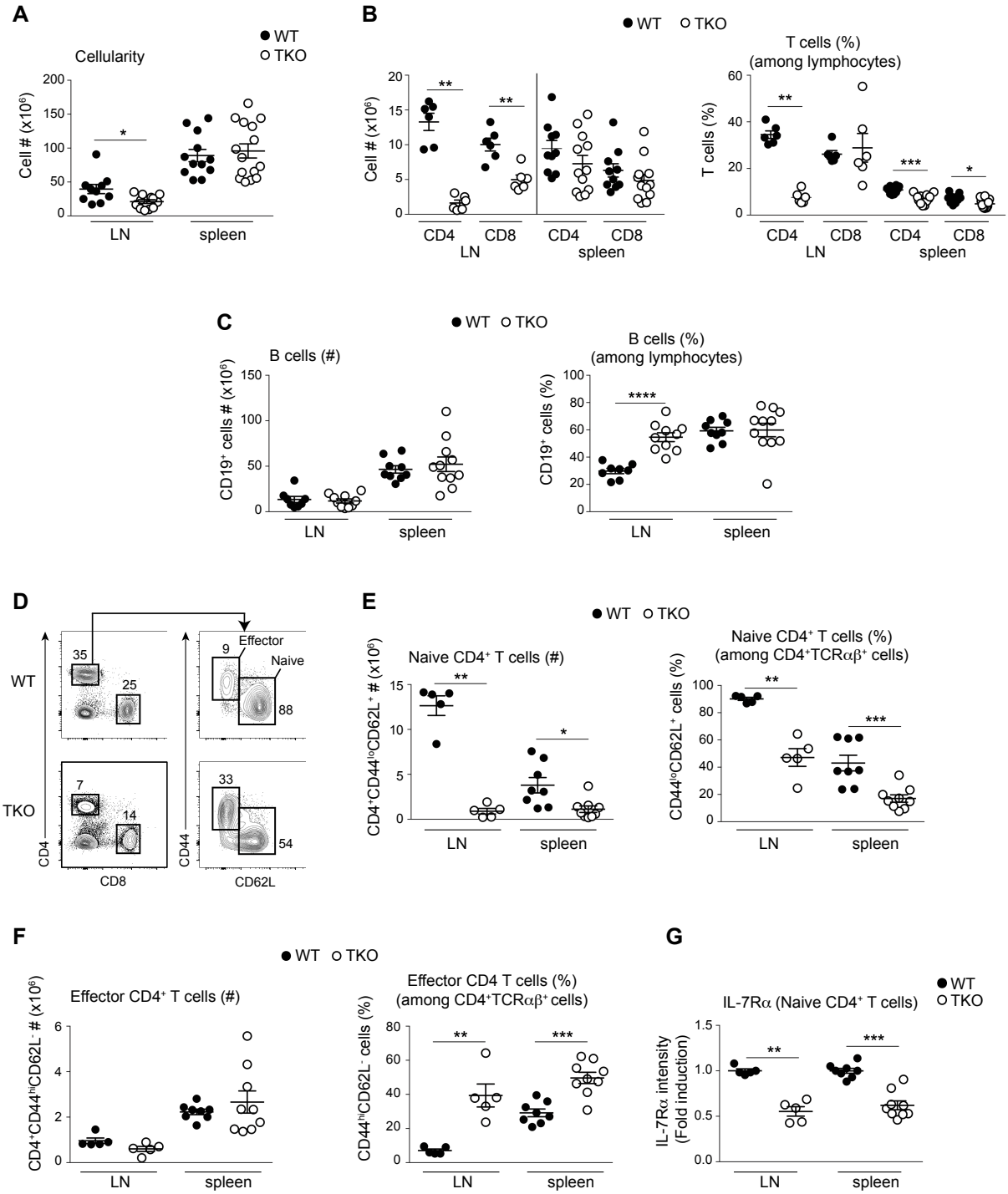

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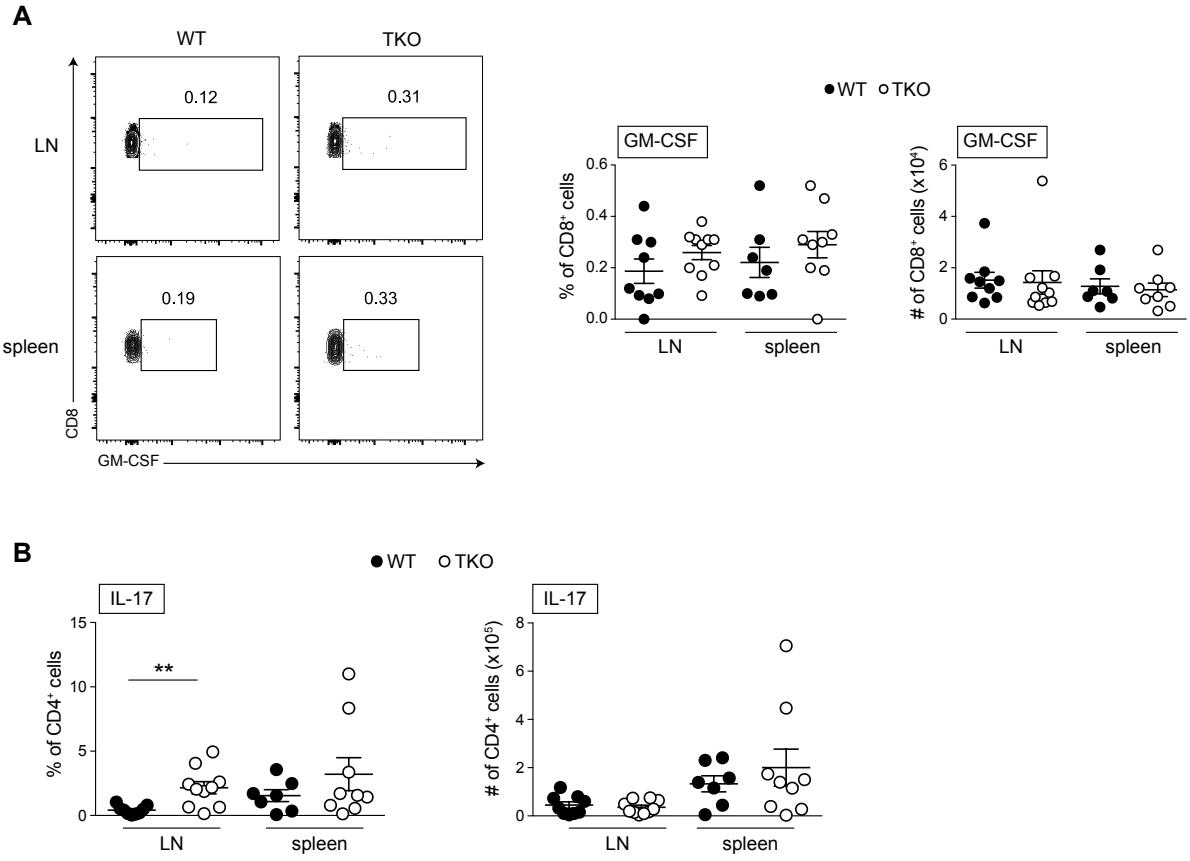

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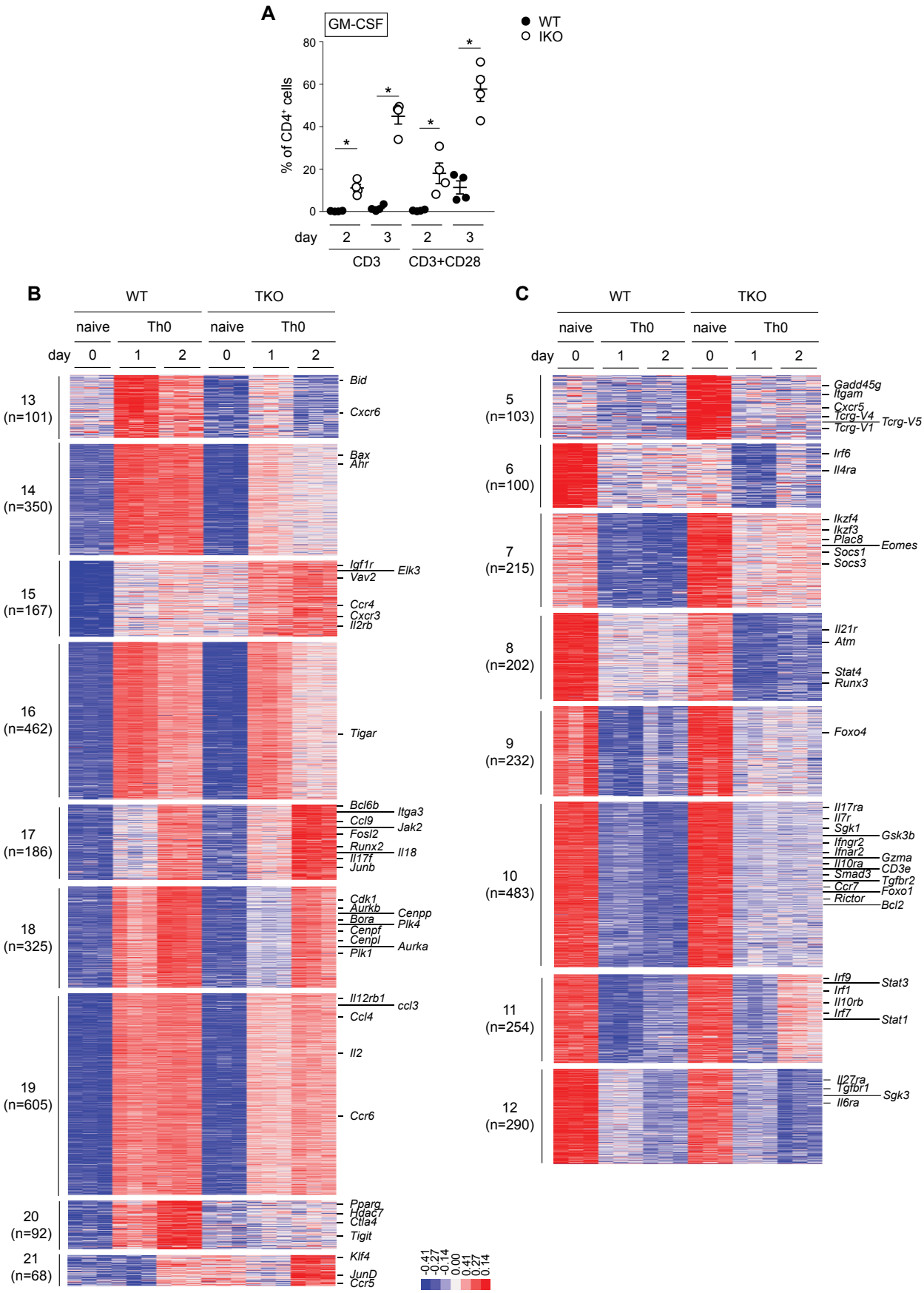
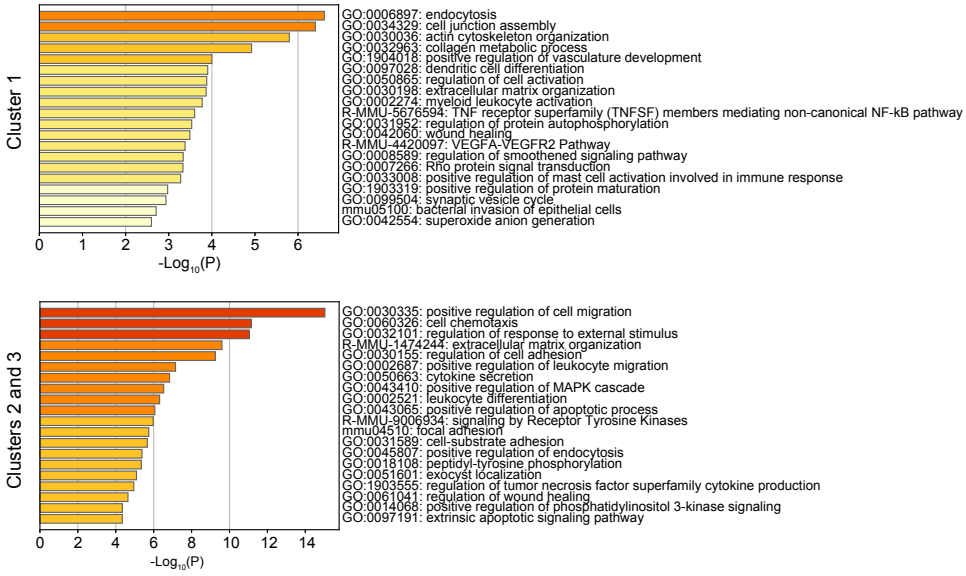


Supplemental Figure 9



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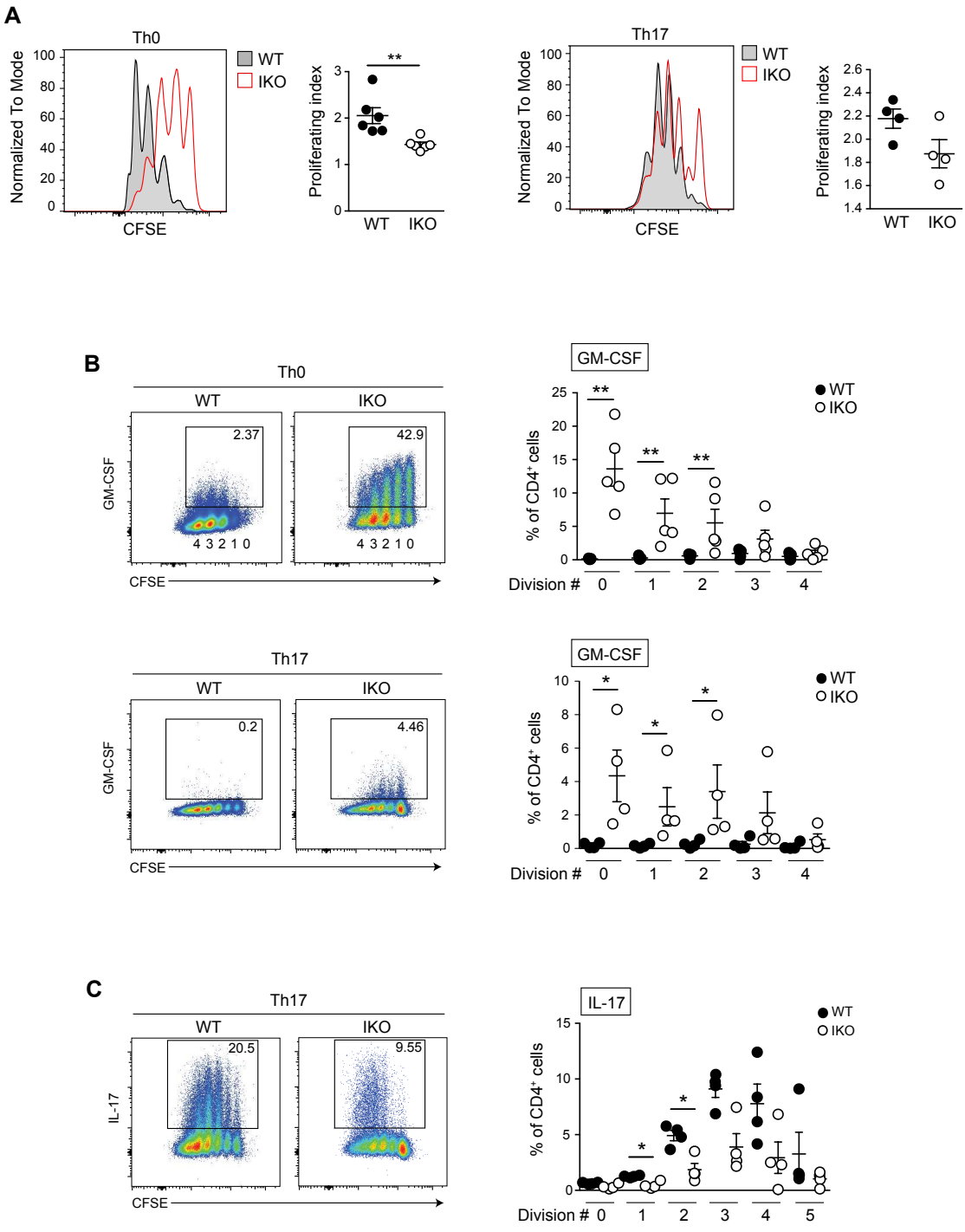

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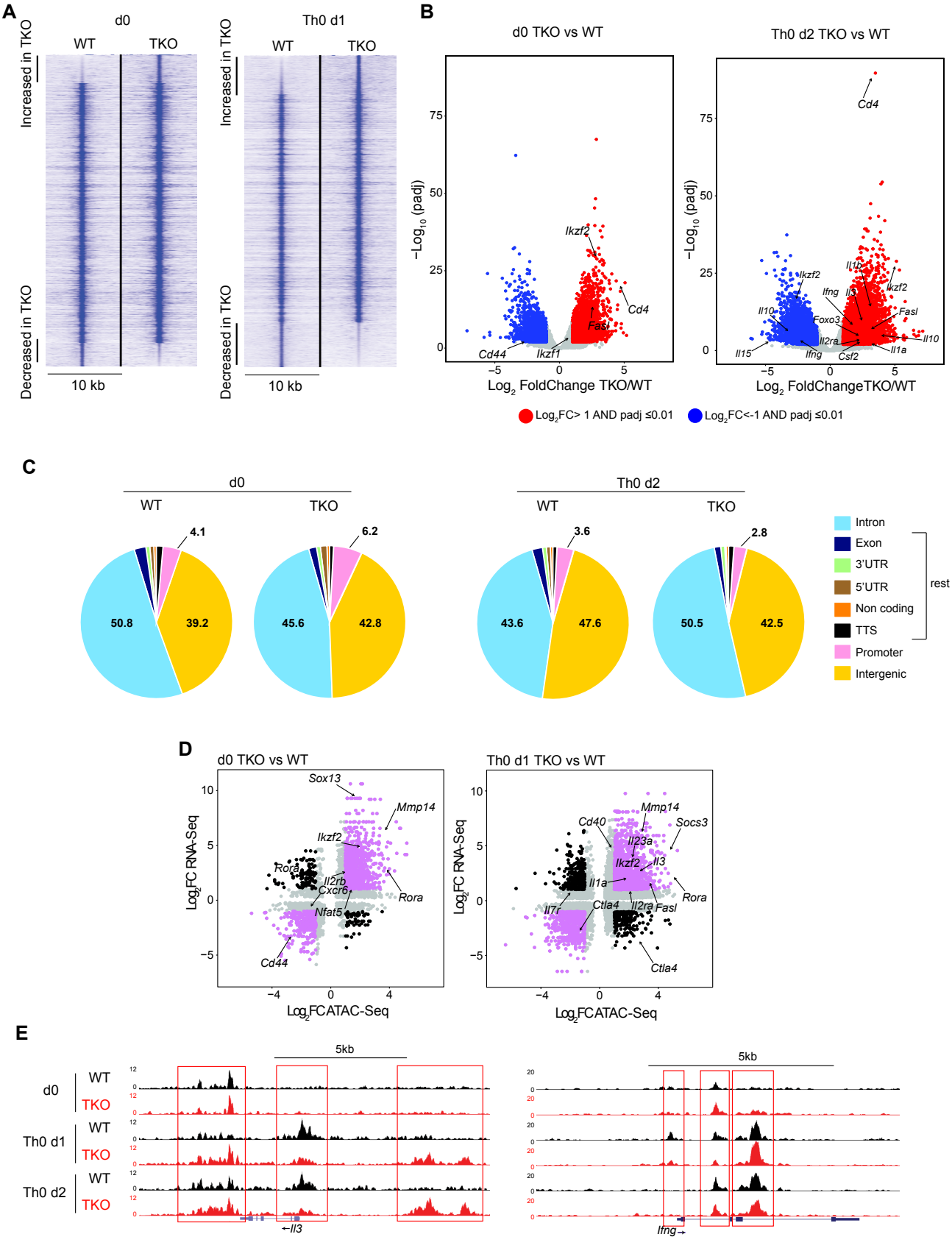

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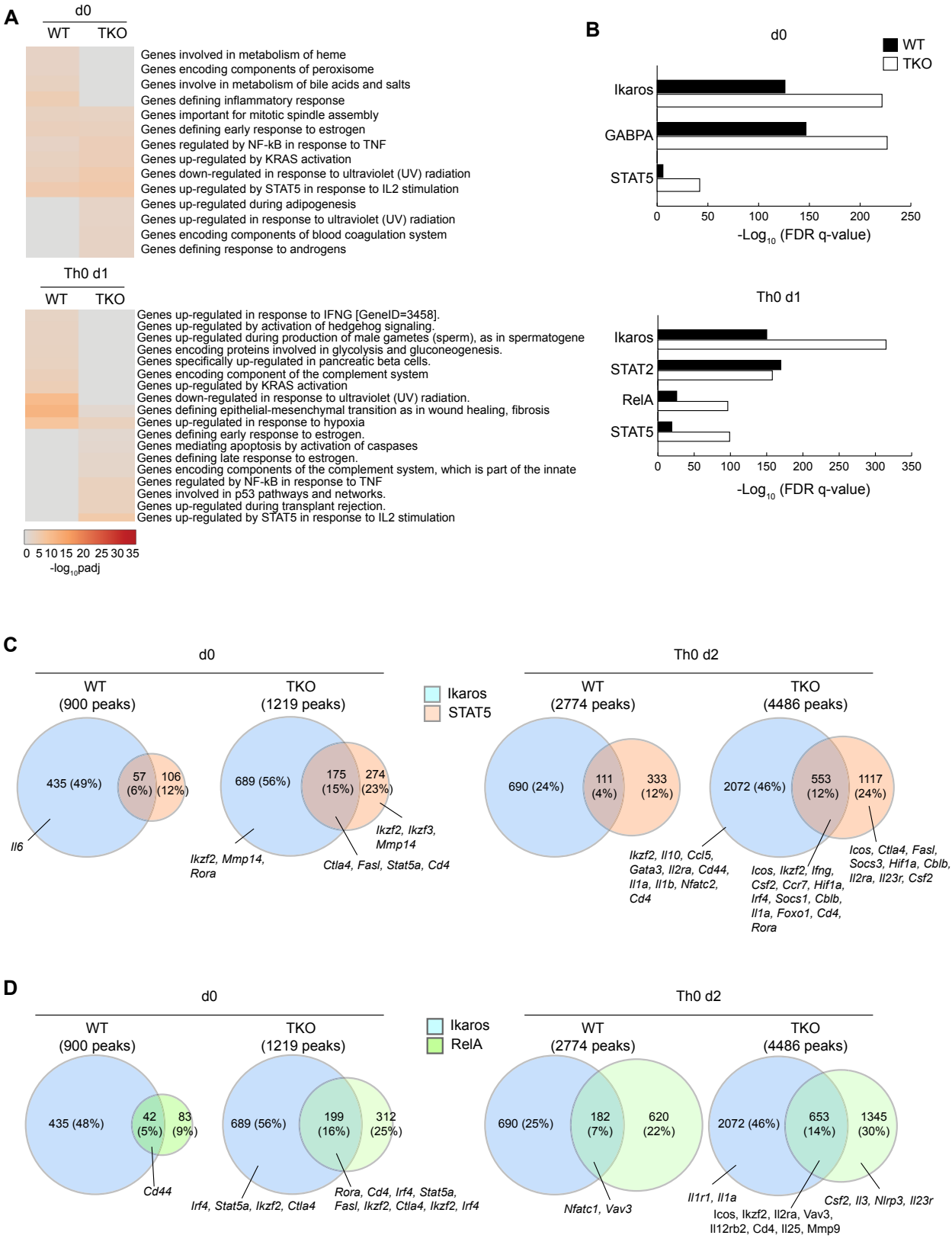

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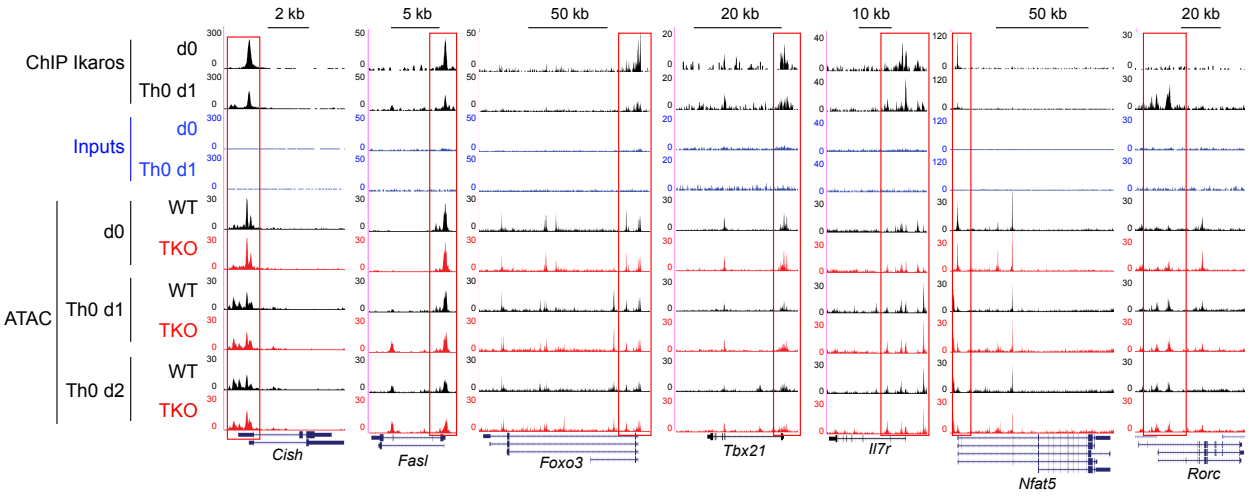

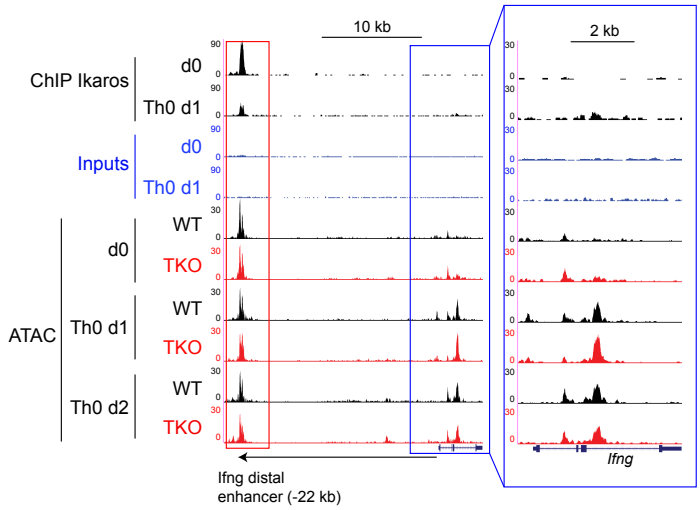

Supplemental Table 1

Name	Primer sequence (5'-3')
m*Csf2* F	TCGTCTCTAACGAGTTCTCCTT
m*Csf2* R	CGTAGACCCTGCTCGAATATCT
m*Ifng* F	CGGCACAGTCATTGAAAGCC
m*Ifng* R	TGTCACCATCCTTTTGCCAGT
m*Il3* F	ATAGGGAAGCTCCCAGAACCT
m*Il3* R	GGATCCACTTCTCCTTGGCT
m*Fasl* F	TTCACCAACCAAAGCCTTAAAG
m*Fasl* R	GCTGGTTGTTGCAAGACTGA
m*RORc* F	TCACCTGTGAGGGGTGCAAG
m*RORc* R	GTTCGGTCAATGGGGCAGTT
m*Il17* F	ATCCCTCAAAGCTCAGCGTGTC
m*Il17* R	GGGTCTTCATTGCGGTGGAGAG
m*L32* F	TTAAGCGAAACTGGCGGAAAC
m*L32* R	TTGTTGCTCCCATAACCGATG
m*Il10* F	CAGTACAGCCGGGAAGACAA
m*Il10* R	TGGCAACCCAAGTAACCCTTA

Part IV

Materials and Methods

Chapter 9

Materials and Methods

9.1 Mice

C57BL/6-Ly5.1 mice were used for co-culture experiments. They express the pan leukocyte marker, CD45.1, while TKO, IKO and the littermate control mice express the CD45.2 allele, C57BL/6-Ly5.2. The mice were kept under SPF conditions.

TKO and IKO mice were described in the manuscript. Animal procedures were approved by the IGBMC ethics committee and the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (2012-096, APAFIS 21777-2019082318 358785v3 and 7760-2016112512394495v4).

9.2 Culture conditions

***in vitro* Th0 culture condition and inhibitors.** Naive $CD4^+$ T cells were obtained as described in the "Materials and Methods" section in the manuscript. Briefly, in the Th0 condition, naive $CD4^+$ T cells ($4x10^4$ cells / well) were activated with anti-CD3 and anti-CD28 (2 µg/ml each) Abs, both pre-coated overnight on a Nunc-immuno 96 well plate (plate-bound) in PBS. Where indicated, neutralizing anti-IL-2 (S4B6) Ab or isotype (30 µg/ml), neutralizing anti-IL-4 and anti-IFNγ Abs (10 µg/ml each) and the pan-caspase inhibitor (QVD-OPh, 20µM) were added. For some experiments, indicated inhibitors were also used as follow: the GSK3 inhibitor (GSK3i CT98014; 0,37µM; Axon Medchem) and the Cyclosporine (CsA; 5nM; Sigma-Aldrich) were added at d0 to the cell culture in IMDM medium supplemented with 10% heat-inactivated FCS, GlutaMAX^{TM}-I, Penicillin/Streptomycin, non-essential amino acids, sodium pyruvate, Hepes and β-mercaptoethanol (IMDM 10% FCS). As control vehicle, DMSO was used. Their inhibitor function is detailed in the Figure 9.1. Where indicated, 40mM of NaCl were added on the culture cell at day 0 (Th0 (NaCl)). At day 3, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each)

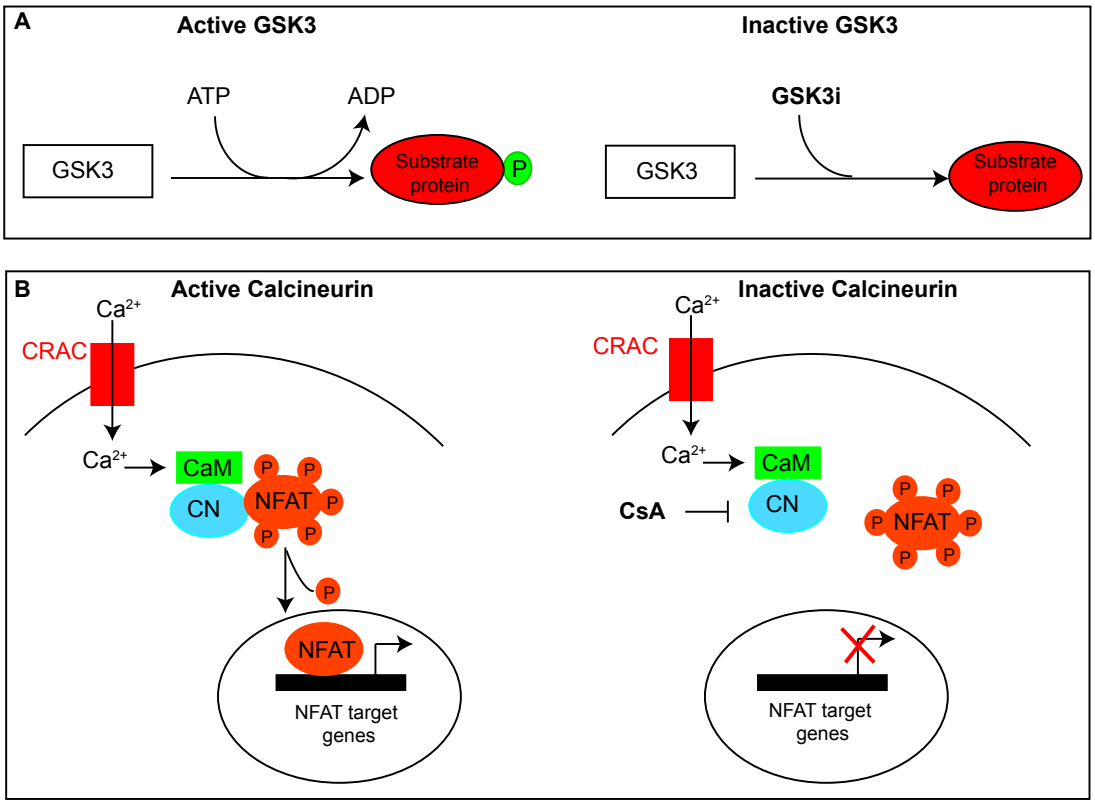

Figure 9.1: **Molecular mechanism of GSK3i and CsA. GSK3i. A)**: GSK3 binds ATP molecules and transfers one phosphate (P) to the target substrate proteins. The GSK3i used is an ATP-competitive molecule. **CsA. B)**: In T cells, following the calcium signaling induction, the activated phosphatase CN dephosphorylates several serine residues of NFAT proteins. NFAT can now enter the nucleus and bind to its target genes. The CsA inhibits the CN activity preventing the dephorphorylation/activation of NFAT.

and GolgiPlug™ (1/1000) for 2h to detect intracellular cytokine expression or for 5h to detect IL-3 intracellular expression.

***in vitro* Th17 culture condition.** For Th17 differentiation, naive $CD4^+$ T cells ($4x10^4$ cells / well) were activated with plate-bound anti-CD3 and anti-CD28 (2 µg/ml each) Abs, in the presence of IL-6 (10 ng/ml) and hTGFβ1 (0.03 ng/ml) for the Th17(TGFβ1) condition, IL-6 (20 ng/ml), IL-1β (20 ng/ml) and IL-23 (40 ng/ml) for the Th17(IL-23) condition (Hasan et al., 2017) or with the addition of 40mM Nacl at day0 to the Th17(TGFβ1) culture for the Th17(NaCl) condition in IMDM 10% FCS. Where indicated, neutralizing anti-IL-2 (S4B6) Ab or isotype (30 µg/ml), neutralizing anti-IL-4 and anti-IFNγ Abs (10 µg/ml each) and the pan-caspase inhibitor (QVD-OPh, 20µM) were added at the culture at d0.

$CD8^+$ T cells: *in vitro* Th0 culture condition. Naive $CD8^+$ T cells ($CD8^+$ $CD44^{low}$ $CD25^-$ $NK1.1^-$ $TCR\gamma\delta^-$) were sorted on either a FACSAria™ II SORP or a FACS ARIA™ FUSION (BD Biosciences) cell sorter. Sort purity was>98%. For Th0

condition, naive $CD8^+$ T cells ($8x10^4$ cells / well) were activated with plate-bound anti-CD3 and anti-CD28 (2 µg/ml each) Abs in IMDM 10% FCS. At day 3, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2 h and stained as described in the "Materials and Methods" of the manuscript.

Supernatant exchange experiments. For supernatant exchange experiments, naive $CD4^+$ T cells were sorted from WT and IKO-tamoxifen injected mice, as described previously. A total number of $4x10^4$ cells cells/well were activated with plate-bound anti-CD3 and anti-CD28 (2 µg/ml each) Abs in Th0 conditions in 200µl of IMDM 10% FCS. The WT and IKO cell cultures were divided into 4 main groups: 1) WT or IKO cells cultured for 3 days without exchange of supernatant; 2) WT or IKO cells cultured for 1 day or 2 days to harvest their respective supernatant in order to replace the supernatant of groups 3 and 4; 3) WT or IKO cells cultured for 1 day or 2 days, subjected to an exchange of their supernatant with the WT supernatant from group 2; 4) WT or IKO cells cultured for 1 day or 2 days, subjected to an exchange of their supernatant with the IKO supernatant from group 2. For the procedure, the supernatant (150µl) from d1 or d2 of WT or IKO cell cultures (group 2) was filtered via a 0.22 µm filter and used to replace the supernatant (150µl) of the receiving cells (groups 3 and 4). QVD-Oph was added at the cell culture at d0 to reduce cell death. At d3, cells from groups 1, 3 and 4 were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h to detect intracellular cytokine expression.

Co-culture experiments. For co-culture experiments, naive $CD4^+$ T cells were sorted from CD45.1 mice, CD45.2 WT and CD45.2 IKO mice, as described previously. A total number of $4x10^4$ cells/well were activated with plate-bound anti-CD3 and anti-CD28 (2 µg/ml each) Abs. Five different culture conditions were performed (Figure 9.2): 1) $4x10^4$ CD45.2 WT cells (blue dots); 2) $4x10^4$ CD45.1 WT cells (green dots); 3) $4x10^4$ CD45.2 IKO cells (red dots); 4) a mix of CD45.1 WT and CD45.2 WT cells and 5) a mix of CD45.1 WT and CD45.2 IKO cells (Figure 9.2). For condition 4 and 5, a different ratio of $CD45.1^+$ and $CD45.2^+$ cells were cultured, keeping the cell number constant at $4x10^4$/well. QVD-Oph was added at the cell culture to reduce cell death. At day 3, cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug™ (1/1000) for 2h and stained as described in the "Materials and Methods" of the manuscript with the addition of anti-CD45.1-PE (A20) and anti-CD45.2-FITC (104.2) (BD Biosciences).

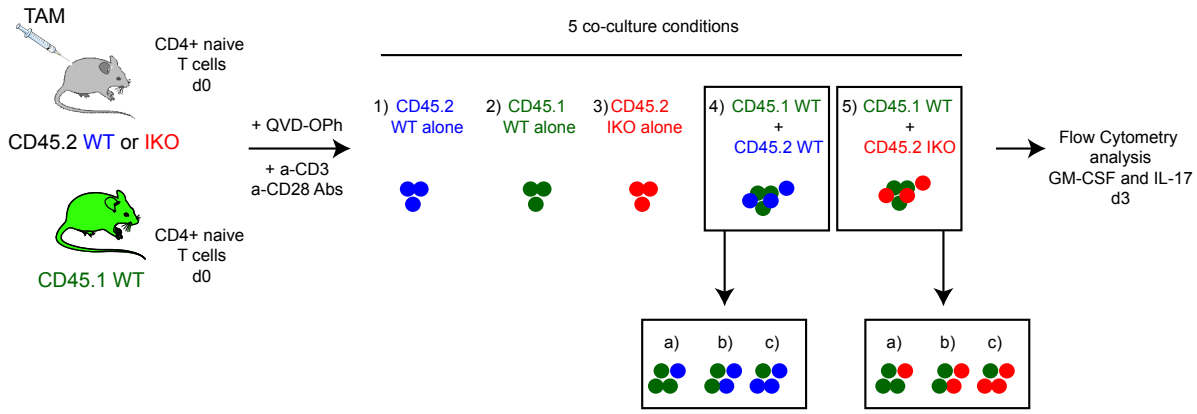

Figure 9.2: **Schema of the co-culture experiments**. Schematic illustration of the co-culture experiments. Five different co-culture conditions are here depicted. In order to unravel the possible effect of an extrinsic factor in the GM-CSF expression in WT and IKO cells, for the culture conditions 4) and 5), CD45.1 and CD45.2 cells were mixed with 3 different cell concentrations in order to have always $4x10^4$ cells/well: a) $3x10^4$ CD45.1 cells + $1x10^4$ CD45.2 WT cells (blue dots) or $1x10^4$ CD45.2 IKO cells (red dots); b) $2x10^4$ CD45.1 cells + $2x10^4$ CD45.2 WT cells (blue dots) or $2x10^4$ CD45.2 IKO cells (red dots); and c) $1x10^4$ CD45.1 cells + $3x10^4$ CD45.2 WT cells (blue dots) or $3x10^4$ CD45.2 IKO cells (red dots).

9.3 Retroviral-infection of murine CD4$^+$ T cells

Plasmids. pMIG was used as retrovirus vectors containing GFP under the regulation of an internal ribosome entry site (IRES). RORγt/pMIGR has been described elsewhere (Zhou et al., 2008) and purchased from Addgene (#24069) (RORγt-IRES-GFP).

Virus production. Platinum-E packaging cells (plat-E) were seeded in 10 ml DMEM plus 10% FCS at $2x10^6$ cells/ml in a 10-cm dish. After 24h, cells were transfected with the retroviral plasmid DNA (10µg) using lipofectamin 2000 (Invitrogen, Grand Island, NY), according to the manufacturer's instructions. The next day, the medium was replaced by 6 ml IMDM plus 10% FCS. The retroviral supernatant was harvested 24h later, filtered on a 0.45 µm filter, and frozen at -80˚C. The medium was replaced with 6 ml IMDM plus 10% FCS for a second harvest again 24h later.

Retroviral-infection. Primary CD4$^+$ T cell retroviral-infection was performed as described in the "Materials and Methods" of the manuscript.

9.4 Immunoprecipitation and immunoblotting

Cells and plasmids. The 293T cells were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin, glutamine, and sodium pyruvate.

C-terminal Myc-tagged murine RORγt plasmid was described elsewhere (Lainé et al., 2015). The murine isoform 1 of Ikaros with a tag HA in its N-terminal was subcloned in the BglII-EcoRI sites of the pTL2 vector (cloning previously done in the laboratory).

Immunoprecipitation experiments. 293T cells ($4x10^6$ in a 10-cm plate) were co-transfected with HA-mIkaros +/- mRORγt-Myc or empty vectors as negative controls (10 µg each) using lipofectamin 2000 (Invitrogen). After 24h, cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.0], 5 mM NaPiP, 1 mM Na3VO4, 20 mM NaPO4 [pH 7.6], 3 mM β-glycerophosphate, 10 mM NaF, 1% Triton X-100 + complete proteases inhibitors (Roche) and Phosphatase inhibitors (Sigma)) and sonicated (15sec on, 20 sec off, 15 min) at 4° C using a bioruptor 200 (Diagenode). The immunoprecipitation of mRORγt-Myc from the whole-cell lysates was performed using an anti-Myc (clone 9E10) Ab overnight at 4° C followed with an incubation with protein A/G magnetic beads (Ademtech) for 3h at 4° C. The immune complexes were separated on SDS-PAGE, transferred on a nitrocellulose membrane, and revealed using anti-Myc and anti–Ikaros Abs by chemiluminescence. Tubulin was used as loading control.

9.5 RT-qPCR

The protocol for the RT-qPCR has been detailed in the "Materials and Methods" of the manuscript. Here, the primers used and not described previously (Table 9.1):

Table 9.1: **Primers used for the RT-qPCR.**

	Forward Sequence 5'-3'	**Reverse Sequence 5'-3'**
m*Eomes*	TACGGCCAGGGTTCTCCGCTCTAC	GGGCCGGTTGCACAGGTAGACGTG
m*Tnf*	CTCCAGGCGGTGCCTATGT	GAAGAGCGTGGTGGCCC
m*Csf2ra*	GGATACACCGGCGAGTCTC	ATCTCGCAGGTCAGGTTCTC
m*Tbx21*	AGGGGGCTTCCAACAATG	AGACGTGTGTGTTAGAAGCACTG
m*Il2*	GAGCAGCTGTTGATGGACCT	AATCCAGAACATGCCGCAGA

9.6 Genotyping

Genomic DNA extraction. The mice genotype was defined performing a PCR on the DNA extracted from mice tails. For the DNA digestion, tails were incubated

overnight at 55°C in a digestion buffer (50mM Tris [pH 8], 100mM NaCl, 100mM EDTA, 1% SDS) supplemented with proteinase K (0.1mg/ml). DNA was then precipitated and purified with NaCl, isopropanol and 70% ethanol. When the pellet was dry, DNA was resuspended in 100µl of TE buffer.

Genotype by PCR. After the extraction of DNA, a PCR was done to define the mice genotype. For the TKO and the littermate control mice, four different primer pair were used: 1) BBO106 + BBO107 to verify the loxP site insertion; 2) BBO107 + BDD40 to verify the presence of an aspecific genomic deletion; 3) BAD496 + BAD497 to check for the presence of the Cre recombinase insertion; 4) the primers ADC253 + ADC254 to amplify the Tec gene were systematically used as a positive control for the presence of the DNA if the Cre was not detected (Table 9.2).

Table 9.2: **TKO primers**.

Primer	**Sequence 5'-3'**
BBO106	GAGGACCAGATATAAGGCAGCTGGG
BBO107	GGCCATCAACGGCATGGAAACGATAA
BDD40	AGCACAGGTTGGACAATACCTGAAA
BAD496	GTTCGCAAGAACCTGATGGACA
BAD497	CTAGAGCCTGTTTTGCACGTTC
ADC253	GGGAGCTGAGATTTAGCATGTG
ADC254	GCATAATATGGCGGTTTGTGG

For the IKO and the littermate control mice, the loxp site insertion and the genomic aspecific deletion were checked with the same primers used in TKO mice (1) BBO106+ BBO107 and 2) BBO107 + BDD40). To control the presence of the Cre recombinase, three different primers were used: F (forward) and R1 (Reverse 1) are found in the Rosa26 locus; while R2 (Reverse 2) is found in the Cre coding sequence (Table 9.3).

Table 9.3: **IKO primers**.

Primer	**Sequence 5'-3'**
RosaF	CTTCCCTCGTGATCTGCAACTC
RosaR1	AAACTCGGGTGAGCATGTCTTT
RosaR2	GTGAAACAGCATTGCTGTCACTT

The total volume of the PCR reaction mix was 25µl and its composition is depicted in the Table 9.4.

The PCR program used is described in the Table 9.5.

Table 9.4: **PCR reaction mix.**

Reagent	Volume for 1 reaction
DNA	1µL
PCR Buffer 10X (Roche; with $MgCl_2$)	2.5µL
dNTP mix (10mM, Thermo Scientific)	0.5µL
Primers (TKO 10µM/IKO 5µM)	TKO 1µL/IKO 3µL F; 0.75µL R1; 1.5µL R2
TAQ polymerase (homemade)	0.6µL
H_2O	up to 25µl

Table 9.5: **PCR program**.

Step	Temperature ° C	Time	Cycle number
Initial denaturation	94	5 min	1
Denaturation	94	15 sec |	
Amplification	60	30 sec |	35
Elongation	72	30 sec |	
Final elongation	72	5 min	1

9.7 *in vivo* mouse model

Psoriasis mouse model

TKO and littermate control (WT) mice at 5-6 weeks of age were anesthetized with 2,5% isofluorane and the Aldara treatment (5% Imiquinod, 3M Pharmaceuticals) was applied topically on the right ear (around 8mg Aldara/ear) for 6 consecutive days (day 0 to day 5), as a control cream (Lanoline cream) was applied on the left ear. 6 days after the first application, mice were sacrificed.

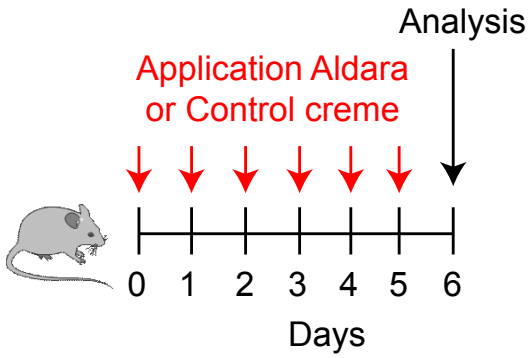

Figure 9.3: **Schematic illustration of the psoriasis mouse model.**

Erythema, scaling and thickening were scored independently on a scale from 0 (absence) to 4 (very marked) as already described elsewhere (Fits et al., 2009). The

ear thickness was measured using a micrometer. The score for the severity of skin inflammation was measured by Psoriasis Area and Severity Index (PASI) and by cumulative PASI score (erythema plus scaling plus thickening) (Fits et al., 2009).

Flow cytometry. The draining lymph nodes (LN) from the ears were isolated. Cells were stimulated with PMA plus ionomycin (0.5 µg/ml each) and GolgiPlug^{TM} (1/1000) for 2h. Then cells were stained with anti-CD4, anti-CD8, anti-CD44, anti-TCRβ, anti-TCR$\gamma\delta$ in PBS 10 % FCS for 15 min on ice, in the presence of an anti-CD16/CD32 (clone 2.4G2) antibody. Then they were fixed for 30 min and permeabilized using the Foxp3 kit (eBiosciences) and stained with anti-GM-CSF and anti-IL-17 Abs in the permeabilization buffer for 30 min on ice. Samples were analyzed on a FACS LSRII (BD Biosciences).

Antibodies. Anti-CD4-AF700 (RM 4-5), anti-CD8-APC-Cy7 (53-6.7) anti-CD44-Pe-Cy7 (IM7), anti-GM-CSF-BV421 (MP1-22E9),anti-TCR$\gamma\delta$-PE (GL3), anti-TCR$\alpha\beta$-PerCPCy5.5 (H57-597), anti-CD16/CD32 (2.4G2) were from Biolegend. Anti-IL-17A-eF660 (eBio17B7) Ab was from eBiosciences.

Histology. Left and right ear skin were cut in three pieces. They were fixed with 4% PFA (paraformaldehyde) for 24h at 4° and embedded in paraffin. 5µm sections were cut using a microtome. The sections were stained with hematoxylin and eosin (H&E).

9.8 Statistics

Data were analyzed using GraphPad Prism. Statistical significance was analyzed by a Mann-Whitney test, an unpaired or paired test to compare two experimental conditions. Unless indicated otherwise, only statistical significance comparing two conditions of interest are mentioned. Graphs and statistics were performed using Prism 7 (*p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 and ****p $\leq$ 0.0001).

Part V

Results

Chapter 10

Results

10.1 CD4$^+$ T cells deficient for Ikaros have a patho genic phenotype

Thanks to our RNA-seq and transcriptome data, we found that Ikaros prevents CD4$^+$ T cells to acquire a pathogenic phenotype. We wanted to confirm the mRNA and the protein expression for some pro-inflammatory genes. Naive CD4$^+$ T WT and IKO cells were sorted and activated *in vitro* in Th0 condition in the presence of QVD-OPh. Protein expression was analyzed by flow cytometry after 3 days of culture and mRNA expression was measured by RT-qPCR at d0 or after 1 or 2 days of Th0 culture.

Consistent with the RNA-seq data, IL-3 and T-bet protein expression were strongly expressed in IKO cells in Th0 condition (Figure 10.1 A and B), as well as the strong increase of *Eomes* mRNA expression in IKO cells compared to WT cells (Figure 10.1 C). *Tnf* mRNA expression was similar in CD3/CD28-activated WT and IKO T cells (Figure 10.1 D right), confirming the RNA-seq data, while surprisingly, TNFα protein expression was substantially increased when Ikaros was lost (Figure 10.1 D left and middle). Altogether, our data suggest that Ikaros regulates the expression of pathogenic genes by different mechanisms. Indeed, Ikaros can regulate the expression of *Csf2*, *Ifng*, *Il3* and *Tbx21* genes at the transcriptional level, while, it seems to regulate TNFα expression via other mechanisms.

The GM-CSFRA is not expressed in WT cells cultured for 3 days in Th17 condition (El-Behi et al., 2011). One hypotheses was that, in the Th0 culture, a strong expression of GM-CSFR in IKO cells could be responsible to maintain the GM-CSF expression via an autocrine mechanism. Indeed, in presence of high concentration of GM-CSF, the STAT5 signaling pathway can be activated (Guthridge et al., 2006) and thus STAT5 could bind to the *Csf2* promoter and positively regulate the GM-CSF expression (Sheng et al., 2014). We could observe that *Csfr2a* mRNA is not

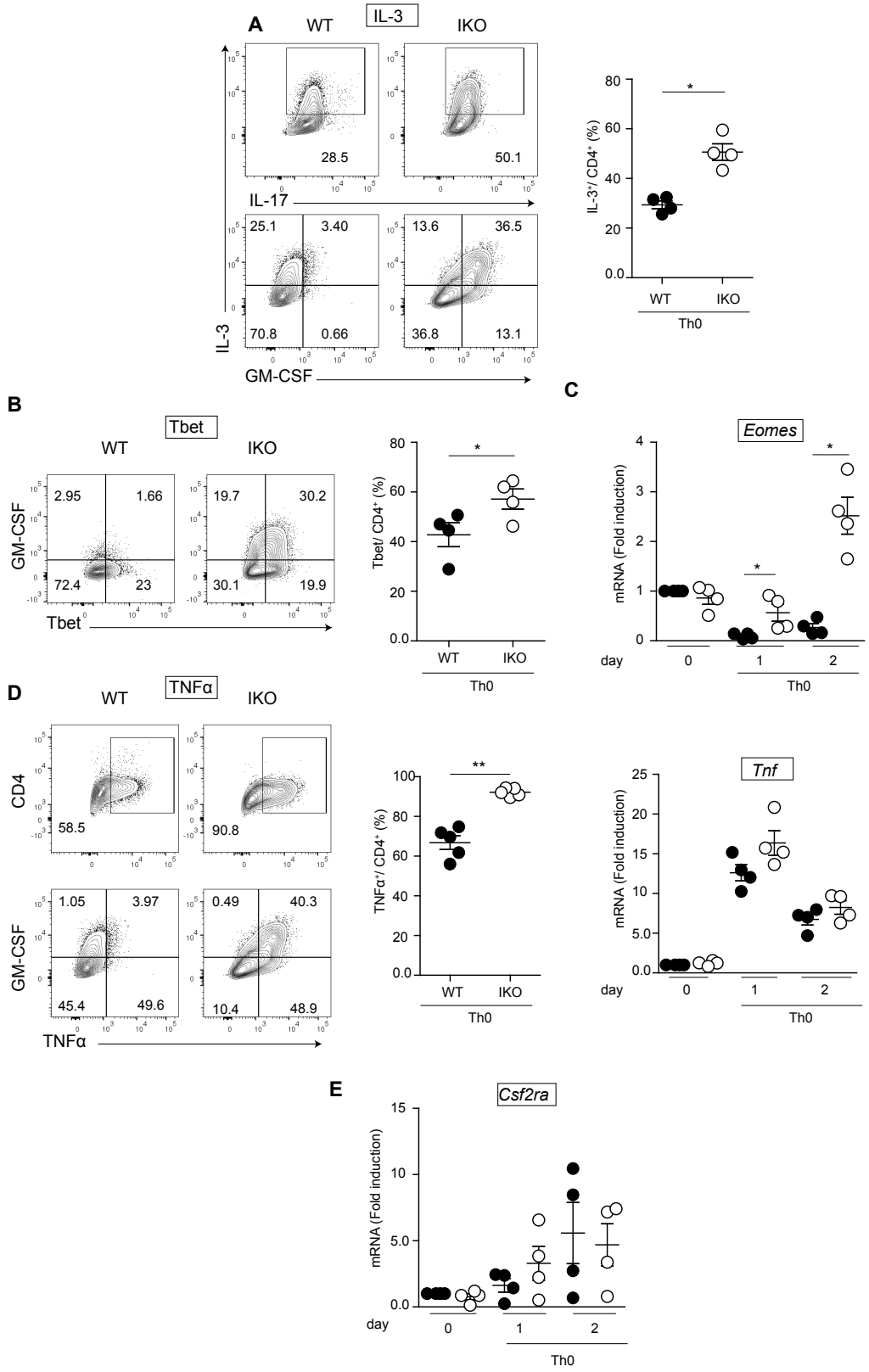

Figure 10.1: **Loss of Ikaros induces a pathogenic phenotype.**

Figure 10.1: **Loss of Ikaros induces a pathogenic phenotype**. **A-E**: Naive LN WT and IKO CD4$^+$ cells from tamoxifen-induced mice were cultured in Th0 conditions with QVD-OPh. **A** Left: Representative contour plots showing GM-CSF and IL-3 expression in WT and IKO CD4$^+$ cells in Th0 condition after 3 days of culture. Right: Graph showing the percentage of IL-3$^+$ cells in WT and IKO LN CD4$^+$ cells cultured as in A left (n=4; mean ± SEM; Mann-Whitney test). **B** Left: Representative contour plots showing GM-CSF and T-bet expression in WT and IKO CD4$^+$ cells in Th0 condition after 3 days of culture. Right: Graph showing the percentage of T-bet$^+$ cells in WT and IKO LN CD4$^+$ cells cultured as in B left (n=4; mean ± SEM, paired test). **C** RT-qPCR analysis of *Eomes* mRNA expression in either naive CD4$^+$T cells (d0) or naive CD4$^+$ T cells cultured in Th0 condition with QVD-OPh for 1 or 2 days (n=4; mean ± SEM; Mann-Whitney test). **D** Left: Representative contour plots showing GM-CSF and TNFα expression in WT and IKO CD4$^+$ cells in Th0 condition after 3 days of culture. Middle: Graph showing the percentage of TNFα$^+$ cells in WT and IKO LN CD4$^+$ cells cultured as in D left. Right: RT-qPCR analysis of *Tnf* mRNA expression in either naive CD4$^+$T cells (d0) or naive CD4$^+$ T cells cultured in Th0 condition with QVD-OPh for 1 or 2 days (n=4; mean ± SEM, Mann-Whitney test). **E** RT-qPCR analysis of *Csf2ra* mRNA expression in either naive CD4$^+$T cells (d0) or naive CD4$^+$ T cells cultured in Th0 condition with QVD-OPh for 1 or 2 days (n=4; mean ± SEM).

expressed at the TN state in both WT and IKO cells, while its expression slightly increases similarly in both WT and IKO cells after activation by a-CD3 and a-CD28 Abs after 1 and 2 days in Th0 condition (Figure 10.1 E), confirming our RNA-seq data. These results indicate that low levels of GM-CSFRα are expressed in activated CD4$^+$ T IKO cells suggesting that the GM-CSF/GM-CSFR signaling could be implicated in the hyper-activation of the STAT5 pathway (Figure 6 of the manuscript).

10.2 CD8$^+$ T cells deficient for Ikaros produce increased levels of GM-CSF, TNFα, but not IFNγ *in vitro*

As Ikaros is also expressed in CD8$^+$ T cells (O'Brien et al., 2014), it was important to determine if the loss of Ikaros in CD8$^+$ T cells also resulted in an increase of pro-inflammatory cytokine production. To analyze this, we sorted naive WT and IKO CD8$^+$ T cells and activated them with a-CD3 and a-CD28 Abs in absence of QVD-Oph (Figure 10.2). At day 3, WT and IKO CD8$^+$ T cells were stimulated with PMA plus ionomycin and GolgiPlug^{TM} for 2h and stained for flow cytometry analysis.

As already described in CD4$^+$ T cells deficient for Ikaros, we noticed more cell death of CD8$^+$ T cells (data not shown) and an increased expression of FasL when Ikaros is lost (Figure 10.2 A). GM-CSF and TNFα expression were also up-regulated

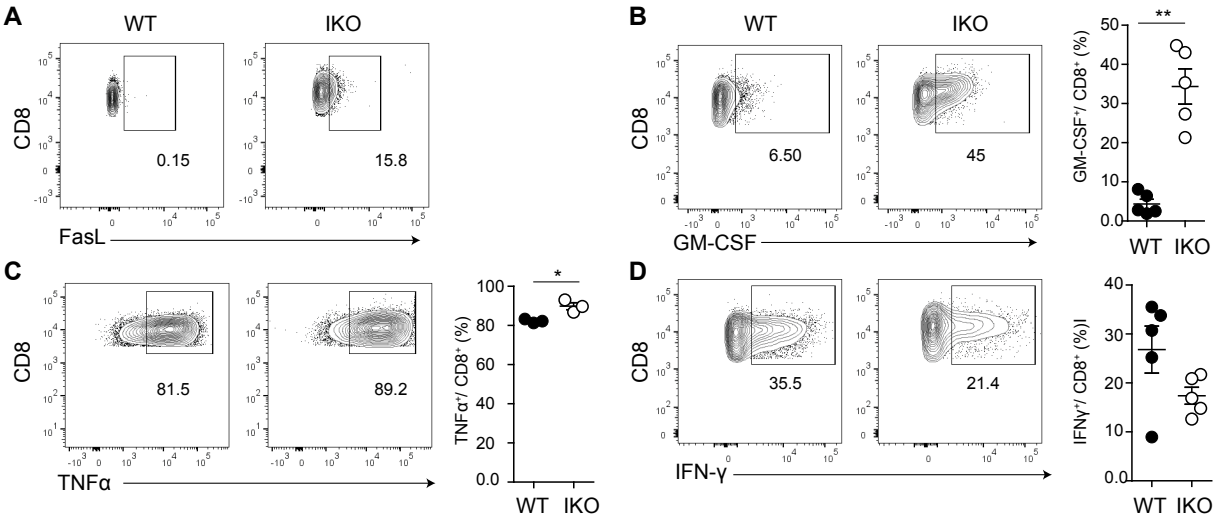

Figure 10.2: **Expression of pro-inflammatory genes in CD8⁺ T cells-lacking Ikaros**. **A-D**: Naive WT and IKO CD8⁺ T cells were sorted and activated with anti-CD3 and anti-CD28 Abs for 3 days in absence of QVD-Oph. Contour plots showing FasL (**A**; n=1), GM-CSF (**B**; n=5; mean ± SEM; Mann-Whitney test), TNFα (**C**; n=3; mean ± SEM; unpaired test) and IFNγ (**D**; n=5) expression in CD8⁺ T cells.

in a similar way as described in CD4⁺ T cells deficient for Ikaros (Figure 10.2 B and C). However, in contrast to CD4⁺ IKO cells (S3 of the manuscript), the IFNγ expression was decreased in CD8⁺ IKO cells (Figure 10.2 D).

These results suggest that CD8⁺ T cells deficient for Ikaros may express some pro-inflammatory cytokines, but they do not recapitulate all the effects observed in CD4⁺ T cells for the production of pro-inflammatory cytokines driven by Ikaros loss.

10.3 GM-CSF expression correlates to Ikaros loss in a dose-dependent way

We found that Ikaros loss leads to an increase expression of GM-CSF and a decreased production of IL-17. We wanted to determine if there was a dose-dependent effect of Ikaros on GM-CSF and IL-17 expression. To do this naive CD4⁺ T cells from the LN of WT (*Ikzf1*$^{f/fCD4Cre-}$), TKO (*Ikzf1*$^{f/fCD4Cre+}$) and Heterozygotes-Hz (*Ikzf1*$^{f/+CD4Cre+}$) mice were purified and cultured under Th0 or Th17 conditions. GM-CSF, IL-17 and Ikaros expression were analyzed after 3 days of culture by flow cytometry.

In both Th0 and Th17 conditions, we observed that Hz CD4⁺ T cells produced a % of GM-CSF that is intermediate between the WT and TKO cells. On the contrary, a total loss of Ikaros seems to be necessary to induce a drastic reduction of IL-17 expression (Figure 10.3). These results indicate that GM-CSF expression is

inversely proportional to Ikaros levels, while IL-17 expression is already strongly expressed when one allele of *Ikzf1* is present.

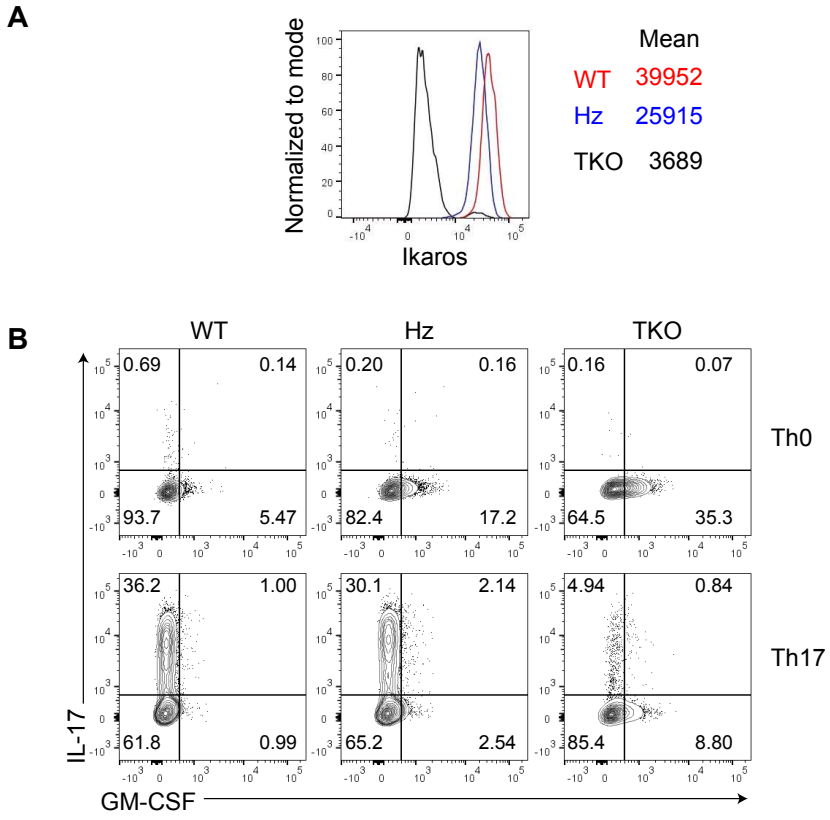

Figure 10.3: **GM-CSF expression depends on the amount of Ikaros expressed**. **A**: Histogram showing Ikaros expression and mean in WT (red), Hz (blue) for Ikaros and TKO (black) CD4$^+$ T cells after 3 days of culture in Th0 conditions (n=2). **B**: Representative contour plots showing IL-17 and GM-CSF expression in WT, Hz and TKO CD4$^+$ T cells after 3 days of culture in Th0 (top) or Th17(TGFβ1) (bottom) conditions in presence of QVD-Oph (n=2).

10.4 GM-CSF expression in WT CD4$^+$ T cells may depend on a membrane-bound factor

One of our hypotheses was that one or several factors secreted by KO cells, such as GM-CSF, IL-2, or IL-23 could be the cause of a strong expression of GM-CSF in KO cells, acting in an autocrine way. In order to investigate this hypothesis, supernatant exchange experiments were performed. Naive CD4$^+$ T cells from WT and IKO-tamoxifen injected mice were sorted and activated in Th0 culture conditions with a-CD3 and a-CD28 Abs in presence of QVD-OPh. Every day the supernatant from WT and IKO culture cells was exchanged with cell-free supernatant collected from WT or IKO cells. GM-CSF, IL-17 and Ikaros expression were analyzed after 3

days of culture by flow cytometry. Very interestingly, we observe that the addition of the IKO supernatant on WT cells in Th0 cultures does not lead to an increase of GM-CSF expression by WT cells and complementary the addition of the WT supernatant on IKO cells does not determine a decreased GM-CSF production (Figure 10.4), suggesting that in our culture conditions, GM-CSF expression does not seem to depend on the secretion of an extrinsic factor.

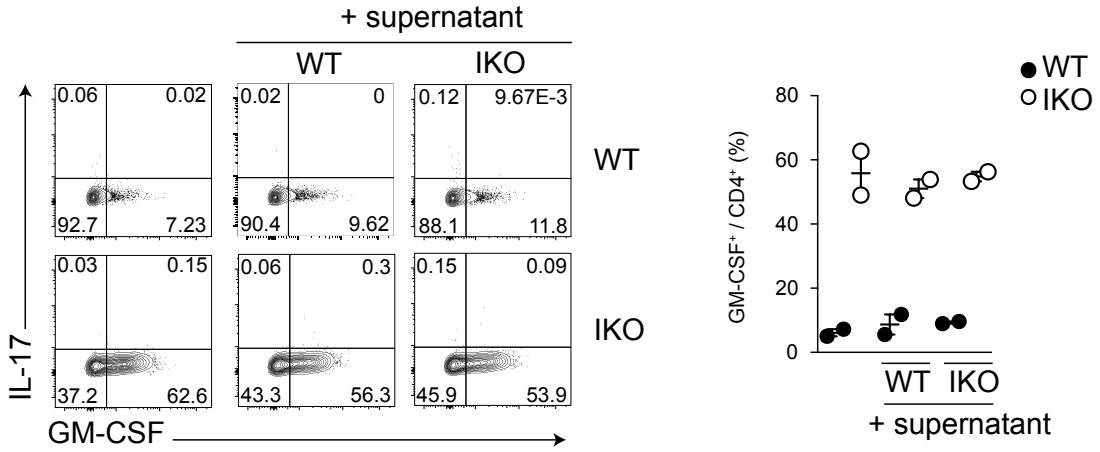

Figure 10.4: **GM-CSF expression does not depend on an extrinsic factor.** Left: representative contour plots showing GM-CSF and IL-17 expression in WT and IKO CD4$^+$ T cells at d3 of Th0 culture upon transfer of WT supernatant on WT/IKO CD4$^+$ T cells or IKO supernatant on WT/IKO CD4$^+$ T cells at d1 and d2. As a control, WT and IKO CD4$^+$ T cells were not subjected to supernatant exchange. Right: percentage of WT and IKO GM-CSF$^+$ cells among CD4$^+$ T cells in Th0 conditions at d3 and cultured as in left (n=2; Mean ± SEM).

To investigate if a cell-cell contact was necessary for GM-CSF expression, we performed co-culture experiments to complement our supernatant exchange experiments. Naive CD4$^+$ CD45.1$^+$ WT cells and naive CD4$^+$ CD45.2$^+$ WT and IKO cells were sorted and activated with a-CD3 and a-CD28 Abs for 3 days in presence of QVD-OPh, as depicted in the Figure 10.5A. Five different conditions of culture were defined (Figure 9.2 in "Materials and Methods" for more details). Briefly, we mixed an increasing number of CD45.1$^+$ WT (1, 2 or 3x10^4 cells) with a decreasing number of CD45.2$^+$ WT or IKO cells (3, 2, or 1x10^4), and vice versa, always keeping a constant cell number (4x10^4) per well. As a control, CD45.1$^+$ WT and CD45.2$^+$ WT or IKO were cultured alone. Cytoplasmic GM-CSF and IL-17 production were analyzed after 3 days of culture by flow cytometry. We observe that the more we add CD45.2$^+$ IKO cells, the more CD4$^+$ CD45.1$^+$ WT cells express GM-CSF, reaching up ≈16% in the presence of 3x10^4 CD45.2$^+$ IKO cells (Figure 10.5 B-top and C), suggesting that IKO cells can influence the GM-CSF production. However, adding CD4$^+$ CD45.1$^+$ WT T cells, even at a high number, with CD45.2$^+$ IKO T cells does not change GM-CSF expression by IKO cells (Figure 10.5 B-bottom), suggesting that

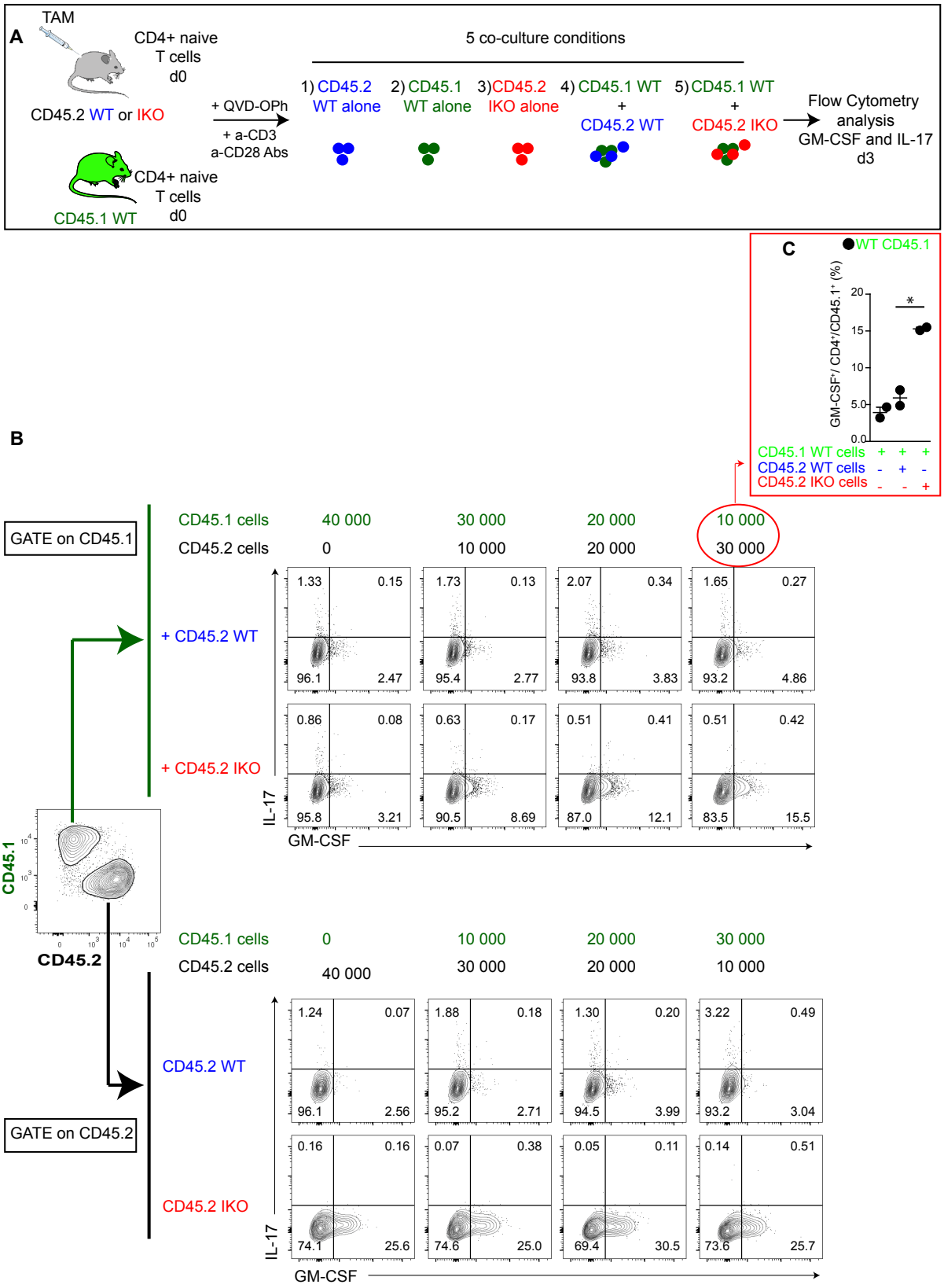

Figure 10.5: **GM-CSF expression in WT CD4⁺ T cells may be stimulated by a membrane-bound factor.**

Figure 10.5: **GM-CSF expression in WT CD4$^+$ T cells may be stimulated by a membrane-bound factor**. **A** Schema of co-coculture experiments. Naive CD4$^+$ CD45.1$^+$ WT cells and CD4$^+$ CD45.2$^+$ WT or IKO T cells were sorted and activated with a-CD3 and a-CD28 Abs in presence of QVD-OPh in Th0 culture condition for 3 days. Cells were mixed in each well into 5 different conditions: 1) CD45.2 WT alone, 2) CD45.1 WT alone, 3) CD45.2 IKO alone, 4) CD45.1 WT + CD45.2 WT at different ratios and 5) CD45.1 WT + CD45.2 IKO at different ratios. **B** up: Representative contour plots of GM-CSF and IL-17 expression in CD4$^+$ CD45.1$^+$ WT cells in culture with CD4$^+$ CD45.2$^+$ WT or CD4$^+$ CD45.2$^+$ IKO cells. Bottom: Representative contour plots of GM-CSF and IL-17 expression in CD4$^+$ CD45.2$^+$ WT or IKO cells in culture with CD4$^+$ CD45.1$^+$ WT cells. **C**: Statistical analysis of the co-culture condition with 10 000 CD4$^+$ CD45.1$^+$ WT cells and 30 000 CD4$^+$ CD45.2$^+$ WT or IKO cells. Percentage of CD4$^+$ CD45.1$^+$ WT cells-expressing GM-CSF. (n=2; mean ± SEM; Unpaired test).

WT T cells cannot influence GM-CSF production in KO T cells. As expected, culturing CD45.2$^+$ WT cells with CD45.1$^+$ WT CD4$^+$ T cells does not change GM-CSF production (Figure 10.5 B).

Together, these data suggest that a membrane-bound factor, rather than a soluble factor, seems to be present on IKO cells and is potentially implicated, at least in part, in the production of GM-CSF by WT cells. This suggest that a juxtacrine, rather than a paracrine signaling, is favored for the expression of GM-CSF in WT cells. These results can not totally exclude the role of cytokines secreted in the close environment that could regulate the GM-CSF expression acting in an autocrine way.

10.5 Polarization of Ikaros-deficient CD4$^+$ T cells under conventional vs pathogenic conditions

Naive CD4$^+$ T cells deficient for Ikaros sorted and cultured under TH17(TGFβ1) culture conditions fail to produce IL-17 and express low levels of GM-CSF (Figure 2 of the manuscript). One possible cause is that the IL-6 and/or TGFβ1 signaling pathways are impaired when Ikaros is lost. The IL-6/STAT3 signaling has been explored and did not seem to be impaired in the absence of Ikaros (Maurer thesis, unpublished data). We therefore questioned if other Th17 culture conditions would result in the same default of production of IL-17 and GM-CSF when Ikaros is lost.

TH17(TGFβ1) vs Th17(IL-23) conditions. We decided to culture naive CD4$^+$ WT and IKO T cells under a cocktail of cytokines that polarize cells towards a pathogenic Th17 phenotype, but that does not include TGFβ1. Therefore, we chose the Th17(IL-23) culture condition (IL-6+ IL-23+ IL-1β) (Ghoreschi et al., 2010). WT CD4$^+$ T cells successfully express IL-17 under both Th17(TGFβ1) and Th17(IL-23) culture conditions (Figure 10.6 A). However, we observed that either the Th17(TGFβ1)

or the Th17(IL-23) cytokine cocktail could not lead to the IL-17 production in CD4$^+$ IKO cells (Figure 10.6 A) indicating that IL-17 expression is not occurring whatever the cytokine cocktail used. Because Th17(IL-23) culture conditions are described to polarize Th17 cells towards a pathogenic phenotype and IL-23 and IL-1β have been described to positively regulate the GM-CSF expression in WT CD4$^+$ T cells (El-Behi et al., 2011), we analyzed the effect of these two cytokines and the Th17(IL-23) culture condition on the GM-CSF expression in WT and IKO cells. The addition of IL-23 or IL-1β alone on the cell culture did not influence the GM-CSF production either in WT or in IKO CD4$^+$ T cells (Figure 10.6 B left). The cell culture under Th17(IL-23) conditions did not increase the GM-CSF expression either in WT or in IKO cells, suggesting that this cocktail of cytokines is not responsible for the GM-CSF expression (Figure 10.6 B right). These results suggest that Ikaros-deficient cells fail to produce IL-17 whatever the exogenous cytokines present in the culture. Moreover, the data indicate that IL-6, IL-23 or IL-1β are not required for a maximal expression of GM-CSF in IKO CD4$^+$ T cells and they are not able to influence the GM-CSF expression in WT CD4$^+$ T cells in our culture conditions.

TH17(TGFβ1) vs Th17(NaCl) conditions. The osmotic stress pathway is another signaling that has been described to be implicated in the IL-17 and GM-CSF regulation (Kleinewietfeld et al., 2013; Wu et al., 2013). The perturbation of the osmotic equilibrium in CD4$^+$ T cells is responsible of an increased expression of IL-17 (Kleinewietfeld et al., 2013), while its role on the GM-CSF expression remains unclear. On one hand, in human CD4$^+$ T cells cultured under Th17 condition, the addition of NaCl induce an increased expression of GM-CSF (Kleinewietfeld et al., 2013). On the other hand, in murine CD4$^+$ T cells cultured under Th0 condition, the addition of NaCl caused a decreased production of GM-CSF (Wu et al., 2013). However, in the two articles it is clear that the addition of NaCl in the cell culture biases the Th differentiation towards pTh17 cells. To investigate the role of the osmotic stress pathway in the GM-CSF and IL-17 expression in IKO cells, naive CD4$^+$ WT and IKO T cells were sorted and cultured under Th0 or Th17(TGFβ1) culture conditions, in presence or not of 40mM of NaCl. As expected, in WT cells, we observed that the addition of NaCl in the Th17 cell culture significantly increased the IL-17 expression (Figure 10.6 C). However, in IKO cells we could not observe any important change in the IL-17 expression (n=1), suggesting that Ikaros is required for IL-17 expression under osmotic stress conditions. In our hands, NaCl addition does not have any major effect on the GM-CSF production in WT cells, while surprisingly reduces GM-CSF expression in CD4$^+$ T cells deficient for Ikaros in Th17, albeit less in Th0 condition. This suggests that the osmotic stress generated by the addition of NaCl in the culture is able to modulate the GM-CSF expression in IKO cells under Th0 or Th17 conditions.

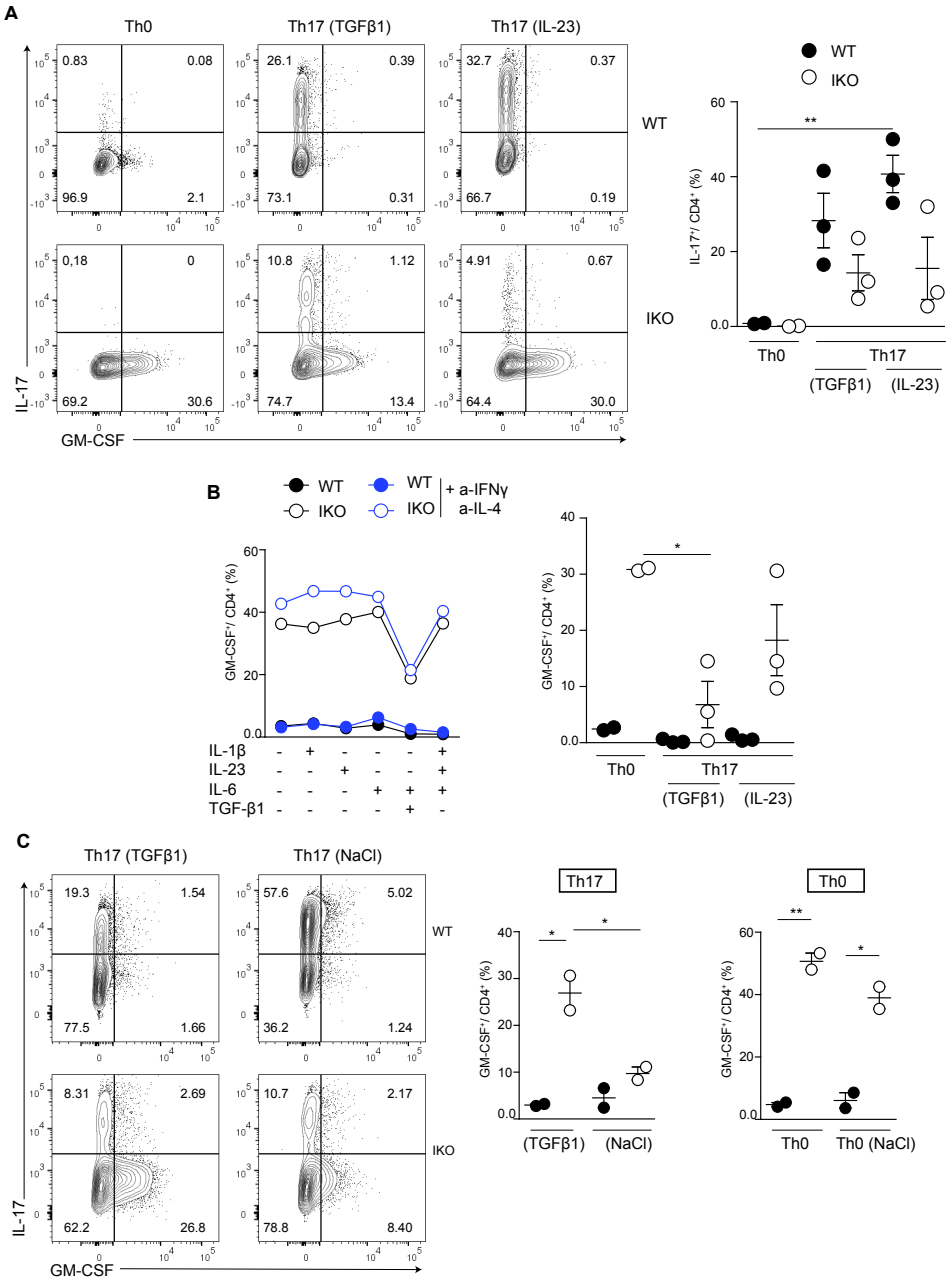

Figure 10.6: **Polarization of Ikaros-deficient CD4⁺ T cells under conventional vs pathogenic conditions. A-B**: Naive LN WT and IKO CD4⁺ cells were cultured in Th0 or Th17(TGFβ1) conditions or in the presence of IL-6 (20 ng/ml), IL-1β (20 ng/ml) and IL-23 (40 ng/ml) for the Th17(IL-23) condition or in the presence of IL-6, IL-1β or IL-23 alone for 3 days with QVD-OPh in the presence or absence of a-IFNγ and a-IL-4 Abs. *Legend continues on the next page.*

Figure 10.6: **Polarization of Ikaros-deficient CD4$^+$ T cells under conventional vs pathogenic conditions. A** left: representative contour plots showing GM-CSF and IL-17 expression in the absence of a-IFNγ and a-IL-4 Abs in WT and IKO CD4$^+$ cells in Th0, Th17(TGFβ1) and Th17(IL-23) conditions. Right: statistical analysis of WT and IKO CD4$^+$ T cells expressing IL-17 in Th0, Th17(TGFβ1) and Th17(IL-23) conditions (n=2-3; mean ± SEM; unpaired test). **B** Left: representative graph showing the proportion of GM-CSF$^+$ cells in WT and IKO LN CD4$^+$ cells cultured as in A in the presence or absence of a-IFNγ and a-IL-4 Abs. Right: proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 3 days of Th0, Th17(TGFβ1) or Th17(IL-23) culture in the absence of a-IFNγ and a-IL-4 Abs (n=2-3; mean ± SEM; unpaired test). **C**: Naive LN WT and IKO CD4$^+$ cells from tamoxifen-induced mice were cultured in Th0 or Th17 conditions in the presence or absence of 40mM of NaCl for 3 days with QVD-OPh in the absence of a-IFNγ and a-IL-4 Abs. Left: representative contour plots showing GM-CSF and IL-17 expression in WT and IKO CD4$^+$ cells in Th17(TGFβ1) and Th17(NaCl) culture conditions. Middle: statistical analysis of the proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 3 days of Th17(TGFβ1) or Th17(NaCl) culture. Right: Statistical analysis of the proportion of WT and IKO GM-CSF$^+$ CD4$^+$ T cells after 3 days of Th0 or Th0(NaCl) culture (n=2; mean ± SEM; unpaired test).

Collectively, our results suggest that the absence of Ikaros leads to a decreased expression of IL-17 that does not depend on the exogenous cytokines, but rather involves an intrinsic mechanism such as via an epigenetic modification or via the expression of a protein that is necessary for the IL-17 expression. In addition, GM-CSF expression in KO cells seems to be differently modulated by exogenous cytokines and the osmotic stress condition. Further investigations will be required to clarify the role of the salt on GM-CSF expression when Ikaros is lost.

10.6 The role of IL-2 in Th0 and Th17 cells deficient for Ikaros

In order to better investigate the possible causes responsible for the IL-17 impaired expression and the increased GM-CSF production in cells deficient for Ikaros, we evaluated the RNA expression of some TFs or cytokines that are considered important for the regulation of IL-17 or GM-CSF expression. The over-expression of T-bet causes the decreased production of IL-17 (Lazarevic et al., 2011). In our Th17 transcriptome analysis, we did not observed an increased expression of *Tbx21* in CD4$^+$ T cells deficient for Ikaros. Indeed, we confirmed these results by RT-qPCR suggesting that T-bet is not implicated in the decreased expression of IL-17 in IKO cells (Figure 10.7 A).

The IL-2 expression can impair the IL-17 expression (Laurence et al., 2007) and,

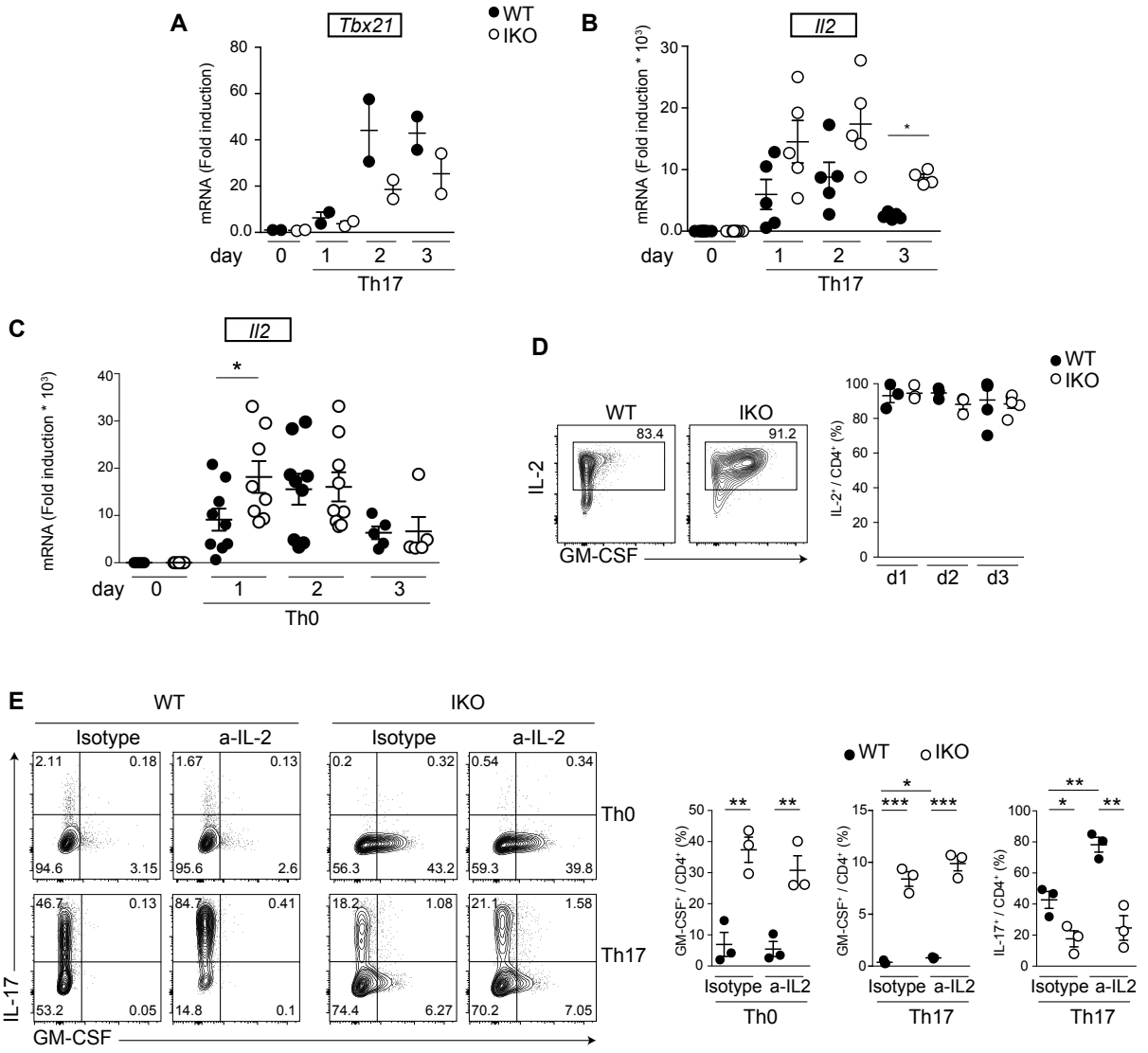

Figure 10.7: **Expression of *Tbx21* in Th17 cells and the role of IL-2 in Th0 and Th17 cells deficient for Ikaros. A-C** RT-qPCR analysis of *Tbx21* (**A**) and *Il2* (**B** and **C**) mRNA expression in either naive CD4⁺T cells (d0) or naive CD4⁺ T cells cultured in Th17 (**A** and **B**) or Th0 (**C**) condition with QVD-OPh for 1, 2 or 3 days (A: n=2; B: n=5; C: n=8. Mann-Whitney test). mRNA expression is represented as -fold induction with respect to the d0 WT sample. The same d0 WT and IKO samples were considered for *Il2* mRNA expression in Th17 and Th0 conditions. **D** left: representative contour plots showing IL-2 and GM-CSF expression at d3 in WT and IKO cells cultured in Th0 conditions with QVD-OPh. Right: proportions of WT and IKO IL-2⁺ CD4⁺ T cells at d1-3 in Th0 cultures with QVD-Oph (n=3-5; mean ± SEM). **E**: Naive LN WT and IKO CD4⁺ cells from tamoxifen-induced mice were cultured in Th0 or Th17 conditions for 3 days with QVD-OPh and a neutralizing anti-IL-2 Ab or isotype control. Left panels: representative contour plots showing GM-CSF and IL-17 expression in WT and IKO CD4⁺ cells. Right panels: proportion of WT and IKO GM-CSF⁺ CD4⁺ T cells after 3 days of Th0 or Th17 culture or IL-17⁺ CD4⁺ T cells after 3 days in Th17 culture (n=3; mean ± SEM; unpaired test).

on the contrary, it has been described to be able to positively regulate the GM-CSF expression via the activation of the STAT5 signaling cascade (Sheng et al., 2014). At the mRNA level, by using RT-qPCR analysis, we observed that *Il2* expression was up-regulated in cells deficient for Ikaros and cultured under Th17 condition (Figure 10.7 B). While in Th0 culture conditions, at the mRNA level, in IKO cells the *Il2* expression was up-regulated only at day 1 (Figure 10.7 C). However, at the protein level, we show that the percentage of $CD4^+$ T cells producing IL-2 in Th0 conditions is comparable between WT and IKO (Figure 10.7 D). Indeed, we asked whether IL-2 deregulation observed at the mRNA level prevents the IL-17 expression and induces the GM-CSF expression in Ikaros null cells. Naive WT and IKO T cells, cultured in Th0 and Th17 conditions, in the presence of anti-IL-2 neutralizing Abs or a control isotype, were measured for IL-17 and GM-CSF production. Interestingly, in the Th17 condition, the anti-IL-2 Ab significantly increased the IL-17 expression in WT cells, as expected, but had no effect on IKO cells (Figure 10.7 E). Surprisingly, the presence of anti-IL-2 neutralizing Abs in Th0 and Th17 conditions did not reduce the GM-CSF expression in IKO cells, while it slightly increased the GM-CSF production in WT cells cultured in Th17 condition (Figure 10.7 E). These data indicate that the decreased expression of IL-17 and the increased expression of GM-CSF in Ikaros null cells seem to be IL-2-independent.
Together, these data suggest that T-bet and IL-2 are not playing a potential negative role of IL-17 regulation in the absence of Ikaros and that IL-2 does not seem to be implicated in the regulation of the GM-CSF expression in our culture conditions.

10.7 Ikaros loss in *in vivo* psoriasis mouse model

In vitro, Ikaros loss promotes the differentiation of $CD4^+$ T cells towards a pathogenic phenotype. However, it is important to determine if these cells have a pathogenic function *in vivo*. We wanted to evaluate specifically the effects of the absence of Ikaros in $CD4^+$ T cells in a Th17-related *in vivo* disease mouse model. To do that, we decided to use the TKO mice. In order to correctly choose the challenge to present to mice, we had to consider that the TKO mice develop a T-ALL from 8-9 weeks of age and that the induction and development of the chosen disease needed to happen in around 1-2 weeks in young mice. For these reasons, the psoriasis mouse model was chosen. This model was a good compromise because the induction of the disease and its study were possible in only 1 week and it led us to study the IL-17 and GM-CSF expression. Indeed, the IL-17 cytokine is part of the principal axis IL-23/Th17 implicated in the induction of the disease, while the role of GM-CSF in the psoriasis induction is not clear and is still a matter of debate.

Aldara was topically applied on the right ear of WT and TKO mice for 6 consec-

utive days. The induction of the disease was measured daily and the T cell immune response was analyzed in WT and TKO mice at the 7th day of the study. From day 2, the inflammation in ears treated with Aldara was visible and continually increased in severity up to the end of the experiment, but surprisingly, without any difference between WT and TKO mice (Figure 10.8 A). The histology analysis showed an increased epidermal thickening in the ear skin that was comparable between WT and TKO mice (Figure 10.8 B). These data suggest that the loss of Ikaros in T cells is not sufficient to influence the progression of psoriasis.

We decided to pursue our study and analyzed the T cell immune response. T lymphocytes from the LN draining the ears were analyzed by flow cytometry and by RT-qPCR. In the LN draining the ears treated with the control creme, as expected, an increased expression of *Csf2* was observed in TKO mice. In the LN draining ears treated with Aldara, WT and TKO mice produced *Csf2* and *Il17* mRNA, without any differences (Figure 10.8 C). The GM-CSF expression was confirmed also by flow cytometry analysis. Indeed, in the LN draining the ears treated with Aldara or with the control cream, we observed that the percentage of cells expressing GM-CSF among $CD4^+$ T cells was more important in TKO cells, however the cell number seems to remain unchanged between WT and TKO mice (Figure 10.8 D). These results suggest that even if the GM-CSF^+ cells are more produced in TKO mice, this is not sufficient for the induction of a more severe disease. These results seem to be in line to what described by Hartwig and colleagues, only at the end of 2018. Indeed, in a psoriasis mouse model they show that Treg cells could prevent the expansion of $CD4^+$ T cells-producing GM-CSF and that GM-CSF production was crucial for the development of a more severe psoriasis when Treg cells were depleted (Hartwig et al., 2018). In our mouse model, we did not analyze the Treg cells, analysis that would be really interesting in order to understand the behavior of Treg cells deficient for Ikaros *in vivo*.

During the psoriasis induction, the innate immune response is first in line to be activated and then production of IL-17 by $\gamma\delta$ T cells is considered a critical point for the development of the disease (Becher and Pantelyushin, 2012). It is only later in the induction of the disease, that the adaptive immune response is activated and leads to the differentiation of $CD4^+$ Th17 cells expressing IL-17. In the TKO mouse model, Ikaros is absent only in $CD4^+$ and $CD8^+$ T cells, while $\gamma\delta$ T cells have not a deletion of Ikaros. Indeed, only few $\gamma\delta$ T cells express CD4 molecules. We therefore explored if the induction of psoriasis in TKO mice was potentially occurring because of the presence of functional $\gamma\delta$ T cells. We found that TKO $\alpha\beta$ $CD4^+$ T cells have a defect in IL-17 production during psoriasis development (Figure 10.8 E). However, $\gamma\delta$ T cells from TKO mice highly express IL-17 to similar levels compared to $\gamma\delta$ T cells from WT mice (Figure 10.8 F). This suggests that $\gamma\delta$ T cells from TKO and WT

mice are equally functional, because not subjected to *Ikzf1* deletion, and are probably responsible for the similar development of psoriasis. This is a good possibility given the importance of γδ T cells in psoriasis, indeed it has been described that *Tcrb*$^{-/-}$ mice are able to develop a similar psoriasis than WT mice, while the psoriasis induction in *Tcrd*$^{-/-}$ mice is much more lower than in WT mice (Pantelyushin et al., 2012).

The development of T-ALL in our model of Ikaros-deficient mice is a limitation to study the pathogenicity of T cells. We are aware that the psoriasis model is not the best one to understand the *in vivo* function of GM-CSF. However, the short time window of the pathologic development and the involvement of pro-inflammatory cytokines in this model seemed to give an alternative to the limitations of our study, which was ended up unsuccessful. Further mouse models, that are more suitable, will be approached in the discussion.

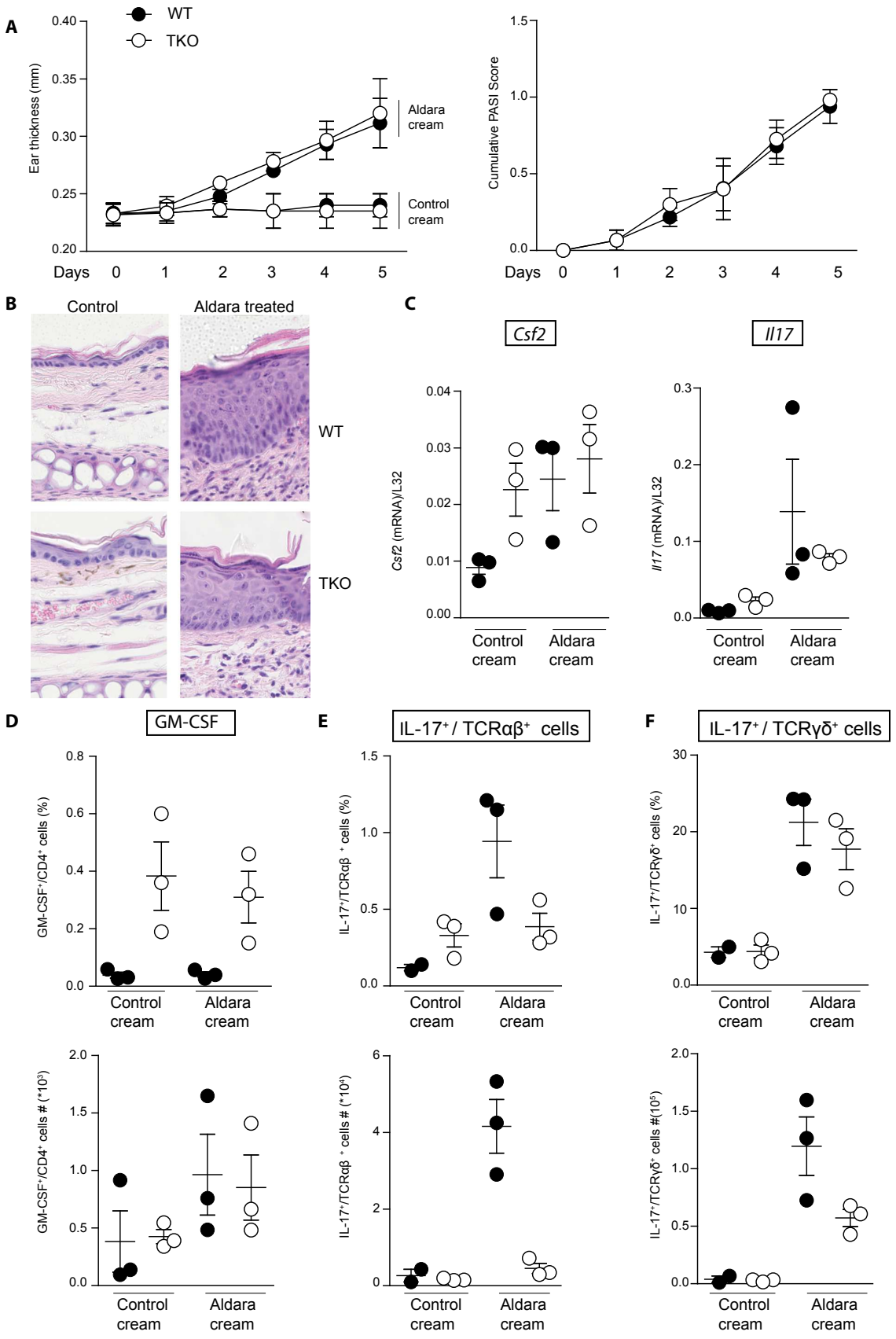

Figure 10.8: **TKO mice develop imiquinod-induced psoriasis similarly to WT mice.**

Figure 10.8: **TKO mice develop imiquinod-induced psoriasis similarly to WT mice.** Aldara cream (containing Imiquinod) was topically applied on the right ear of WT and TKO mice for 6 consecutive days, while the control cream (Lanoline) was applied on the left ear. The induction of the disease was monitored daily and the T cell immune response was analyzed in WT and TKO mice at the 7th day of the study. **A** left: Ear thickness of the control- and Aldara-cream-treated ears is represented at the indicated days. Right: the cumulative PASI score (ear erhytema, scaling and thickness) is shown for the Aldara-treated ear. Each dot represents the mean score ± SD. The results are from 2 independent experiments and a total of 7 WT and 7 TKO mice. **B**: representative H&E staining of the control or Aldara cream-treated ears of WT and TKO mice. **C-F**: At the 7th day of the procedure, WT and TKO mice were sacrificed and the LN draining the ears were analyzed (n=3; mean ± SEM). **C**: RT-qPCR analysis of *Csf2* (left) and *Il17* mRNA expression (right) in the draining LN from the Aldara or control cream-treated ears. Relative mRNA levels were calculated using L32 as internal control. Each dot represents 1 mouse. **D-F**: Cells from the LN draining the treated-ears were stimulate with PMA, Ionomycine and GolgiPlug for 2h and the intracellular expression of GM-CSF and IL-17 in αβ and γδ T cells was analyzed by flow cytometry. The top graphs show the percentage of GM-CSF$^+$ (**D**), IL-17$^+$ among TCRαβ cells (**E**) and IL-17$^+$ among TCRγδ cells (**F**), while the bottom graphs show the corresponding cell numbers (n=3; mean ± SEM; Mann-Whitney test).

Part VI

Discussion

Chapter 11

DISCUSSION

In this work we reveal a crucial role for Ikaros in modulating T cell differentiation through modifications of the chromatin accessibility and of the transcriptional program. We show that Ikaros is required 1) to promote the cTh17 differentiation and the IL-17 expression and 2) to suppress the development of pathogenic T cells via its inhibitory effect towards pro-inflammatory cytokine expression. These conclusions are the results of a the analysis of three high throughput techniques (ATAC-seq, RNA-seq and ChIP-seq) in WT and Ikaros deficient (TKO) $CD4^+$ T cells cultured under Th0 conditions, in combination with the transcriptome study that was previously done in cTh17 conditions and the analysis of protein production by flow cytometry. Globally, by using these complementary techniques, we can affirm that in the absence of Ikaros, activated $CD4^+$ T cells over-express a group of pro-inflammatory markers that resemble those expressed by pTh cells, such as IFNγ, TNFα, GM-CSF, IL-3, IL-1 and IL-23. I will integrate the discussion of the manuscript with the results presented here and I will only briefly approach the effect of the loss of Ikaros in TN cells and mostly focus on understanding the mechanisms that lead Ikaros to regulate the IL-17 and GM-CSF expression.

11.1 Does Ikaros loss irreversibly change the fate of naive T cells?

Ikaros loss leads to important modifications in the T cell transcription program already in unstimulated $CD4^+$ T cells (TN) (444 genes down-regulated and 986 genes up-regulated in TN TKO vs TN WT cells Figure in Annex 12.1), confirming the important role of Ikaros in the regulation of T cell development and differentiation. Moreover, it has been suggested that the loss of Ikaros leads to an increased TdT expression (Trinh et al., 2001), a DNA polymerase participating in the VDJ recombination process, that may be responsible of important modifications in the TCR

diversity repertoire in TN cells deficient for Ikaros. Indeed, the loss of Ikaros could be considered as an irreversible or reversible event. Indeed, the loss of Ikaros could be considered a critical loss that completely and irreversibly changes the fate of the cells. In accord with this hypothesis, we showed that in TN cells, the loss of Ikaros impaired the expression of IL-7R (Figure S6G of the manuscript), suggesting that the survival mechanism of these cells could be drastically damaged. However, we did not study further the identity of TN cells, their TCR repertoire and the impact of Ikaros loss on their fate or their reaction to challenges and thus we do not have any observation that could corroborate this hypothesis. Alternatively, it is possible that the loss of Ikaros could cause changes in TN cells that are instead reversible after the re-expression of Ikaros. One important observation that may support this hypothesis is that with the re-expression of Ikaros in TKO cells (Figure 2D of the manuscript), the IL-17 expression is restored and the GM-CSF expression is decreased suggesting that maybe the absence of Ikaros does not irreversibly change the gene expression profile of cells. However, if the re-expression of Ikaros could globally restore a normal phenotype in naive $CD4^+$ T cells remains still an open question that would be interesting to approach. Other important questions still remain unanswered, such as: are naive T cells deficient for Ikaros characterized by a different TCR diversity than WT cells? And if this was found to be true, this TCR diversity could be associated to the development of a different immune response in KO mice?

11.2 Ikaros inhibits GM-CSF expression

In this work we mostly focused our attention on the molecular mechanism that leads Ikaros to regulate the GM-CSF expression. Indeed, GM-CSF is an old cytokine that is acquiring more and more interest in research for its powerful pro-inflammatory abilities in human diseases. Given that the CD3/CD28 activation was sufficient and necessary to produce important levels of GM-CSF in $CD4^+$ KO cells (Figure 3 of the manuscript), we decided to mostly use the Th0 condition to dissect the role of Ikaros in regulating the GM-CSF expression. Here, I am discussing two main mechanisms by which Ikaros could repress GM-CSF production involving extrinsic and intrinsic factors.

Is Ikaros able to regulate the GM-CSF expression via an extrinsic or membrane-bound factor?

Our results show that the supernatant from IKO culture did not impact GM-CSF expression in WT cells and vice versa, suggesting that a secreted extrinsic factor

responsible for the GM-CSF secretion in this context may not exist (Figure 10.4). Consistently, we clearly show that IL-4, IFNγ and IL-2 are not necessary for the GM-CSF production (Figure 10.7 and S3E of the manuscript). Moreover, we demonstrate that neither IL-1β nor IL-23 seem to influence the GM-CSF production in WT or IKO cells in our culture conditions (Figure 10.6), however, only experiments with Abs blocking these two cytokines would complete the understanding about their role on the GM-CSF expression. IL-7 is a cytokine described to activate STAT5 and able to induce GM-CSF expression in WT cells (Sheng et al., 2014). In our hands, the addition of IL-7 without CD3/CD28 activation is unable to induce the GM-CSF production in both WT and IKO cells indicating that the IL-7 signaling alone without T cell activation is not sufficient for the expression of GM-CSF (Figure 3 of the manuscript). Moreover, in our culture condition, CD3/CD28 activated T cells do not produce IL-7 (results from RNA-seq analysis), indicating that IL-7 does not seem to have a role in the GM-CSF expression in WT or KO cells cultured under Th0 conditions. However, it is important to mention that the supernatant exchange experiments have an important limitation. Indeed, we exchanged the majority of the WT or IKO supernatant, but we could not control the local concentrations of cytokines secreted by WT or IKO cells. These cytokines produced in the close cell environment could potentially act in an autocrine rather than in a paracrine way.

The co-culture experiments complemented our previous data helping us to establish if a cell proximity between WT and KO cells is required to modulate GM-CSF expression and lead to two important observations (Figure 10.5):

1) a cell-cell contact between WT and IKO CD4$^+$ T cells seems to be required for the increased GM-CSF expression by WT cells, suggesting that IKO cells express a membrane-bound molecule that seems to be able to increase the GM-CSF expression by WT cells. Among the membrane-bound factors, FasL and TNFα are two good candidates because they exist as soluble and membrane-bound forms and their expression is up-regulated in CD4$^+$ T cells deficient for Ikaros (FasL both at the mRNA and protein levels, while TNFα only at the protein level). By using neutralizing anti-FasL Abs, we demonstrated that the FasL signaling is implicated in the GM-CSF expression in WT cells (Figure S4 of the manuscript), suggesting that FasL could be the factor responsible also for the increased expression of GM-CSF in WT cells co-cultured with IKO cells. The membrane TNFα has already been described to influence GM-CSF expression in thymocytes (Grell et al., 1995). Co-culture experiments where we pre-treat the IKO CD4$^+$ T cells with blocking-FasL or -TNFα Abs would allow us to test the co-culture conditions without the trigger by the FasL or TNFα produced by IKO cells and would give us more insight about their role in the enhanced expression of GM-CSF in WT cells. Indeed, it would be inter-

esting to identify the responsible membrane factor in order to study it and evaluate its ability to regulate the GM-CSF expression by WT cells also in *vivo* models.

2) The presence of WT cells on the co-culture conditions did not influence the GM-CSF expression by IKO cells and WT cells co-cultured with IKO cells could increase the GM-CSF expression, however they did not reach the same expression level of GM-CSF produced by IKO cells, suggesting that the membrane-bound factor is not sufficient for the GM-CSF expression. Indeed, the loss of Ikaros causes important modifications at the transcriptional level indicating that an intrinsic factor seems more likely implicated in the GM-CSF production in IKO cells and I will detail better this point on the next section.

How Ikaros regulate the GM-CSF expression?

Ikaros could modulate the GM-CSF expression in an intrinsic way 1) via the direct regulation of the *Csf2* gene or 2) via an indirect mechanism.

1) Direct regulation of *Csf2* by Ikaros.

The *Csf2* gene is a complex region and many TFs have been described to play a role in its regulation (Sheng, 2015), however Ikaros has not been described as a candidate TF until now. In the discussion of the manuscript, we have already detailed the hypotheses that lead Ikaros to directly inhibit the GM-CSF expression. In order to validate the repressive function of the Ikaros binding on the CNSa, one possibility would be to use a luciferase reporter gene containing the CNSa and the *Csf2* promoter regions. Indeed, we would expect that the co-expression of this luciferase reporter with BRG1 would increase the luciferase activity, as already shown (Wurster et al., 2011), while the co-expression of the luciferase reporter with Ikaros would importantly decrease the luciferase activity.

Ikaros has been described to be able to associate to protein complexes that are able to modulate the chromatin accessibility (Kim et al., 1999; Koipally and Georgopoulos, 2002). Our ATAC-seq data show an increased chromatin accessibility at level of the *Csf2* enhancer and the CNSb (Figure 5 of the manuscript), where the role of CNSb in regulating the GM-CSF expression has not been confirmed yet (Wurster et al., 2011), while ChIP-seq analyses of these regions do not find an Ikaros binding, suggesting that Ikaros may be not directly responsible for these chromatin modifications. However, whether Ikaros, without binding, could modulate the chromatin accessibility in these specific regions remains an interesting open question and one possible answer could be that Ikaros may be directly responsible for remodeling the chromatin state on these regions by binding on distal regulatory regions (Klemm

et al., 2019).

2) Indirect mechanisms by which Ikaros represses GM-CSF expression.

STAT5. It is possible that the loss of Ikaros causes a modification in the expression of a particular TF or in the activation of a signaling cascade that leads to the increased GM-CSF expression. This is the case for the STAT5 signaling. STAT5 has been described to positively regulate GM-CSF expression in WT $CD4^+$ T cells via its direct binding on the *Csf2* promoter (Sheng et al., 2014). Moreover, ChIP-seq analyses show that STAT5 is also able to bind the CNSa region in WT $CD4^+$ T cells stimulated with IL-2 (Li et al., 2017). In the manuscript, we show that STAT5 pathway is hyper-activated in cells deficient for Ikaros after 2 days of Th0 culture and by using inhibitory molecules, STAT5i, we observe an important reduction of GM-CSF expression in IKO cells (Figure 6 of the manuscript). Considering these data, we can propose two complementary hypotheses of how Ikaros could regulate the GM-CSF expression via the STAT5 signaling: 1) in cells deficient for Ikaros, the hyper-activated STAT5 could directly bind and positively regulate the *Csf2* regulatory regions such as the promoter or the CNSa; 2) STAT5 has been described to antagonize Ikaros by regulating the same genes (Katerndahl et al., 2017; Heizmann unpublished data). Indeed, STAT5 could compete with Ikaros itself for the binding on the CNSa of *Csf2*. Thus one possible mechanism is that, in WT cells, Ikaros "wins" for the binding on the CNSa inhibiting the GM-CSF expression, while in cells deficient for Ikaros, STAT5 binds the CNSa instead, promoting the GM-CSF expression. The mechanisms allowing the hyper-activation of the STAT5 signaling in IKO cells remain to be understood. In this context, IL-7 does not seem to be implicated in the activation of the STAT5 signaling. Moreover, IL-2 neutralization using anti-IL-2 Ab did not lower GM-CSF expression in IKO cells, ruling out a potential implication of IL-2 in GM-CSF expression. In view of our results, STAT5, but not IL-2, is critical for GM-CSF production in IKO cells. However, it will be important to determine if the neutralization of IL-2 decreases STAT5 phosphorylation in IKO cells.

GM-CSFR could be a possible candidate implicated in the STAT5 sustained activation in IKO cells, because of the increased expression of GM-CSF (Guthridge et al., 2006). In our hands, $CD4^+$ T cells cultured in Th0 conditions express low levels of *Csf2ra* mRNA after 1 and 2 days of culture without significant differences between WT and IKO cells (Figure 10.1 E). However, since Ikaros-deficient cells express high levels of GM-CSF, the expression of GM-CSFR, even if it is low, could be sufficient to transmit a downstream signal through the activation of the STAT5 cascade, leading to the activation of a positive feedback loop. By using neutralizing

GM-CSF Abs, we could know if GM-CSFR is important for the STAT5 signaling activation in IKO cells.

NF-kB. NF-kB is another crucial pathway that plays an important role in the GM-CSF production in IKO cells and that could be responsible for the direct regulation of *Csf2* gene. NF-kB has been described to positively regulate the GM-CSF expression by binding on the CNSa region in stimulated $CD4^+$ T cells promoting the BRG1 recruitment (Wurster et al., 2011) and on the *Csf2* promoter (Holloway et al., 2003). Moreover, NF-kB seems to share many target genes with Ikaros (Katerndahl et al., 2017). By inhibiting the NF-kB signaling we observe a drastic reduction in the GM-CSF expression in $CD4^+$ T IKO cells (Figure 6 of the manuscript) and our ChIP-seq data show that Ikaros directly binds the CNSa in d0 and Th0d1 cells. These data raise two main hypotheses of how Ikaros could regulate GM-CSF expression via the NF-kB signaling: 1) first, Ikaros could influence the GM-CSF expression via the direct regulation of the *Csf2* gene by competing with NF-kB for the binding on the CNSa region; 2) the chromatin of the *Csf2* promoter is accessible in KO and WT cells in the naive and activated conditions with slight differences, however a more specific study for the presence of repressive or activator marks would be interesting to understand if more drastic differences were present at the level of the organization of the chromatin between WT and KO cells at the level of the promoter. Indeed, it is possible that in absence of Ikaros, the *Csf2* promoter is characterized by more activator marks that could allow the premature NF-kB binding leading to the increased expression of GM-CSF. The study of the NF-kB signaling cascade and a NF-kB ChIP-seq in WT and KO cells could help to better dissect the role of this pathway in the expression of GM-CSF in $CD4^+$ T cells deficient for Ikaros.

NFAT. The *Csf2* enhancer activity is mostly regulated by NFAT, and its optimal activation seems to require the cooperation between NFAT and AP-1 proteins (Johnson et al., 2004a). Our ATAC-seq results at day 2 show a more accessible chromatin in the *Csf2* enhancer region in TKO cells compared to WT cells, suggesting that NFAT, AP-1 or other TFs could have a role in the increased expression of GM-CSF in KO cells. Our data focus on the NFAT function and show that the use of CsA could only partially reduce the GM-CSF expression in IKO cells indicating that NFAT does not seem to be the crucial TF involved in the *Csf2* gene activity. Interestingly, the use of GSK3i, responsible to lead a sustained activation of NFAT, generates an even extreme outcome with a drastic reduction of the GM-CSF expression in cells deficient for Ikaros. GSK3 is known to have a variety of substrates and the action of the GSK3i on the GM-CSF production in WT and IKO cells could be explained by its effect on different signaling pathways than NFAT, such as its inhibitory action towards the NF-$\varkappa$B or STAT signaling (Beurel and Jope, 2008; Beurel et al., 2010). In conclusion, these data suggest that an increased activity of

NFAT may not be responsible for the increased GM-CSF expression in CD4$^+$ T cells deficient for Ikaros. However, NFAT could eventually participate in the regulation of the GM-CSF expression with other TFs, such as AP-1.

RORγt. How RORγt regulates the expression of GM-CSF remains unclear. In one study, RORγt seems to be crucial for the GM-CSF expression (Codarri et al., 2011). Indeed, CD4$^+$ T cells from RORγt-deficient mice cultured with anti-IFNγ and anti-IL-12 Abs produced lower amounts of GM-CSF than WT cells. In addition, the enforced expression of RORγt in WT CD4$^+$ T cells increased the percentage of GM-CSF$^+$ IL-17$^+$ cells (Codarri et al., 2011). Moreover, the screening of the *Csf2* promoter revealed a conserved binding site for RORγt, suggesting that RORγt could directly regulate the GM-CSF expression (Codarri et al., 2011). However, another study observed that RORγt-deficient CD4$^+$ T cells cultured under Th17(TGFβ1) or Th17(IL-23) conditions produced similar or increased quantities of GM-CSF than WT cells (El-Behi et al., 2011). In our hands, RORγt is less expressed in IKO cells cultured under Th0 or Th17(TGFβ1) conditions (Figure **??** A), while GM-CSF production is high. This is in line with the hypothesis of El Behi and colleagues where RORγt seems to be non necessary for the GM-CSF expression. Moreover, even if the enforced expression of RORγt in CD4$^+$ WT T cells confirms the results of Codarri and colleagues demonstrating an increased expression of GM-CSF$^+$ IL-17$^+$ cells. Very interestingly, in cells deficient for Ikaros, the over-expression of RORγt does not significantly influence the GM-CSF expression (Figure **??**). These results suggest that RORγt may be able to regulate GM-CSF expression by different mechanisms depending on the polarizing cytokines in the environment. In addition,they reveal that when Ikaros is lost, RORγt does not seem to be important for the regulation of the GM-CSF expression. However, the lack of Ikaros could also potentially override the effect of RORγt on GM-CSF expression, underlining the importance of Ikaros to fine-tune GM-CSF production in T cells.

Metabolism. In recent years, the metabolic pathway has become an important subject in research because of its ability to shape the differentiation of Th cells. Among the genes implicated in the metabolism, *Cd5l* is an interesting one. *Cd5l* is considered a regulatory gene and its down-regulation is associated to a pTh17 phenotype *in vivo* mouse model experiments (Wang et al., 2015). Indeed, CD5L/AIM regulates the lipid metabolism and modulates the activity of TFs that are dependent to cholesterol ligands, such as RORγt, by promoting the polyunsaturated fatty acid (PUFA) generation and by decreasing the lipid containing saturated fatty acids (SFA). Indeed, its expression restrains the production of RORγt ligands, thus limiting the expression of RORγt target genes such as IL-17 and IL-23R, while cells deficient for CD5L show an increased expression of IL-17, IL-23R and other pro-inflammatory cytokines typical of the pTh17 phenotype. Really interestingly, the

addition of SFA to WT cell culture could bias the differentiation of WT cells towards a pathogenic profile expression with an increased expression of *Csf2* (Wang et al., 2015). Our RNA-seq results indicate that *Cd5l* is strongly decreased in CD4$^+$ T cells deficient for Ikaros activated for 2 days under Th0 conditions, suggesting that the lipid metabolism could be modified in IKO cells. It would be interesting to determine if the addition of PUFA in the culture of CD4$^+$ IKO T cells could contribute to modulate the T cell differentiation towards cells with a less pathogenic profile and in particular with a decreased expression of GM-CSF. However, more in detail, the mechanism that leads CD5L to inhibit the GM-CSF secretion has not been investigated yet.

Osmotic stress. The osmotic stress pathway is another signaling that has been involved in favoring the pathogenic T cell polarization *in vitro* and *in vivo* mouse model (Kleinewietfeld et al., 2013; Wu et al., 2013). However, its role in the regulation of the GM-CSF expression remains unclear. In humans, it has been described that NaCl stimulates the *in vitro* pTh17 differentiation via the activation of the NFAT5 signaling and SGK1 (Kleinewietfeld et al., 2013). Very interestingly, the microarray data of human naive CD4$^+$ T cells cultured under Th17 condition in presence of NaCl show an increased expression of *CSF2* associated to a decreased expression of *IKZF1* (Kleinewietfeld et al., 2013). Also in mice, NaCl stimulates the *in vitro* and *in vivo* pTh17 differentiation inducing a severe form of EAE probably via the activation of SGK1. However, in murine CD4$^+$ T cells cultured under Th0 condition, the addition of NaCl seems to decrease the GM-CSF expression (Wu et al., 2013). We found that the addition of NaCl to the Th0 or Th17 culture cell conditions does not have a crucial role in the GM-CSF expression in WT cells. However, in cells deficient for Ikaros, the presence of NaCl decreases the GM-CSF expression with a more striking result in cells cultured under Th17 conditions (Figure 10.6) suggesting that in absence of Ikaros the osmotic stress could regulate the GM-CSF expression depending on the cytokine environment. Indeed, only the Th17 cell transcriptome analysis showed an increased expression of *Nfat5* in IKO cells in comparison to WT cells, indicating that the osmotic stress could be more implicated in the Th17 condition. Moreover, our ChIP-seq data reveal the presence of an Ikaros peak on the promoter of *Nfat5* in WT naive T cells (Figure S13 of the manuscript). Although the activity of NFAT5 and the functional inhibition of NFAT5 transcription by Ikaros need to be determined, one hypothesis could be that Ikaros directly represses NFAT5 expression and that in absence of Ikaros, the increased expression of NFAT5 and its yet to-be-determined activity could be responsible for the increased expression of GM-CSF in Th17 IKO cells. If the inhibition activity of Ikaros towards the NFAT5 transcription and activity is confirmed, the NFAT5 pathway could be considered responsible for the GM-CSF increased expression in IKO cells.

However, it is not clear yet which mechanism could activate NFAT5 in presence of NaCl and thus understanding the role of Ikaros in the GM-CSF expression in these conditions remains still enigmatic.

Fas-FasL pathway. As the Fas-FasL pathway has been described to be implicated in death cell and in pro-inflammatory cytokine production via NF-$\varkappa$B pathway stimulation (Cullen et al., 2013; Henry and Martin, 2017), we decided to investigate the role of this pathway in the production of GM-CSF in cells deficient for Ikaros. By using Ab blocking FasL, we demonstrate that the activity of FasL is crucial for the GM-CSF production only in WT cells (Figure S4 of the manuscript). These results suggest that FasL is not implicated in the GM-CSF expression in absence of Ikaros, however it could be possible that the absence of Ikaros overrides the Fas-FasL signaling leading to an hyper-activation of the pathway itself without the necessity of the trigger by the FasL binding. It would be interesting to deeper dissect the Fas-FasL pathway in order to understand its role in cells deficient for Ikaros. The inhibition of the NF-$\varkappa$B canonical pathway activated via the Fas-FasL signaling by cIAP inhibitors molecules, or by inhibiting the scaffold function of RIPK1 or caspase 8, could give more insight into the mechanism regulating the GM-CSF expression via the Fas-FasL signaling in IKO cells.

Proliferation. The role of Ikaros in T cell proliferation remains unclear as Ikaros was first shown to repress (Avitahl et al., 1999) and later to promote T cell proliferation (Lyon De Ana et al., 2019). We wanted to understand if the GM-CSF expression was dependent or independent to T cell proliferation. Our results, using IKO mice, show that proliferation of KO $CD4^+$ T cells under Th0 and Th17 conditions was strongly impaired (Figure S10 of the manuscript), confirming the recent results of Lyon de Ana and colleagues. Consistent with our CFSE data, the transcriptome analyses show that in $CD4^+$ T cells deficient for Ikaros the genes in the cluster 18 are composed by cell-cycle related genes and they are remarkably less induced in activated IKO cells (Figure S1 of the manuscript). Very interestingly, our results show that in absence of Ikaros, the cells that express the most GM-CSF are the ones that divided only few times (Figure S10 of the manuscript). By using the GSK3i, we could reduce the GM-CSF expression in IKO cells, but also the proliferation defect of the IKO cells (Figure **??** C). These data may suggest that the GM-CSF expression and the proliferation defect are two linked events in IKO cells, however, if this is a *sine qua non* remains unclear and needs to be further investigated. In order to dissect these two phenomena, we need to understand if only blocking the proliferation in WT cells could be sufficient to have an increased GM-CSF expression or if restoring proliferation in IKO cells is always associated to a decreased GM-CSF expression.

In the figure 11.1, I am representing the possible mechanisms that lead Ikaros to regulate the GM-CSF expression in CD4⁺ cells.

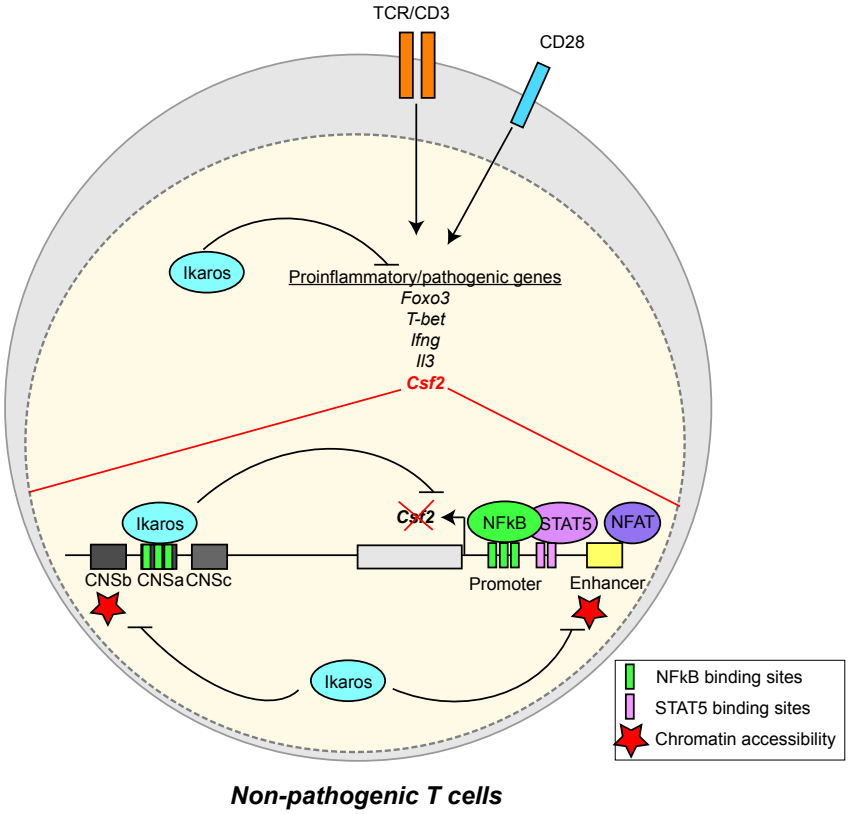

Figure 11.1: **Possible mechanisms that lead Ikaros to regulate the GM-CSF expression.**

11.3 Ikaros promotes cTh17 cell differentiation

The function of Ikaros in Th17 polarization has already been studied but remained unclear. cTh17 polarization is impaired in CD4⁺ T cells of mice carrying a germline *Ikzf1* null mutation (Wong et al., 2013), while IL-17 expression is enhanced in cells missing ZF4 of Ikaros (Heller et al., 2014) and is normally produced in total CD4⁺cells of *Ikzf1*$^{f/fdLckCre}$ mice (Lyon De Ana et al., 2019). Our results are more in line with the work of Wong and colleagues. The different results in IL-17 production between the *Ikzf1*$^{f/fdLckCre}$ and the *Ikzf1*$^{f/fCD4Cre}$ or the *Ikzf1*$^{f/fRosa26CreER}$ mouse models are probably due to the cells used for the *in vitro* cell differentiation and the effects of the *Ikzf1* deletion. Indeed, in our study a pool of naive CD4⁺ T cells is sorted and then activated in cTh17 cell polarizing conditions. In the other work, total CD4⁺ T cell are purified, thus in this case the pool is composed of naive and activated T cells which could introduce a bias in the Th17 cell polarization. Moreover, our results show that only the complete loss of Ikaros cause an impaired IL-17 expres-

sion (Figure 10.3), and in *Ikzf1*$^{f/fdLckCre}$ mice, Ikaros does not seem to be completely lost in CD4$^+$ T cells, suggesting that the little levels expressed may be sufficient to guarantee a normal IL-17 expression. Here I discuss further the possible role of Ikaros in IL-17 production.

Ikaros promotes the IL-17 expression under cTh17 cell culture conditions

RORγt. As RORγt expression is crucial for the IL-17 production (Ivanov et al., 2006), we analyzed its role in the decreased IL-17 expression in cells deficient for Ikaros. Our data indicate that *Rorc* mRNA and protein levels are slightly less induced in Ikaros null CD4$^+$ T cells stimulated in Th17 conditions suggesting that Ikaros may promote *Rorc* gene expression (Figure **??**). Strikingly, we show that exogenous RORγt could enhance the IL-17 expression in WT cells, as expected, but could not rescue IL-17 production in Ikaros null cells (Figure **??**), suggesting that RORγt could not accomplish its direct function in increasing the IL-17 expression in absence of Ikaros. One hypothesis is that Ikaros could regulate RORγt function. However, we could speculate that the functionality of RORγt is not completely impaired in IKO cells, as other RORγt-target genes are normally or even strongly expressed, this is the case for IL-23R. Alternatively, Ikaros could be required to guide RORγt on *Il17* promoter. This seems to be a possible hypothesis, given that we found that, in 293T cells, Ikaros and RORγt can interact together or at least are part of the same protein complex (Figure **??**). However, this interaction needs to be confirmed in murine cells in Th17 culture conditions. RORγt and Ikaros have also been described to be able to interact with the SWI/SNF and the NURD complexes (Lee et al., 2020; Johnson et al., 2004b). Indeed, one possibility is that in CD4$^+$ T cells, the absence of Ikaros could prevent the correct positioning of the nucleosome remodeling complexes on the *Il17* promoter resulting in a decreased expression of IL-17, even when RORγt is over-expressed. By cloning the *Il17* promoter with lucifearse assays, we could confirm if the presence of Ikaros alone or the synergy between Ikaros and RORγt could be important in promoting the IL-17 expression. Given the possibility that Ikaros interacts with RORγt, it would be interesting to study if Ikaros interacts directly with RORγt by forming an hetero-dimer or if they are only part of the same protein complex and they interact with other proteins. Moreover, an Ikaros ChIP-seq in Th17 cells could help us to understand if Ikaros can directly bind *Il17* regulatory regions in activated cells even if no Ikaros peaks was detected in the *Il17* promoter in CD4$^+$ T cells at the naive state. While the RORγt ChIP-seq in Th17 cells will clarify if RORγt is still able to bind the *Il17* promoter in CD4$^+$ cells deficient for Ikaros.

Other TFs. As the absence of Ikaros determines important changes in the gene transcription, we decided to investigate the role of some TFs implicated in the Th17 cell differentiation. T-bet expression has been described to be responsible for the down-regulation of IL-17 (Lazarevic et al., 2011). Our data show that $CD4^+$ T cells deficient for Ikaros and cultured in cTh17 conditions do not up-regulated *Tbx21* mRNA, suggesting that this is not the mechanism responsible for the decreased expression of IL-17 in KO cells. High concentrations of TGFβ1 induce the differentiation of $Foxp3^+$ iTreg cells (Zhang et al., 2008). One possibility was that KO cells do not produce IL-17 because of a switch towards the Treg cell differentiation. Our data clearly demonstrate that in absence of Ikaros, the reduced expression of IL-17 in $CD4^+$ T cells cultured under Th17 condition is not caused by the increased expression of Foxp3 (Figure S3 of the manuscript). Indeed, the loss of Ikaros abrogates Foxp3 expression. A reduced expression of Foxp3 was also observed in cells missing ZnF4 suggesting that probably it is the DNA-binding domain of Ikaros that is important for the Foxp3 expression (Heller et al., 2014).

The different members of the Ikaros family can interact between each other and can bind common target genes. Aiolos and Eos, two members of the Ikaros family, have been described to influence the Th17 cell development (Quintana et al., 2012; Rieder et al., 2015). Aiolos has been reported to promote Th17 cell development by silencing IL-2 expression (Quintana et al., 2012), while Eos is described to have the opposite role and to inhibit the Th17 cell differentiation by promoting the IL-2 production (Rieder et al., 2015). In T-cells deficient for Ikaros cultured in cTh17 conditions, we observe the up-regulation of Aiolos and Eos (data of transcriptome and RNA-seq analysis). In our hands, these mechanisms described are not implicated in the reduced expression of IL-17 in Ikaros-deficient cells, indeed, by using Ab neutralizing IL-2, we show that IL-2 seems not to be responsible for the impaired production of IL-17 (Figure 10.7). However, experiments in KO mice for Ikaros and Eos or Ikaros and Aiolos would definitely exclude these mechanisms as implicated in the failed expression of IL-17 cells in IKO cells. Helios has not been described to have a role in the IL-17 expression, but analyzing the transcriptome data, we identified Helios as up-regulated in IKO cells. By using the DKO mice, double KO for Ikaros and Helios (data not shown), we demonstrate that Helios is not responsible for the reduced expression of IL-17 in Ikaros-deficient mice. Our results do not exclude the hypothesis that the failure in the IL-17 expression is caused by a TF or protein that is under or over-expressed. Indeed, we need to investigate more in detail other TFs crucial for the IL-17 expression such as Ahr and Batf.

TGFβ1. Our results show that, in cells deficient for Ikaros cultured under cTh17 conditions, neither the presence of IL-6 alone nor the addition of different concentrations of TGFβ1 have an effect on the expression of IL-17 (Figure 2 of the

manuscript). The IL-6 signaling and the STAT3 activation have been already studied in T cells deficient for Ikaros and they do not seem to be impaired (Maurer thesis, unpublished data). In a previous work in our laboratory, Ikaros has been described to antagonize the TGFβ1 signaling in common dendritic progenitors cells (Mastio et al., 2018). However, in T cells, our results suggest that the loss of Ikaros would rather leads to an increased threshold for the activation of the TGFβ1 pathway. Some observations supporting this hypothesis are: 1) TGFβ1 signaling has been described to inhibit IL-2 production (Laurence et al., 2007), indeed, in IKO cells cultured under Th17 condition, we observe the increased expression of IL-2 (Figure 10.7); 2) the failure of IKO cells to produce IL-17; 4) the failure in the Foxp3 expression in cells deficient for Ikaros; 4) TGFβ1 signaling inhibits the GM-CSF expression in T cells (El-Behi et al., 2011) and our results show that little doses of TGFβ1 are sufficient to inhibit the GM-CSF expression in WT cells, while in IKO cells, higher concentrations of TGFβ1 are necessary to show the same inhibitory effect on the GM-CSF production (Figure 2 of the manuscript). Further experiments are required to investigate in more detail the TGFβ1/SMAD pathway in IKO cells. Interestingly, the failure of IL-17 production in IKO cells also occurred in Th17 culture conditions-independent to TGFβ1 signaling, such as Th17(IL-23) and Th17(NaCl) conditions (Figure 10.6), indicating that exogenous cocktail of cytokines can not influence the IL-17 production in IKO cells and that Ikaros possibly controls a more intrinsic mechanism.

Chromatin landscape. Ikaros seems to modify the chromatin accessibility at the level of the *Il17* regulatory regions (Wong et al., 2013). Indeed, one interesting possibility that we did not explore is that the absence of Ikaros could drastically modify the chromatin state at the level of the *Il17* regulatory regions preventing the binding of the TFs necessary to induce the IL-17 expression, even if they are over-expressed, as we showed for RORγt. Moreover, we show that the re-expression of Ikaros rescue the IL-17 expression in IKO cells, suggesting that the impaired production of IL-17 is a reversible event. Indeed, it would be interesting to evaluate the modifications in the chromatin landscape in IKO cells cultured under Th17 conditions before and after the re-expression of Ikaros. Ikaros may regulate the chromatin landscape by directly binding on the *Il17* regulatory regions, but ChIP-qPCR analyses suggest that this is not the case (Wong et al., 2013) or another possibility is that Ikaros could remodel the chromatin state by binding on distal regulatory regions. A deeper study of the chromatin state by histone marks ChIP-seq and ATAC-seq analysis in WT and IKO Th17 cells would clarify the role of Ikaros in the differentiation of Th17 cells.

In the figure 11.2, I present a scheme of the possible mechanisms implicated in

the IL-17 expression and that could be impaired in CD4⁺ cells deficient for Ikaros.

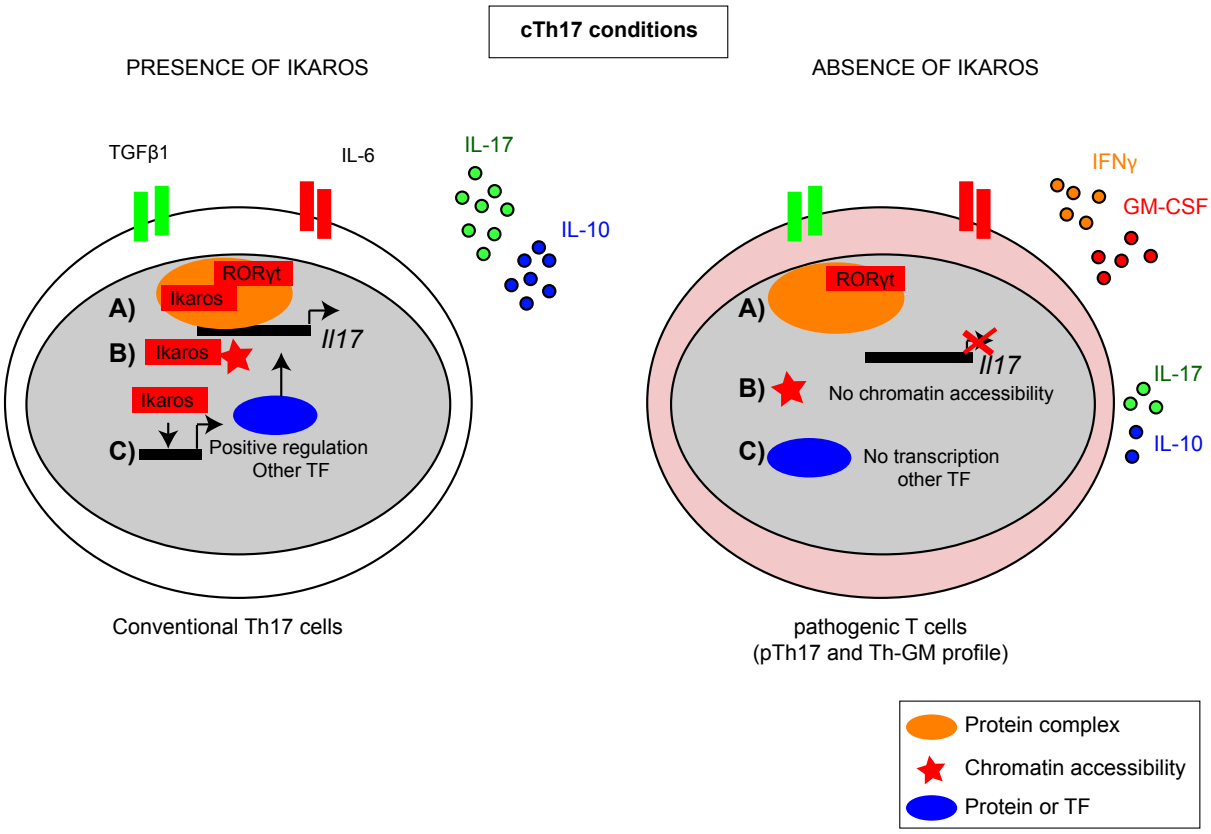

Figure 11.2: **Possible mechanisms responsible for the impaired IL-17 expression**. **A**): Ikaros interacts directly with RORγt or is part of the same protein complex. In absence of Ikaros, RORγt can not bind the *Il17* promoter. **B**): Ikaros regulates the chromatin accessibility at the level of the *Il17* promoter. In absence of Ikaros, the *Il17* promoter is not accessible for the binding of TFs such as RORγt. **C**): Ikaros promotes the transcription of a protein/TF that is indispensable for the IL-17 expression. In absence of Ikaros, this protein or missing TF is not present preventing the expression of IL-17.

11.4 Ikaros loss and the *in vivo* mouse model

It is important to understand how Ikaros regulates the pathogenic T cell profile *in vivo* mouse model and if these cells deficient for Ikaros have a pathogenic function *in vivo*, especially in Th17-related diseases. To address this question, we decided to use the psoriasis model. Psoriasis is an excellent model to study the IL-17/IL-23 signaling. To choose the mouse model, we had to take in consideration that the TKO mice develop a T-ALL from 8-9 weeks of age, thus the induction and development of the disease needed to happen in a close window of time. Surprisingly, psoriasis was equally induced in WT and TKO mice. With this *in vivo* model we made three

important observations: 1) *in vivo* CD4$^+$ αβ T cells where Ikaros is deleted are not able to produce IL-17; 2) the IL-17 produced by the CD4$^-$ CD8$^-$ γδ T cells, where the deletion of Ikaros did not happen, is sufficient for the induction of the disease; 3) at the steady state, *Csf2* mRNA and GM-CSF expression are already increased in CD4$^+$ T cells from the draining LN of the control treated ear of TKO mice and this expression is not sufficient to induce a more severe psoriasis in mice deficient for Ikaros. A more suitable mouse model is necessary to unravel the pathogenic role of GM-CSF *in vivo*.

pTh17 cells and high GM-CSF expression are considered important for the pathogenesis of MS and the most common mouse model used to mimic MS is EAE. It will therefore be important to evaluate the development and function of CD4$^+$ T cells deficient for Ikaros in *in vivo* mouse models, such as EAE. The expected reduced IL-17 expression *in vivo* in CD4$^+$ T cells deficient for Ikaros does not seem to compromise the EAE mouse model, indeed, the IL-17 neutralization has already been described not to limit the pathogenicity of Th17 cells in the EAE induction (Hofstetter et al., 2005). On the contrary, EAE is the perfect model to study the GM-CSF expression, indeed, GM-CSF-deficient mice are highly resistant to the EAE induction (McQualter et al., 2001) and more in detail the cells expressing the more GM-CSF during the EAE are CD4$^+$ T cells (Komuczki et al., 2019). We are currently collaborating with Anne Dejean (Toulouse) to induce a passive EAE model using CD4$^+$ cells lacking Ikaros. Indeed, we would expect that the pro-inflammatory cytokine profile of CD4$^+$ T cells that have lost Ikaros would be sufficient to induce a more severe or faster EAE disease compared to WT CD4$^+$ T cells. However, some parameters are important to keep in mind. The *in vivo* CD4$^+$ lymphocytopenia observed in Ikaros deficient cells or the increased expression of FasL (confirmed only in *in vitro* cell culture) could determine a reduced infiltration of CD4$^+$ T cells in the CNS preventing the appearance of the disease. Another important variable to consider and that could influence the EAE induction is the migration ability of activated T cells into the CNS and the chemokine expression of activated T cells deficient for Ikaros. Cell migration and cell adhesion are pathways enriched in the cluster of genes up-regulated in the transcriptome and RNA-seq of Ikaros deficient cells (Figure 1 and 3 of the manuscript), but they have not been further studied. At the mRNA level, some chemokines important for the EAE induction, such as *Ccr6* and *Ccr4* expression are comparable between WT and mutant cells, while *Ccl20* and *Ccl5* are up-regulated in mutant activated CD4$^+$ T cells. In the development of the EAE mouse model, these pitfalls could be eventually avoided in a passive transfer model by the injection of CD4$^+$ T cells into the CNS (McGeachy et al., 2007). Currently, we have already decided to inject mice with QVD-OPh in order to reduce the cell death of Ikaros deficient mice. We hope that these experiments will help us to

determine if CD4$^+$ cells lacking Ikaros show a pathogenic function *in vivo*.

11.5 Final conclusions

All the data presented in this work are results of mouse studies. An important question is whether we can apply some of these results to human cells. Indeed, in humans the loss of Ikaros has been mostly associated to CVID and B-ALL diseases, but its association to inflammatory diseases, such as MS or RA remains unclear. Many studies are needed to understand the role of Ikaros in humans and for this reason, a collaboration with Patrick Lalive and the Neurology Department in Geneva is ongoing. The GM-CSF and Ikaros expressions will be tested on new diagnosed MS patients and we will analyze if a reduced Ikaros expression is associated to an increased expression of GM-CSF or to more severe inflammatory symptoms in MS patients and if the expression of Ikaros could be associated to a specific response to therapy. Moreover, lately, the GM-CSF secreted by T cells has been described in particular to amplify the Graf-Versus-Host Disease (GVHD) by recruiting donor DCs in the intestinal tract of mice, however its role in this disease remains unclear (Piper et al., 2020). In collaboration with the Hematology Department in Geneva, I will analyze if the severity of the GVHD after stem cell transplantation could be related to the GM-CSF and Ikaros expression in human hematologic diseases. Indeed, if a correlation between the reduced/loss Ikaros expression and the increased pro-inflammatory cytokine production was found, it would mean that Ikaros could be considered with a new prognostic/therapeutic value. Thus, if this was the case, in an inflammatory-type disease, reduced or absent Ikaros expression in CD4$^+$ T cells could be considered as a negative prognostic factor inducing the use of a more aggressive therapy. A more complex question for the future is if Ikaros could be considered as a therapeutic target because in this case we would need to re-express Ikaros in CD4$^+$ T cells. Indeed, if we confirmed that the loss of Ikaros is responsible for the increased pathogenicity of T cells in the *in vivo* EAE mouse model and that its loss is associated to a more aggressive disease in MS patients, it would be amazing to genetic engineering CD4$^+$ T cells deficient for Ikaros from EAE-induced mice to over-express Ikaros. Indeed, this would probably lead to the reduced expression of pro-inflammatory cytokines and would influence the CD4$^+$ T cell polarization *in vivo* decreasing their pathogenic potential clearing the possibility to use this strategy in humans. Another field where we could transfer the experiments done in mice to humans is the field of CAR-T cells, considered among the new promising therapies. The purpose of this new therapy is to engineer the TCR in order to target specific antigen, such as antigens specific to cancer cells. One critical secondary effect of this new therapy is the cytokine release syndrome where pro-inflammatory

cytokine such as GM-CSF, IFNγ or TNFα can be released by the activated engineered T cells. One hypothesis is that by engineering the CAR-T in order to couple their activity to target specific antigens with the over-expression of Ikaros, we could eventually induce a reduced induction of cytokine release syndrome. However, the use of gene therapy and CAR-T cells in autoimmune and inflammatory diseases remains still a challenging field, and moreover, currently, the manipulation of Ikaros expression seems to be an even more challenging issue. Indeed, given the crucial role of Ikaros in the differentiation of many T cell subtype, the fine-tuned modifications in Ikaros expression could also lead to a modification of the T cell response that need to be further studied.

Part VII

Annex

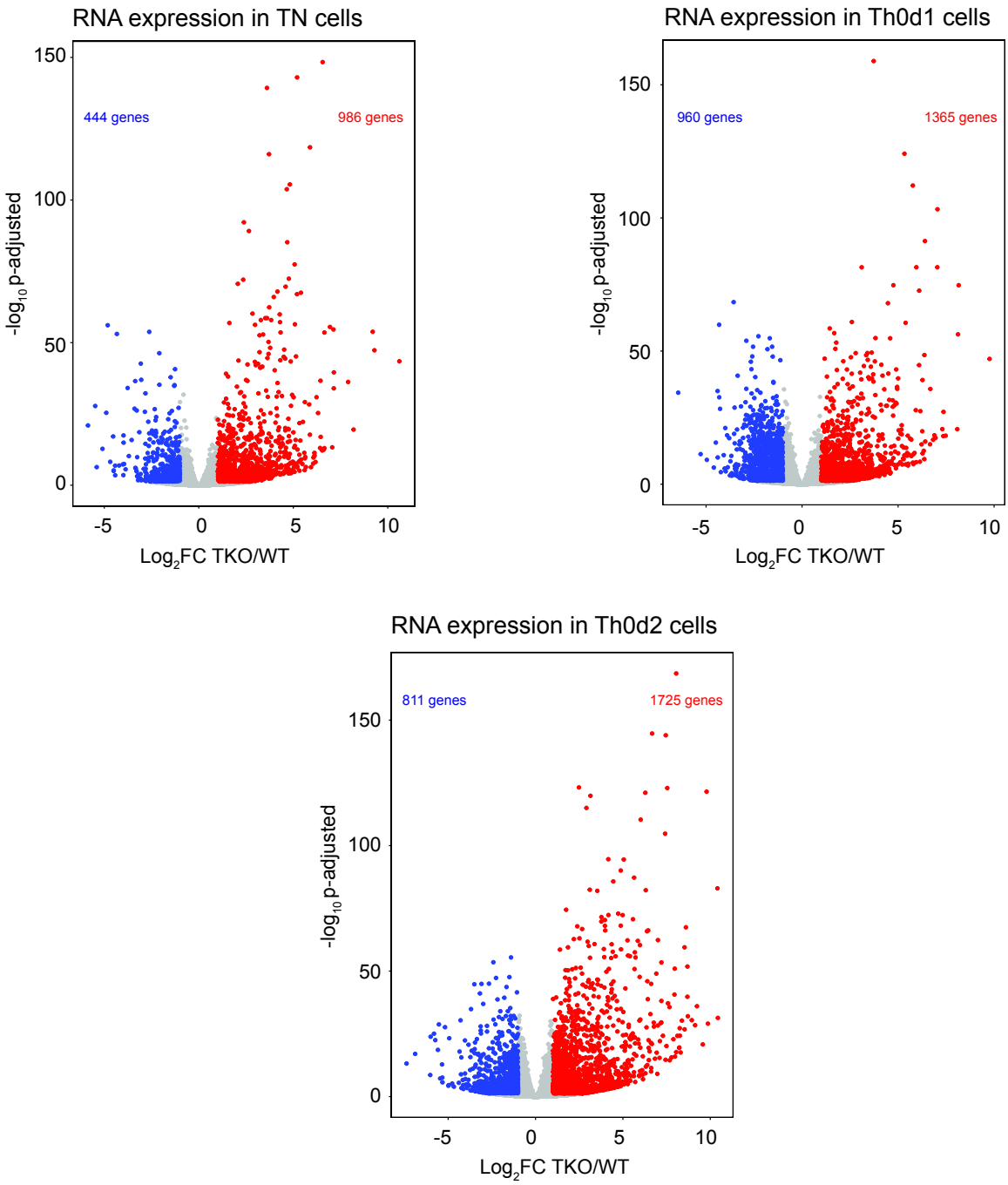

Figure 12.1: **Integration RNA-seq analysis results.** Volcano Plot showing log_2FC TKO/WT identified by RNA-seq in naive T cells (TN) and in-vitro-differentiated T cells at day 1 (Th0 d1) and day 2 (Th0 d2). Up- and down-regulated genes with changes significantly greater than 1-fold are highlighted in red and blue, respectively (padj$\leq$ 0.05). In TN, all genes considered: 15435; blue dots: 444; red dots: 986. In Th0d1, all genes considered: 15435; blue dots: 960; red dots: 1365. In Th0d2, all genes considered: 18339; blue dots: 811; red dots: 1725.

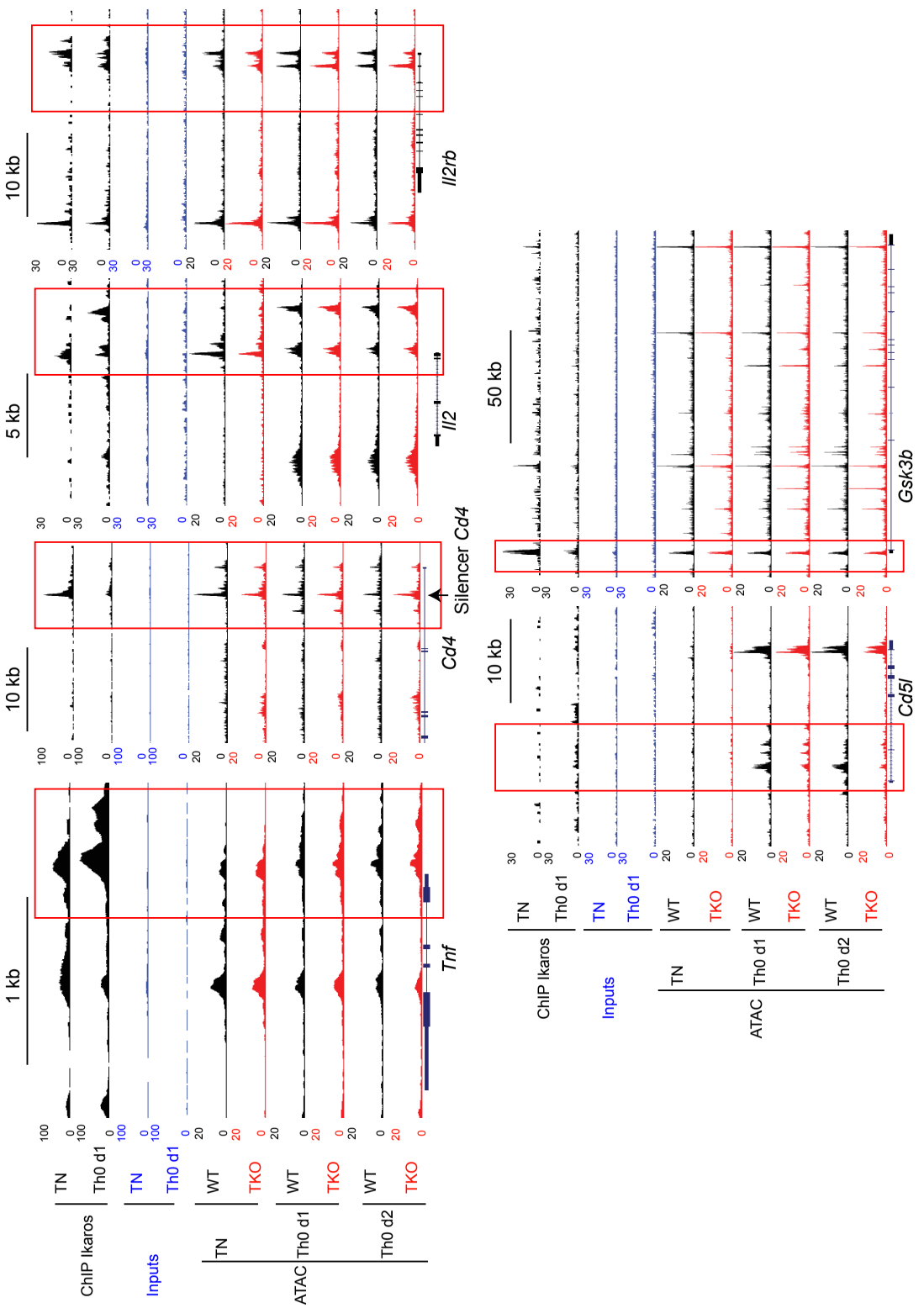

Figure 12.2: **Integration ChIP-seq and ATAC-seq analysis results.** UCSC Genome track views of ChIP-seq and ATAC-seq results. The more interesting peaks close to the TSS are highlighted in a red box.

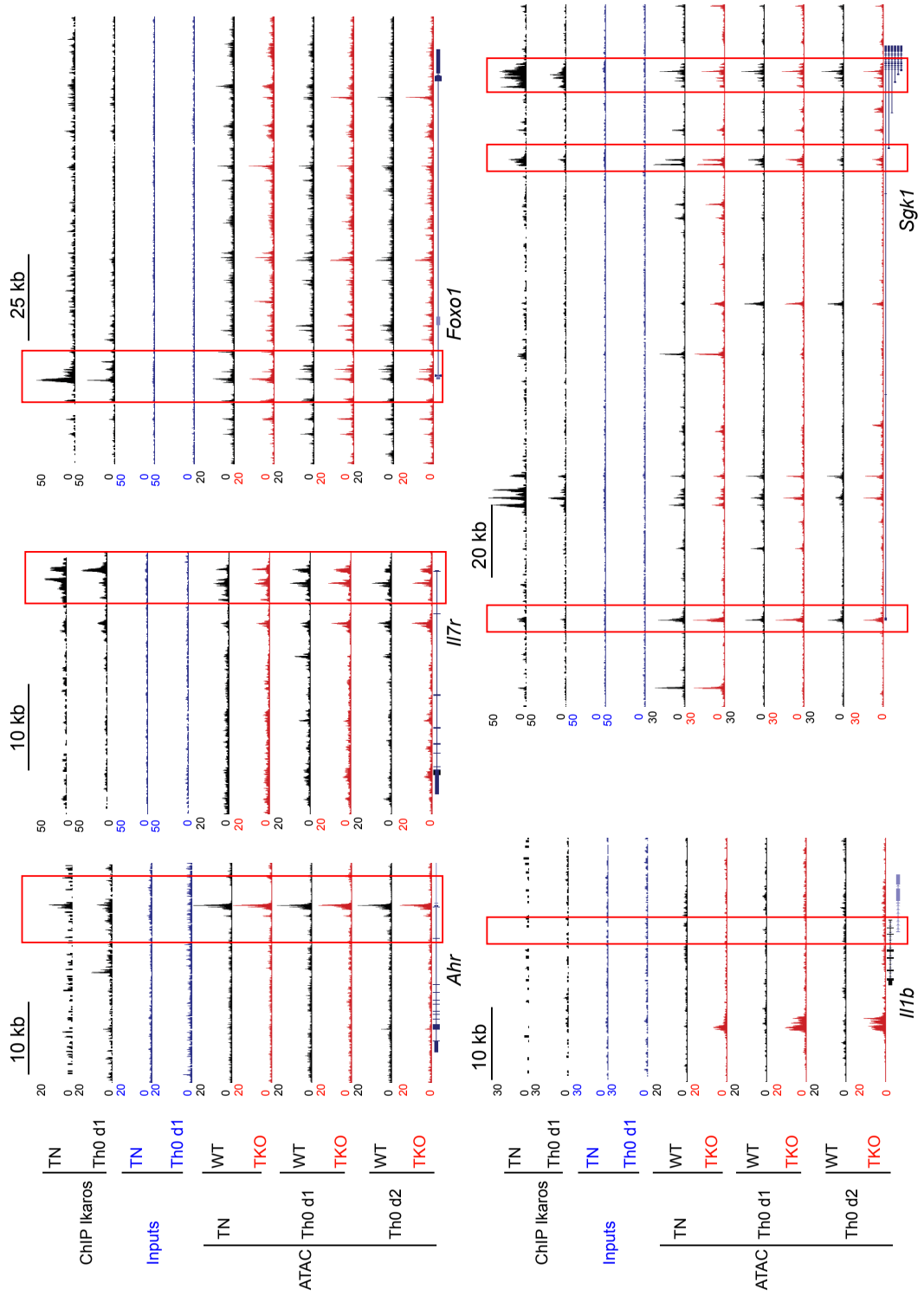

Figure 12.3: **Integration ChIP-seq and ATAC-seq analysis results.** UCSC Genome track views of ChIP-seq and ATAC-seq results. The more interesting peaks close to the TSS are highlighted in a red box.

Part VIII

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Rôle d'Ikaros dans l'expression du GM-CSF dans la pathogenicité des lymphocytes T CD4$^+$

Résumé: Les cellules Th17 sont importantes dans la protection de l'organisme au cours de la réponse immunitaires contre des infections bactériennes ou fungique. Cependant, les cellules Th17 peuvent être impliquées dans des pathologies autoimmunes et inflammatoires et ont été classifiées en fonction de leur dégré de pathogénicité. Nous avons mis en evidence qu'Ikaros est un facteur de transcription qui induit la différentiation des lymphocytes Th17 conventionnelles et réprime l'expression des gènes pathogéniques (par exemple *Csf2*, qui code pour GM-CSF, *Il3*, *Ifng*). Nos résultats démontrent que l'expression de GM-CSF est une conséquence directe de la perte d'Ikaros *in vitro* et qu'elle est indépendant de RORγt et de la voie de signalisation de FasL. De plus, la stimulation par le TCR-CD3 et le coréceptoeur CD28 est fondamentale pour l'expression du GM-CSF. Enfin, nos expériences de séquençage à haut-débit (RNA-seq, ATAC-seq et ChIP-seq) permettent de renforcer le rôle d'Ikaros en tant que gardien du profil pathogénique des lymphocytes T en agissant comme un répresseur de la transcription et de l'accessibilité de la chromatine.
Mots clés : Ikaros, cellules Th17, gènes pathogéniques

Role of Ikaros in the regulation of GM-CSF expression and in the pathogenicity of CD4$^+$ T cells

Abstract: Th17 cells are key players to protect host against fungal and bacterial infections, but they are also important in the onset of autoimmune and inflammatory diseases. Th17 cells are classified according to their degree of pathogenicity. However, the factors controlling their pathogenicity remain poorly understood. Here, we show that, under conventional Th17 polarizing condition, the transcription factor Ikaros is critical to promote IL-17 production and repress the expression of genes that form the pathogenic signature of Th17 cells (e.g *Csf2*, encoding GM-CSF, *Il3*, *Ifng*). I clearly show that the induction of GM-CSF is a direct consequence of acute Ikaros loss *in vitro* and is independent of FasL and RORγt signaling. Interestingly, T cell activation via the TCR-CD3/CD28 complex is sufficient and necessary to trigger the GM-CSF expression. The combination of 3 high throughput analysis (RNA-seq, ATAC-seq and ChIP-seq) emphasizes that Ikaros is an essential guardian of T cell pathogenic profile acting as a general repressor of the transcription and a chromatin remodeling factor.
Keywords : Ikaros, Th17, pathogenic genes, GM-CSF.