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Allison Voisin, Yenkel Grinberg-Bleyer. The many-sided contributions of NF- $\kappa$ B to T-cell biology in health and disease. Thomas S. Postler; Lorenzo Galluzzi. Signal Transduction in Cancer and Immunity, 361, Elsevier, pp.245-300, 2021, International Review of Cell and Molecular Biology, 978-0-12-823757-1. 10.1016/bs.ircmb.2020.10.006 . hal-03846759

**HAL Id: hal-03846759**

**<https://hal.science/hal-03846759>**

Submitted on 18 Aug 2023

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# **The many-sided contributions of NF- $\kappa$ B to T-cell biology in health and disease.**

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## **Abstract**

T cells (or T lymphocytes) exhibit a myriad of functions in immune responses, ranging from pathogen clearance to autoimmunity, cancer and even non-lymphoid tissue homeostasis. Therefore, deciphering the molecular mechanisms orchestrating their specification, function and gene expression pattern is critical not only for our comprehension of fundamental biology, but also for the discovery of novel therapeutic targets. Among the master regulators of T-cell identity, the functions of the NF- $\kappa$ B family of transcription factors have been under scrutiny for several decades. However, a more precise understanding of their pleiotropic functions is only just emerging. In this review we will provide a global overview of the roles of NF- $\kappa$ B in the different flavours of mature T cells. We aim at highlighting the complex and sometimes diverging roles of the five NF- $\kappa$ B subunits in health and disease.

## **Keywords**

T lymphocytes, NF- $\kappa$ B, immunology, immunotherapy, signal transduction

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### 1-The NF- $\kappa$ B signalling pathway(s) in T cells

Originally discovered in B cells, NF- $\kappa$ B has been the topic of extensive studies in many fields of biology. It is now clear that NF- $\kappa$ B exerts crucial roles in nearly all mammalian cell types, with diverse functions in cell growth, survival, development, inflammation and immunity. The NF- $\kappa$ B family of transcription factors comprises five subunits that all share a Rel Homology Domain (RHD) and have been historically split into two pathways: RelA (p65), c-Rel (encoded by *Rel*) and p105/p50 (NF- $\kappa$ B1) belong to the canonical pathway, whereas RelB and p100/p52 (NF- $\kappa$ B2) form the alternative pathway (Hayden and Ghosh, 2011). These proteins form homo- or heterodimers to regulate gene transcription. In the resting state, NF- $\kappa$ B subunits are retained in the cytosol by Inhibitor of kappaB (I $\kappa$ B) proteins through their ankyrin repeats, which interact with the RHD of the subunits. Importantly, p100 and p105 also possess ankyrin repeat in their C-terminal portion, and thus act as inhibitors prior to activation. {INSERT FIGURE 1 HERE}

In T cells, canonical pathway activation is classically triggered by antigen recognition and co-receptor stimulation (385). Engagement of the T-Cell Receptor (TCR) and its co-receptor CD3 by its specific major histocompatibility complex (MHC)+antigenic peptide, leads to the recruitment of a series of kinases resulting in the activation of Protein Kinase C, isoform Theta (PKC $\theta$ ) (Figure 1). Importantly, PKC $\theta$  activation can also be driven by engagement of the CD28 co-stimulation receptor or some members of its family such as Inducible CoStimulator (ICOS). Upon binding its ligand CD80/CD86 molecules expressed by antigen presenting cells (APC), CD28 drives activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway, leading to the recruitment of 3-phosphoinositide dependent tyrosine kinase 1 (PDK1) at the immunological synapse. PDK1, in turn, activates PKC $\theta$ . PKC $\theta$  then phosphorylates CARMA1 (caspase recruitment domain, CARD, membrane-associated guanylate kinase, MAGUK, protein 1, encoded by *card11*), allowing its association with B-cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (Malt1) to form the CBM 'signalosome'. This scaffold recruits the IKK complex composed of I $\kappa$ B kinases (IKK) $\alpha$ ,  $\beta$  and  $\gamma$  (also called NEMO). IKK $\beta$  directly phosphorylates I $\kappa$ B $\alpha$ , triggering its proteasomal degradation, and p105 is

processed into its active form p50 by the 20S proteasome. Together, this enables the nuclear translocation of canonical NF- $\kappa$ B subunits. Importantly, canonical pathway activation can be triggered, or sustained in complement to TCR/CD28 signals, by the engagement of several members of the TNFRSF family, such as CD137 (4-1BB) or CD134 (OX-40). In this case, activation of the IKK complex can occur through TNF receptor-associated factor (TRAF)2 and independently of the CBM complex, or through the 'classical' PI3K/PDK1/CBM axis.

Engagement members of the TNFRSF family can also lead to the activation of the alternative NF- $\kappa$ B pathway (Hayden and Ghosh, 2011). For instance, engagement of TNFR2 leads to the recruitment of TRAFs (in particular TRAF3) and Cellular Inhibitor of Apoptosis Proteins (cIAPs) that at steady-state constantly direct the NF- $\kappa$ B-inducing kinase (NIK) for degradation. In turn, NIK can accumulate and phosphorylate IKK $\alpha$ ; IKK $\alpha$  then processes I $\kappa$ B into p52, enabling nuclear translocation of p52 and RelB. Importantly, association of canonical and alternative subunits have been largely described. This crosstalk relies both on TNFRSF surface receptors with the ability of triggering two signalling cascades, as well as on signalling intermediates that are shared between the two pathways, NIK and IKK $\alpha$  being the best examples. Therefore, it is becoming increasingly evident that the simplified view of two separate or divergent NF- $\kappa$ B pathways should be revised in favour of specific subunit dimerization and function (Oeckinghaus et al., 2011).

Once in the nucleus, NF- $\kappa$ B dimers actively suppress or induce transcription by binding their consensus sequence to target genes. Importantly, among these genes, inhibitors of the NF- $\kappa$ B pathway such as I $\kappa$ B $\alpha$  or the A20 deubiquitinase, are among the first target genes to be transcribed, thus providing a critical negative feedback to constrain aberrant NF- $\kappa$ B activation.

To date the lack of full-spectrum analysis of NF- $\kappa$ B subunits binding to their DNA targets, for instance using Chromatin Immuno-precipitation followed by deep sequencing (ChIP-Seq), hinders the description of subunits or dimer-specific binding sequences. However, major advances in the past decades, in particular using knock-out (KO) mouse models and patients with NF- $\kappa$ B mutations, have provided some insight into the complex and multifaceted roles of NF- $\kappa$ B subunits in T-cell function. In this chapter our aim was to provide a global overview of this knowledge. Of note, because this was largely described

elsewhere, we will not discuss the roles of NF- $\kappa$ B in the initial steps of T-cell development in the thymus, but rather its function in peripheral mature T-cell subsets.

## **2-A short overview of T-cell flavours and functions in health and disease**

T-cell immunity essentially covers every type of immune response, from pathogen clearance to autoimmunity and cancer. In addition, novel roles for T cells in theoretically non-immune-related processes are emerging, such as tissue homeostasis and repair upon injury. This wide range of functions relies on the existence of a myriad of T-cell subsets, many of which are under the control of NF- $\kappa$ B activity—at least in part.

Every T-cell subset originates from a common progenitor that differentiates in the bone marrow and then migrates to the thymus, where it undergoes a series of selection processes and specialises into a specific flavour. More precisely, at the CD4<sup>-</sup>CD8<sup>-</sup> double negative stage, progenitors turn on the expression of the TCR composed either of alpha and beta chains, or of gamma and delta chains. Once committed,  $\alpha\beta$  T cells are subjected to positive and negative selection processes that eliminate >98% of cells; surviving  $\alpha\beta$  T cells differentiate into CD4<sup>+</sup> single positive (SP), CD8<sup>+</sup> SP or Natural-Killer T (NKT) cells. Among CD4<sup>+</sup> SP cells, aside from conventional CD4<sup>+</sup> T cells (Tconv), a critical subset of cells expressing the Foxp3 transcription factor can arise: regulatory T (Treg) cells, the functions of which will be largely described in section 4.5. Strikingly, NF- $\kappa$ B is involved in nearly each of these differentiation steps (reviewed in Gerondakis and Siebenlist, 2010).

Once in the periphery, naïve CD4<sup>+</sup> Tconv cells can then be polarised into specialised subsets with specific functions. Depending on the tissue, the cytokine milieu and the cellular environment, Tconv cells specialise into Thelper (TH)1, TH2, TH9, TH17, T follicular helper (TFH) or peripheral (or 'induced') Treg cells (pTreg) (Saravia et al., 2019). It is believed that CD8<sup>+</sup> T cells can be polarised into similar subsets, though their main function remains that of cytotoxic 'killer' cells (Annunziato et al., 2015). Similarly, TH1-TH2- and TH17-like NKT cells have been described, but their differentiation seems to occur directly in the thymus (Bendelac et al., 2007).

Each T-cell population displays a specific pattern of expression of transcription factors, cytokines and homing receptors, and is involved in the clearance of specific pathogens or in the maintenance of immune (and non-immune) homeostasis. Importantly, under the influence of genetic and environmental factors, these cells can reveal their deleterious facet and drive systemic or tissue-restricted autoimmunity.

Because of these multi-layered functions, it is absolutely critical to understand the molecular regulation of T-cell functions. Here we will emphasize the critical and diverse cell-autonomous functions of NF- $\kappa$ B activity in mature T cells.

### **3-NF- $\kappa$ B in T-cell homeostasis: proliferation, survival, cytokine expression**

The study of NF- $\kappa$ B in T-cell biology originates from a series of papers in the 90's, describing the phenotype of T cells in mice harbouring germline deletions of NF- $\kappa$ B subunits. Indeed, upon TCR/CD28 stimulation *in vitro*, *Nfkb1*, *Rela* and *Rel*-deficient T cells exhibit a defect in proliferation (Doi et al., 1997; Köntgen et al., 1995; Sriskantharajah et al., 2009); this phenotype is even more pronounced in double mutant cells (Grumont et al., 2004; Zheng et al., 2003). This function in murine T-cell proliferation, was confirmed in patients carrying loss-of-function mutations in *REL* or *IKBKB* (encoding IKK $\beta$ ) (Beaussant-Cohen et al., 2019; Pannicke et al., 2013). Interestingly, at odds with the latter report, mouse T cells lacking IKK $\beta$  show normal proliferation *in vitro*, though their homeostatic expansion was impaired upon transfer to lymphopenic recipients (Schmidt-Supprian et al., 2003; Marc Schmidt-Supprian et al., 2004). The maintained proliferation *in vitro* could be explained by conserved basal NF- $\kappa$ B activity, perhaps because residual IKK $\alpha$ /NEMO association and phosphorylation of I $\kappa$ B $\alpha$ . In agreement with this, *Ikbka* (IKK $\alpha$ )<sup>-/-</sup> T cells show defects both in *in vitro* proliferation assays, and upon transfer in Rag1<sup>-/-</sup> animals (Chen et al., 2015).

In fact, this proliferative defect relies on different mechanisms including cell cycle, survival and cytokine production. First, canonical NF- $\kappa$ B is clearly required to ensure cell survival, as it is critical for the expression of the anti-apoptotic protein Bcl-xL, while down-regulating the expression of p73 (Saibil et al., 2007; Wan and DeGregori, 2003). Importantly, chronic activation of canonical NF- $\kappa$ B is also deleterious, as illustrated by the massive apoptosis following stimulation of murine T cells carrying a constitutively active version of IKK $\beta$  (Krishna et al., 2012).

Second, NF- $\kappa$ B1, RelA and/or c-Rel are required for the upregulation of c-Myc upon TCR/CD28 stimulation and for the ensuing initial increase in cell size (Grumont et al., 2004; Zheng et al., 2003). Myc overexpression in *RelA-Rel* double KO cells could rescue the phenotype. Finally, c-Rel and NF- $\kappa$ B1- but not RelA- are required for the production of Interleukin (IL)-2 that is essential for T-cell proliferation *in vitro*. Consequently, the proliferative defect of *Rel*-deficient T cells can be rescued by adding exogenous IL-2 to the culture (Beaussant-Cohen et al., 2019; Grumont et al., 2004; Saibil et al., 2007). Collectively, this demonstrates the essential role of canonical NF- $\kappa$ B subunits in T-cell homeostasis, which is further documented by the decreased proportion of CD44<sup>hi</sup> activated/memory T cells in the lymphoid tissues of *Nfkb1*-mutant or *IKK $\beta$*  conditional KO mice (Schmidt-Supprian et al., 2003; Sriskantharajah et al., 2009). Interestingly, a study using *Bcl10*-deficient mice suggested that the requirement of the CBM/NF- $\kappa$ B axis is more prominent in naïve CD4<sup>+</sup> than memory CD4<sup>+</sup> T cells, suggesting that the main involvement of NF- $\kappa$ B occurs during the initial steps of T-cell activation (So et al., 2011).

Conversely to this prominent role of canonical NF- $\kappa$ B activation, the alternative pathway mostly seems expendable for CD3/CD28-driven naïve T-cell proliferation, as observed in *Map3k14* (NIK)-deficient mice (Cao et al., 2015; Rizk et al., 2019) or in patients with loss-of-function mutations in *NFKB2* (Klemann et al., 2019). However, in the case of OX-40-induced T-cell proliferation, NIK becomes integral to T-cell function (Cao et al., 2015). This suggests that engagement -and thus, function- of specific subunits relies on the physiological or inflammatory settings.

In addition to T-cell survival and proliferation at steady-state, canonical NF- $\kappa$ B activation is involved in the expression of a series of cytokines following TCR engagement. As described above, c-Rel is essential for IL-2 expression, both through direct binding to its promoter and through the upregulation of AP-1, which also mediate IL-2 production (Köntgen et al., 1995; Shapiro et al., 1996). This was recently confirmed in a patient with a mutation in the *REL* locus (Beaussant-Cohen et al., 2019). IFN $\gamma$  and TNF expressions are also altered by *IKK $\beta$*  ablation in mice and humans (Pannicke et al., 2013; Marc Schmidt-Supprian et al., 2004). Overall, this suggests that NF- $\kappa$ B is also involved in the effector functions of T cells, which leads us to the next sections on the diverse roles of the NF- $\kappa$ B pathways in T-cell specification and function.

#### **4-NF- $\kappa$ B in CD4<sup>+</sup> T cells** {INSERT FIGURE 2 HERE}



## 4.1 TH1 cells

TH1 cells, characterised by their high level of IFN $\gamma$  expression, are central actors of the response to intracellular pathogens. In other settings, they were shown to be involved in the pathogenesis of several autoimmune conditions, whereas their presence is generally of good prognosis in cancer (Barnes and Amir, 2017; Chalmin et al., 2018). Polarisation toward the TH1 lineage is first driven by TCR engagement and IFN $\gamma$  signalling through STAT1, which leads to the expression of the master transcription factor T-bet. T-bet, in turn, increases the expression of the IL-12 receptor. IL-12R stimulation then activates the STAT4 pathway, leading to increased and stabilised expression of IFN $\gamma$ . NF- $\kappa$ B has long been described as an important mediator of the TH1 response (Figure 2).

A seminal paper from 1999 first described a dramatic reduction of *in vivo* 'type 1 immunity', i.e. IFN $\gamma$  production in response to the OVA antigen administered intra-peritoneally in a Complete Freund Adjuvant solution, in mice bearing a dominant negative form of I $\kappa$ B $\alpha$  in T cells (I $\kappa$ B $\alpha$ -( $\Delta$ N)-Tg) (Aronica et al., 1999). This TH1 impairment was next confirmed *in vitro* by the same group using this transgenic strain and the IKK inhibitor PDTC (Corn et al., 2003). Interestingly, PKC $\theta$  seems dispensable for TH1 generation *in vivo* upon challenge with *L. major* (Marsland et al., 2004). IFN $\gamma$  expression is preserved in *CARMA1*-deficient TH1 cells (Molinero et al., 2012). T-bet expression is also unimpaired in *Malt1*<sup>-/-</sup> T cells submitted to TH1 polarisation. Together, this suggests a PKC $\theta$ /CBM complex-independent function of NF- $\kappa$ B in TH1 polarisation.

In an elegant study using mice carrying conditional ablation (cKO) of RelA in T cells, Balasubramani et al. showed that RelA-deficient naïve T cells failed to produce IFN $\gamma$  under *in vitro* TH1 polarisation conditions (Balasubramani et al., 2010). Mechanistically, RelA was shown to bind to different conserved non-coding sequences (CNS) in the *Ifng* locus, originally made accessible by T-bet itself (Balasubramani et al., 2010; Sica et al., 1997). In addition to RelA, c-Rel is also implicated in TH1 polarisation *in vitro* (Hilliard et al., 2002), although paradoxical results were later described (Corn et al., 2005; Reinhard et al., 2011; Vasanthakumar et al., 2017). Furthermore, *Nfkb1*-deficient T cells exhibit a normal TH1 differentiation capacity (Artis et al., 2003; Das et al., 2001). This suggests that IFN $\gamma$  expression in TH1 cells is driven by a heterodimer composed of RelA as an obligate member, and either p50 or c-Rel that may have redundant functions. Surprisingly, *IKK $\beta$* -deficient murine T cells secrete normal amounts of IFN $\gamma$  (Marc Schmidt-Supprian et al., 2004). However, this result could be attributed to the experimental

conditions, since cells were stimulated with anti-CD3 but not anti-CD28, whereas both anti-CD3 and anti-CD28 (or antigen-presenting cells) are classically used. Thus, canonical NF- $\kappa$ B regulates IFN $\gamma$  expression in cells undergoing TH1 differentiation, and acts in concert with T-bet to mediate the whole TH1 identity (Hertweck et al., 2016), in particular to sustain IL-2 expression (Balasubramani et al., 2010). Bcl-3, by binding to p50 and c-Rel at the RORc locus, prevents TH17 transdifferentiation into TH1 cells (Tang et al., 2014). Canonical NF- $\kappa$ B may also be involved at the level of T-bet expression during the initial steps of TH1 polarisation, as observed with I $\kappa$ B $\alpha$ -( $\Delta$ N)-Tg murine cells and human PBMCs treated with a RelA decoy oligodeoxynucleotide (ODN) (Corn et al., 2003; McCracken et al., 2007).

Activation of alternative NF- $\kappa$ B subunits may also be important for TH1 cells. Total T cells isolated from *Relb*<sup>-/-</sup> animals exhibited lower IFN $\gamma$  expression in TH1-polarising conditions (Corn et al., 2005). However, naïve *Nik*<sup>-/-</sup> and *Nfkb2*<sup>-/-</sup> T cells show intact TH1 differentiation *in vitro* (Hofmann et al., 2011; Li et al., 2011; Speirs et al., 2002). More studies are therefore required to unravel the putative contribution of the alternative NF- $\kappa$ B pathway during TH1 polarisation.

Taken together, this exhaustive literature suggests that RelA has a unique, non-redundant and critical function in the expression of IFN $\gamma$  once TH1 cells have entered their polarisation programme driven by T-bet. Other NF- $\kappa$ B subunits seem to be either expendable or have redundant roles.

## 4.2 TH2 cells

TH2 cells, characterised by their expression of GATA-binding protein 3 (GATA3), produce IL-4, IL-5 and IL-13. Their involvement in the protection from helminth infection is well-known, and they are critical mediators of many allergic inflammatory conditions. Mechanistically, their polarisation is first induced by IL-4, which triggers STAT6 activation, leading to GATA3 expression (Walker and McKenzie, 2018). NF- $\kappa$ B was proposed to act at different levels in the polarisation process and effector functions.

Historically, the role of the NF- $\kappa$ B pathway was assessed in TH2-mediated *in vivo* responses to helminth infections or to an airway antigen (usually OVA). Downstream of TCR signalling, PKC $\theta$  seems to be important for both the clearance of the helminth *Nippostrongylus brasiliensis* and the development of allergic airway inflammation (Marsland et al., 2004). *Carma1*-deficient mice display a similar phenotype, with decreased IL-4, IL-5 and IL-13 expression in the lungs of challenged mice (Medoff et al., 2006). This can be rescued by the transfer of WT CD4<sup>+</sup> OVA-specific TH2 cells, suggesting a cell-intrinsic

function of the NF- $\kappa$ B pathway in TH2 differentiation *in vivo*. This is further supported by the spontaneous TH2-mediated inflammation observed in mice with constitutive CARMA1 activation (Blonska et al., 2012). Interestingly, in mice bearing a conditional deletion of CARMA1 in activated T cells (OX40-CRE-mediated deletion), allergic responses and IL-4 production are maintained, suggesting a role of the CBM/NF- $\kappa$ B axis during the initial steps of activation but not in later phases (Ramadas et al., 2011). In line with these reports, *Nfkb1*-deficient mice are fully resistant to allergic airway inflammation, with major decreases in Th2 cytokines in the lungs (Das et al., 2001; Yang et al., 1998). T cells isolated from these animals display reduced IL-4, but not IL-5, production. Similarly, lung inflammation is reduced in *Rel<sup>-/-</sup>* mice, though the T-cell phenotype was not assessed in this study (Donovan et al., 1999). This suggests a prominent role for the canonical pathway in the pathogenic allergic process. Owing to the lack of adequate mouse models, the role of RelA has not been assessed yet. However surprisingly, full inhibition of this pathway in the I $\kappa$ B $\alpha$ ( $\Delta$ N)-Tg mouse strain, does not impair OVA-induced lung inflammation (Aronica et al., 1999).

In contrast to this substantial amount of *in vivo* data, the role of NF- $\kappa$ B in *in vitro* differentiated TH2 cells is poorly described. *Carma1<sup>-/-</sup>* splenocytes stimulated with anti-CD3/CD28 and IL-2, display impaired expression GATA3, IL-4 and IL-5 (Blonska et al., 2012). Similarly, T cells from *IKK $\beta$*  cKO mice exhibit decreased IL-4 expression, while IL-5 or IL-10 remain unchanged compared to WT cells (Marc Schmidt-Supprian et al., 2004). However, under TH2 polarising conditions, *Carma1<sup>-/-</sup>* naïve CD4<sup>+</sup> T cells have a normal capacity to differentiate into IL-4 producing cells (Molinero et al., 2012). Interestingly, *Bcl3*-deficient cells do not differentiate well into TH2 cells, with impaired expression of GATA-3, IL-4, IL-5 and IL-13 (Corn et al., 2005). SMAC mimetics, that subvert activation of the canonical pathway and favour activation of the alternative subunits, significantly increase the capacity of mouse T cells to be polarised into TH2 cells (Rizk et al., 2019). A recent and very exhaustive study using a loss-of-function CRISPR-screen approach, investigated the genes involved in TH2 polarisation of murine T cells at the whole genome level (Henriksson et al., 2019). This confirmed the role of Bcl-3 in GATA3 expression, and highlighted the roles of RelA and NF- $\kappa$ B2 in GATA3 and IL-4 expression, respectively. Individual KO of other NF- $\kappa$ B subunits and upstream molecules did not show significant differences in TH2 polarisation.

At the molecular level, it was shown that RelA could bind to the *Il4* promoter in human T cells, and that RelA, but not c-Rel, overexpression induced IL-4 secretion *in vitro* (Li-Weber et al., 2004). NF- $\kappa$ B may

also cooperate with STAT-6 to stabilise the expression of the chromatin regulator SATB1, which in turn sustains expression of GATA-3 in human cells (Khare et al., 2019).

Altogether, these studies suggest a complicated and perhaps multifaceted role for NF- $\kappa$ B in TH2 cells. Additional work, using for instance mice lacking specific NF- $\kappa$ B subunits in T cells, is therefore required.

### 4.3 TH9 cells

TH9 cells form a recently discovered subset, characterised by a high level of IL-9 expression, which is involved in allergic responses and may exert potent anti-tumour functions (Kaplan, 2013). Both mouse and human Th9 cells depend on the transcriptional regulator PU.1 (Chang et al., 2010), although IL-9 production may occur independently of PU.1 in some settings (Xiao et al., 2012).

*In vitro* polarisation toward TH9 cells is classically achieved by stimulation with TGF $\beta$  and IL-4 in addition to TCR and CD28 triggering. Interestingly, these signals sustain extensive and prolonged translocation of all NF- $\kappa$ B subunits in mouse TH9 cells, higher than that observed in other TH subsets (Lu et al., 2018). This suggests an important role for NF- $\kappa$ B in the polarisation process. In fact, it was shown that both p50 and RelA could bind to the *Il9* promoter and induce gene expression in total human T cells (Early et al., 2009) and murine differentiated TH9 cells (Jash et al., 2012). Subsequently, silencing of RelA by siRNA reduces IL-9 production *in vitro* (Jash et al., 2012). Canonical NF- $\kappa$ B subunits might also be directly involved in PU.1 expression, as shown in human acute myeloid leukaemia cells (Bonadies et al., 2010). In patients carrying *TNFAIP3* (A20) mutations, a spontaneous increase in circulating TH9 cells was detected, reinforcing the role of the canonical NF- $\kappa$ B pathway (Zhou et al., 2016).

In addition, stimulation of several members of the TNFRSF family can enhance TH9 polarisation. In a remarkable study, Xiao *et al* showed that stimulation through OX40, using transgenic APCs or agonistic antibodies, massively increases IL-9 producing cells *in vitro*, independently of PU.1 (Xiao et al., 2012). Interestingly, this phenomenon is dependent on the alternative, but not the canonical NF- $\kappa$ B pathway. Indeed increased IL-9 expression is abolished in *Nfkb2*<sup>-/-</sup> but not I $\kappa$ B $\alpha$ -( $\Delta$ N)-Tg cells, and can be enhanced by overexpression of RelB and p52. At the molecular level, RelB but not RelA binds the *Il9* locus in differentiated mouse TH9 cells, and promotes the formation of a 'superenhancer', allowing sustained expression of the cytokine (Xiao et al., 2018, 2012). In the same line, SMAC mimetics increase TH9 cells *in vitro*, likely through enhanced RelB and p52 activity, similar to what was observed with TH2

cells (Rizk et al., 2019). Moreover, the TNF/TNFR2 pathway increases the number of TH9 in polarisation assays, in an NF- $\kappa$ B-dependent manner (Jiang et al., 2019).

In conclusion, distinct NF- $\kappa$ B subunits appear to be involved in the two different TH9 lineages: 'regular' TH9 differentiation requires RelA, whereas OX-40-induced TH9 cells are dependent on TRAF6-driven p52 and RelB activation.

#### 4.4 TH17 cells

TH17 cells are well-known guardians against extracellular infections, but also play a deleterious role in the pathogenesis of different inflammatory and autoimmune disorders, such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (Sandquist and Kolls, 2018). TH17 cells that are all identified by their expression of retinoic acid-related orphan receptor  $\gamma$  (ROR $\gamma$ t, encoded by *Rorc*) and IL17A/F, come in different flavours depending on their differentiation process. Polarisation upon culture with TGF- $\beta$  and IL-6 gives rise to a poorly inflammatory phenotype, whereas the addition of 'danger signals' such as IL-1 $\beta$  or IL-23 drives the differentiation of highly inflammatory cells producing GM-CSF and even IFN $\gamma$ . The role of the NF- $\kappa$ B pathways in TH17 differentiation and function has drawn particular attention in the past decade.

Downstream of the TCR/CD28 signals, the role of the CBM complex in this process is undeniable. Naïve T cells sorted from *Carma1*- and *Malt1*-deficient mice exhibit a profound impairment in TH17 polarisation, with decreased IL-17A, IL-17F and GM-CSF secretion, and decreased ROR $\alpha$  but not ROR $\gamma$ t expression (Brüstle et al., 2012; Molinero et al., 2012). Similar results were obtained with I $\kappa$ B $\alpha$ -( $\Delta$ N)-Tg cells, and by using an IKK $\beta$  chemical inhibitor (Molinero et al., 2012). *In vivo*, mice with *Ikkb* depleted T cells, show impaired IL-17A expression in the context of Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) (Greve et al., 2007). Besides, IKK $\alpha$  seems to have an NF- $\kappa$ B-independent function in this process, as it can directly phosphorylate ROR $\gamma$ t in S376 to potentiate its activity (He et al., 2017). In addition, it was proposed that IKK $\alpha$  could bind to the *Il17a* promoter to allow proper recruitment of the transcription machinery (Li et al., 2011). Thus, canonical NF- $\kappa$ B activation seems to be integral to the TH17 lineage. In line with these results, RelA and c-Rel play a critical role in cell-autonomous TH17 differentiation. ROR $\gamma$ t and *Il17a* expression levels are drastically impaired in TH17-polarised *Rel<sup>-/-</sup>* T cells, as well as in cells obtained from *Rela<sup>-/-</sup>* fetal liver

chimera mice (Chen et al., 2011; Ruan et al., 2011). Interestingly, this c-Rel-dependency can be masked by the addition of IL-2, which inhibits TH17 development (Visekruna et al., 2010). Mechanistically, these subunits directly control *Rorc* expression, and *Rorc* overexpression fully rescues IL-17A production, suggesting that NF- $\kappa$ B is dispensable for *I17a* gene expression *per se* (Ruan et al., 2011). Another report inquired the role of RelA in TH17 polarisation, but this discrepancy could be attributed to the addition of TNF (that activates NF- $\kappa$ B through a CBM-independent mechanism) to the culture (Powolny-Budnicka et al., 2011). In contrast with RelA and c-Rel, NF- $\kappa$ B1 seems to be dispensable for TH17 differentiation, as IL-17A and ROR $\gamma$ t expression levels remain unchanged in TH17 polarized form *Nfkb1*-deficient cells (Chang et al., 2009; Okamoto et al., 2010). Interestingly, constitutive activity of p50 in mice, drives *in vivo* TH17 expansion in a cell-extrinsic manner (Chang et al., 2009). Finally, as shown in other contexts, aberrant canonical NF- $\kappa$ B activation might be deleterious, as *USP18*<sup>-/-</sup> T cells, that display uncontrolled p50 and RelA activation, have a reduced capacity to differentiate into TH17 cells (Liu et al., 2013).

The role of the alternative NF- $\kappa$ B pathway in TH17 cells remains a matter of debate. Cells isolated from *NIK*-deficient animals display a moderate reduction in their capacity to polarise into TH17, likely since NIK enables proper phosphorylation of STAT-3 independently of NF- $\kappa$ B (Jin et al., 2009; Rizk et al., 2019). Mice with conditional ablation of RelB in T cells exhibit normal IL-17A production (Powolny-Budnicka et al., 2011), whereas, interestingly, mice carrying a non-processable version of p100 (*Nfkb2-lym* strain), show a specific impairment in GM-CSF expression, while other TH17 markers remain unchanged. However, more recently, a series of reports established the alternative pathway, especially RelB, as a potent inhibitor of TH17 differentiation. Indeed, SMAC mimetics, which increase nuclear translocation of RelB and p52, reduce IL-17A expression in mouse and human TH17 cells in a NIK-dependent manner (Rizk et al., 2019). Accordingly, overexpression of RelB, and to a lesser extent p52, in mouse T cells, reduce IL-17A expression (Rizk et al., 2019; Xiao et al., 2016). At the molecular level, it was proposed that OX-40 signalling could drive the activation of RelB and its binding to the *I17* locus. In turn, RelB could recruit repressive histone modifiers and therefore inhibit *I17a* expression (Xiao et al., 2016). Interestingly, this inhibitory function could be achieved independently of NF- $\kappa$ B1 and NF- $\kappa$ B2.

Non-conventional I $\kappa$ B proteins, such as I $\kappa$ B $\zeta$  (encoded by *Nfkbiz*) or I $\kappa$ BNS (encoded by *Nfkbid*), may also help TH17 differentiation. *Nfkbiz*<sup>-/-</sup> mouse T cells display a profound defect in IL-17A production

upon TH17 polarisation *in vitro*. Of note, levels of ROR $\alpha$  and ROR $\gamma$ t remain intact. It was proposed that I $\kappa$ B $\zeta$  may, on the one hand, interact with RelA, and on the other hand, associate with ROR $\gamma$ t and ROR $\alpha$  to activate the *Il17a* promoter (Okamoto et al., 2010). A similar defect in IL-17A expression was found in *Nfkbid*<sup>-/-</sup> TH17 cells (Annemann et al., 2015). At odds with these results, Bcl3 is expendable for TH17 differentiation, but represses ROR $\gamma$ t expression in TH1-polarised cells (Tang et al., 2014).

In addition, indirect evidence exposes NF- $\kappa$ B as a possible regulator of IL-22 expression by TH17 and/or TH22 cells. IL-22 expression is reduced in CARMA1-deficient TH17 cells (Molinero et al., 2012). Moreover IL-22 production by ILC3 and dendritic cells was shown to be dependent on NF- $\kappa$ B, with RelA binding to the *Il22* promoter, suggesting that a similar mechanism may occur in T cells (Fumagalli et al., 2016; Victor et al., 2017).

Taken together, it is now clear that the canonical NF- $\kappa$ B pathway, by controlling ROR $\gamma$ t expression, is critical to the initial specification to TH17 lineage, whereas RelB inhibits IL-17A expression. These functions have major consequences *in vivo* in pathological situations, which will be presented below in section 7.

#### **4.5 Follicular helper T cells**

Follicular helper T (TFH) cells are a highly specialised CD4<sup>+</sup> T cell subpopulation that supports the generation of germinal centres (GC) and provides B cells with critical signals promoting antibody class switching, generation of high affinity antibodies, and memory formation. TFH cells are characterised by their elevated expression of the chemokine receptor CXCR5, the transcription factor Bcl-6, costimulatory molecules ICOS, and PD-1, and the secretion of IL-21 (Crotty, 2019).

Several studies have revealed a requirement for canonical NF- $\kappa$ B signalling in TFH differentiation at different levels. Patients with loss-of-function mutation of *NEMO* (Ma et al., 2016, 2015) or *NFKB1* (Gonzalez-Granado et al., 2018) display reduced numbers of circulating TFH, whereas patients with IKK $\beta$  gain-of-function mutation show an increase in circulating TFH (Cardinez et al., 2018). Consistently, *Nfkb1*-deficient TCR-transgenic mice immunised with their cognate Ag, display impaired upregulation of CXCR5, leading to a severe defect in the generation of TFH and GC B cell responses (Serre et al., 2011). In addition, c-Rel binds to the *Il21* promoter to promote its expression in T cells (Chen et al., 2010). *Rel*-deficient mice have impaired IL-21 production, leading to defective development of TFH and

GC B cells upon immunisation (Chen et al., 2010; Liu et al., 2016). Interestingly, RelA seems to be dispensable for TFH differentiation in the context of viral infection (Vasanthakumar et al., 2017).

NIK-deficient mice display impaired TFH differentiation (Hu et al., 2011), and a similar phenotype was detected in patients with *NIK* loss-of-function mutations (Willmann et al., 2014). However, this was not due to a T-cell intrinsic deficiency, but likely to an impaired ICOSL expression in B cells, which is crucial for TFH differentiation (Akiba et al., 2005; Hu et al., 2011).

Altogether, these findings highlight the diverse roles of NF- $\kappa$ B family members in regulating TFH development and function. Additional investigations are required to fully understand the molecular regulation and therapeutic implications of these NF- $\kappa$ B-dependent mechanisms.

#### **4.6 Foxp3<sup>+</sup> regulatory T cells** {INSERT FIGURE 3 HERE}

Regulatory T cells can differentiate both in the thymus (*thymic* tTreg) and in the periphery -or *in vitro*- from naïve Foxp3<sup>-</sup> T cells (*peripheral* pTreg or *induced* iTreg). As illustrated by the systemic autoimmune syndrome observed in *Foxp3*-mutant mice and patients (*Scurfy* and IPEX syndromes, respectively), Treg cells are essential for the maintenance of peripheral immune tolerance (Josefowicz et al., 2012). Recently, they were also suggested to participate in tissue homeostasis in non-lymphoid organs such as the adipose tissue. Nonetheless, Treg cells can exert deleterious functions by preventing pathogen clearance and inhibiting anti-tumour immunity. Therefore, understanding the transcriptional regulation of Treg cell development and identity has major implications for the development of immunotherapeutic agents. For this reason, we have chosen to highlight the critical roles of NF- $\kappa$ B both in the development of Treg cells, and in their homeostasis and function in the periphery (Figure 3).

##### *4.6.1 NF- $\kappa$ B in Treg cell development*

Development of tTreg cells in the thymus is a 2-step process (Lio and Hsieh, 2008). Following TCR and CD28 engagement, naïve cells differentiate into CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> Treg progenitors. Importantly, these cells also express several members of the TNFR family, such as GITR, TNFR2 and OX-40 (Mahmud et al., 2014). Then, IL-2 or, to a lesser extent, IL-15, engage the STAT-5 pathway and give rise to Foxp3<sup>+</sup> mature Treg cells (Lio and Hsieh, 2008). The integral role of NF- $\kappa$ B in tTreg cell development in the



thymus has been largely described. The CBM/canonical pathway axis is required for the arising of Treg progenitors, as illustrated in *Cd28*-, *Carma1*-, *Bcl10*-, *TAK1*- or *Rel*-deficient mice (Anderson et al., 2006; Deenick et al., 2010a; Isomura et al., 2009; Molinero et al., 2009; Vang et al., 2010; Wan et al., 2006). This cell-intrinsic role was later confirmed in mice with conditional deletion of *RelA* and/or *Rel* in T cells (Oh et al., 2017). Importantly, canonical NF- $\kappa$ B is required for *Foxp3* expression *per se*. *RelA* and *Rel*-deficient Treg progenitors fail to convert into mature Treg *in vitro* in the presence of IL-2 (Oh et al., 2017). Both *RelA* and *Rel*, but not p50 or *RelB*, bind to the *Foxp3* promoter and initiate its expression (Ruan et al., 2009; Soligo et al., 2011). C-*Rel*, as an homodimer, also binds to the CNS3 region of the *Foxp3* locus to sustain gene expression in developing tTreg cells (Zheng et al., 2010). It was proposed that I $\kappa$ BNS could associate to NF- $\kappa$ B subunits at the *Foxp3* promoter as well as CNS3 (Schuster et al., 2012). Confirming these reports, constitutive activation of canonical NF- $\kappa$ B in IKK $\beta$ <sup>CA</sup> mice drives massive expansion of tTreg cells (Long et al., 2009). Interestingly, it is likely that strong NF- $\kappa$ B activation in Treg progenitors is mediated by TNFR signalling, as shown *in vitro* and *in vivo* (Mahmud et al., 2014), thereby suggesting that NF- $\kappa$ B is involved in both TCR-dependent and TCR-independent steps of tTreg development.

In contrast to tTreg, NF- $\kappa$ B seems mainly expendable for the polarisation of naïve T cells in pTreg and iTreg. Cells derived from *Carma1*<sup>-/-</sup> and I $\kappa$ B $\alpha$ -( $\Delta$ N)-Tg mice display a normal capacity to differentiate into FoxP3<sup>+</sup> cells *in vitro* (Molinero et al., 2011). Confirming this data, the ablation of *RelA* or c-*Rel* alone does not impact iTreg development (Oh et al., 2017). However, deletion of both subunits inhibits the appearance of Foxp3<sup>+</sup> iTreg cells, perhaps because of a defective cell survival and/or proliferation. Conversely, enforced activation of the canonical NF- $\kappa$ B pathway in IKK $\beta$ <sup>CA</sup> cells, prevents the conversion into iTreg cells due to spontaneous expression of IFN $\gamma$  and IL-17 even in iTreg polarising conditions (Molinero et al., 2011).

In summary, *RelA* and c-*Rel* are critical for tTreg development in the thymus, whereas NF- $\kappa$ B1, NF- $\kappa$ B2 and *RelB* are largely expendable (Deenick et al., 2010a; Grinberg-Bleyer et al., 2018). To date, no strong evidence supports a role for NF- $\kappa$ B in pTreg or iTreg differentiation.

#### 4.6.2 NF- $\kappa$ B in mature Treg cells

The development of cKO mice, using different flavours of the *Foxp3*-CRE deleter strain, has provided scientists with a powerful tool to precisely interrogate the function of the NF- $\kappa$ B pathway in mature Treg cells without affecting their thymic differentiation. A number of studies now support the critical role of the NF- $\kappa$ B pathway in peripheral Treg homeostasis and function.

Indeed, conditional ablation of either *Carma1*, *Bcl10*, *Malt1*, *IKK $\beta$*  or *Rela* in mature Treg cells, gives rise to a common phenotype (Cheng et al., 2019; Di Pilato et al., 2019; Heuser et al., 2017; Oh et al., 2017; Rosenbaum et al., 2019), namely a multifocal and lethal autoimmune syndrome, likely mediated by effector T cells. *Rel* cKO mice develop a later and milder autoimmunity, while strikingly, *Rela/Rel* double cKO animals succumb 3 weeks after birth, mimicking the *Foxp3*<sup>-/-</sup> mice that are devoid of functional Treg cells (Sharma et al., 2009). This clearly demonstrates the integral function of canonical NF- $\kappa$ B signalling in mature Treg cells, to maintain immune homeostasis. In contrast, aberrant alternative NF- $\kappa$ B activation seems deleterious for mature Treg cells. Constitutive activation of NIK in Treg cells, leads to spontaneous autoimmunity (Polesso et al., 2017). Similarly, *Nfkb2* cKO mice develop intestinal inflammation due to aberrant RelB activity; *Relb* ablation itself does not induce any pathology or defects in Treg homeostasis (Grinberg-Bleyer et al., 2018; Li et al., 2018).

Aside from this evident role in preventing autoimmunity, NF- $\kappa$ B activity in Treg also seems to be crucial for their function as inhibitors of protective anti-tumour immunity. Conditional ablation of any member of the CBM complex in Treg cells allows enhanced immune responses and reduced tumour burden in settings of transplanted melanoma, thymoma or colon adenocarcinoma (Cheng et al., 2019; Di Pilato et al., 2019; Rosenbaum et al., 2019). Surprisingly, forced activation of NF- $\kappa$ B1 and RelA using the IKK $\beta$ <sup>CA</sup> transgene, fails to rescue tumour growth in *Carma1* and *Bcl10* cKO animals (Di Pilato et al., 2019; Rosenbaum et al., 2019). This suggests either a NF- $\kappa$ B-independent mechanism, or a c-Rel-specific dependent function. Downstream of the CBM complex, daily treatment of mice with the IKK $\beta$  inhibitor KINK-1, improves anti-tumour immunity in a transplanted model of melanoma (Heuser et al., 2017). Finally, conditional ablation of *Rel*, but not *RelA*, in Treg cells, significantly delays melanoma growth in a CD8<sup>+</sup> T-cell-dependent manner (Grinberg-Bleyer et al., 2017). Moreover, chemical inhibition of c-Rel using pentoxifylline (PTXF), inhibits melanoma growth (Dua et al., 2007). This xanthine derivative also potentiates the effects of anti-PD-1 checkpoint blockade therapy to prevent tumour progression

(Grinberg-Bleyer et al., 2017). These results were recently confirmed using intra-tumoural injection of PTXF and immunotherapy, as well as another c-Rel inhibitor (Gao et al., 2009; Li et al., 2020).

These pathological conditions indicate that NF- $\kappa$ B plays a role in peripheral Treg 'homeostasis' (proliferation, survival, maintenance of cell identity) and/or in their suppressive function. Interestingly, despite their role in Treg cell development in the thymus, the ablation of RelA and c-Rel alone does not impact Treg cell numbers in secondary lymphoid tissues (He et al., 2017; Oh et al., 2017). However, in the competitive environment of mixed BM chimeras and Foxp3<sup>CRE/+</sup> heterozygous females, the presence of RelA confers a selective advantage for Treg expansion (Heuser et al., 2017). Moreover, complete inhibition of the canonical NF- $\kappa$ B pathway through deletion of *IKK $\beta$*  or both *Rela* and *Rel*, leads to a dramatic reduction in the number of Treg cells in the periphery (Oh et al., 2017). This could be attributed to a decreased expression of Bcl-xL in NF- $\kappa$ B-deficient Treg cells. Conversely, NF- $\kappa$ B2 restrains Treg expansion in a cell-intrinsic manner (Grinberg-Bleyer et al., 2018). Interestingly, patients with *NFKB1* mutations exhibit reduced numbers of circulating Treg cells in the blood (Vasanthakumar et al., 2017).

The canonical NF- $\kappa$ B pathway has a prominent role in maintaining the integrity of Treg cell identity, in particular the subset of activated/effector cells. In the periphery, Treg cells can be found in two states: resting Treg (rTreg) are 'naïve' cells, the identity of which is controlled by Foxo1/3 transcription factors (Luo et al., 2016), have been proposed to control effector cell priming in the lymph nodes; they can give rise to activated or effector Treg cells (aTreg or eTreg) that are required for suppressing immune responses in the tissues and in the tumour environment. It is now established that the canonical NF- $\kappa$ B pathway is critical for the acquisition of the aTreg phenotype, together with other transcription factors such as IRF4. Indeed, the aTreg cell subset is dramatically impaired in mice carrying conditional ablation of *Carma1*, *Bcl10* or *Rel* in mature Treg cells (Cheng et al., 2019; Di Pilato et al., 2019; Grinberg-Bleyer et al., 2017). RelA also seems to be implicated in aTreg cell biology in a non-inflammatory competitive environment (Vasanthakumar et al., 2017). Interestingly, this aTreg phenotype can be promoted by certain members of the TNFRSF family, such as TNF/TNFR2, GITR/GITRL, 4-1BB/4-1BB-L, which are all potent activators of the canonical NF- $\kappa$ B pathway (Lubrano di Ricco et al., 2020; Vasanthakumar et al., 2017). Importantly, NF- $\kappa$ B directly binds to and controls the expression of the master transcription factor *Blimp1*, which maintains the gene expression pattern of aTreg cells (Vasanthakumar et al., 2017). Consequently, *Carma1*, *Rel* and *Rela*-deficient Treg exhibit reduced expression of a number of aTreg

genes, such as TIGIT, Klrp1 or Ebi3 (Di Pilato et al., 2019; Grinberg-Bleyer et al., 2017; Heuser et al., 2017). Consistent with an inhibitory role of the alternative NF- $\kappa$ B pathway, cells arising from *NIK* and *Nfkb2*-cKO mice are enriched in aTreg, and these cells are hyperproliferative *ex vivo* (Grinberg-Bleyer et al., 2018; Lu et al., 2005).

In addition, canonical NF- $\kappa$ B is essential for maintaining the expression of many Treg hallmark genes, i.e. genes that are specifically expressed by Treg but not Tconv cells. In mice lacking both *Rel* and *RelA* specifically in Treg cells, these express significantly lower levels of *Foxp3* itself, as well as *Ikzf2* (Helios) or *Id3*, proteins that have been involved in Treg homeostasis (Miyazaki et al., 2014; Oh et al., 2017). This was further confirmed in the context of tamoxifen-induced ablation of NF- $\kappa$ B in mature Treg cells *in vivo* (Oh et al., 2017). Strikingly, these Treg cells express significant levels of Thelper-associated genes, including the inflammatory cytokines IFN $\gamma$ , IL-17A or TNF. This is in line with the Treg phenotype detected in *Carma1* cKO animals (Di Pilato et al., 2019). Thus, NF- $\kappa$ B maintains the Treg phenotype while preventing their conversion into Tconv cells. ChIP-Seq analyses show that *RelA* directly binds to the majority of Treg signature genes, with a significant overlap with *Foxp3*-bound loci (Heuser et al., 2017; Oh et al., 2017). This cooperation between *Foxp3* and NF- $\kappa$ B was documented at the *GITR* locus (Tone et al., 2014). NF- $\kappa$ B was later shown to be an essential partner of *Foxp3* to ensure high expression of many Treg-signature genes (Kwon et al., 2017). This demonstrates that canonical NF- $\kappa$ B signalling was instrumental in establishing the Treg-lineage transcriptome.

In contrast to this critical function in Treg cell identity, NF- $\kappa$ B signalling is mainly dispensable for the function of Treg cells in *in vitro* suppression assays. Indeed, ablation of *IKK $\beta$* , *Rel* or *Rela* does not impair Treg cell capacity to inhibit effector T cells in short-term cultures (Grinberg-Bleyer et al., 2017; Heuser et al., 2017; Isomura et al., 2009). *Carma1*<sup>-/-</sup> or *Nfkb2*<sup>-/-</sup> Treg cells display a slight reduction of their suppressive function *in vitro* (Di Pilato et al., 2019; Long et al., 2009). However, the picture is radically different in *in vivo* suppression assays, i.e. the ability of Treg cells to suppress colon inflammation induced by the transfer of Tconv cells in immunodeficient animals (usually *RAG1* or *RAG2*<sup>-/-</sup> mice). This assay does not specifically measure the direct inhibitory function of Treg cells but also takes in account their ability to expand, survive and migrate to inflamed tissues in lymphopenic recipients. In this setting, perturbation of the NF- $\kappa$ B pathway has major consequences, since ablation of

either *Rela*, *Rel* or their upstream signalling pathway abolishes the ability of Treg cells to suppress colitis (Grinberg-Bleyer et al., 2017).

Importantly, the vast majority of these conclusions arise from studies conducted in mice, and the precise roles of NF- $\kappa$ B in human Treg cells remain to be established. It was recently shown that *NFKB1*-deficient human Treg cells exhibit a normal suppressive function *in vitro* (Ziegler et al., 2020). Interestingly, canonical NF- $\kappa$ B inhibition during iTreg induction even improves their function in subsequent assays. This highlights the need for further investigations in human cells to fully apprehend their therapeutic potential.

Altogether, these extensive studies place NF- $\kappa$ B, particularly its canonical pathway, as a central regulator of Treg development and identity, with major consequences in the suppression of pathogenic and protective immunity.

## 5- NF- $\kappa$ B in CD8<sup>+</sup> T cells

As developed in section 3 of this chapter, NF- $\kappa$ B is crucial for T-cell biology. However, relatively little is known about intracellular signalling events that specifically control the genetic programme of CD8<sup>+</sup> T cells, as the large majority of studies have been performed in unseparated T cells or focused only on the CD4<sup>+</sup> population. The present section will specifically focus on components of NF- $\kappa$ B signalling that have been shown to impact CD8<sup>+</sup> T cell biology (Figure 4). {INSERT FIGURE 4 HERE}

### 5.1 The PKC $\theta$ /CBM/IKK axis: of mice and men.

The role of the TCR/CBM/IKK axis in the function of CD8<sup>+</sup> T cells in mice is relatively unclear. *In vitro*, *PKC $\theta$ <sup>-/-</sup>* and *Bcl10<sup>-/-</sup>* but not *Malt1<sup>-/-</sup>* naïve CD8<sup>+</sup> T cells, fail to upregulate CD44 and CD25 and Bcl-xL upon TCR stimulation, unless massive doses of anti-CD3 are used (Kingeter and Schaefer, 2008; Saibil et al., 2007), and IL-2 secretion is impaired. This leads to a minor reduction in T-cell proliferation and increased cell death, which can be rescued by either increasing the level of stimulation or adding exogenous IL-2 to the culture. However, this intermediate phenotype does not preclude a role for canonical NF- $\kappa$ B in CD8<sup>+</sup> T cells, as I $\kappa$ B $\alpha$  phosphorylation and RelA translocation to the nucleus following TCR stimulation, is partially maintained in PKC $\theta$ /CBM-deficient cells, in contrast to what is observed in CD4<sup>+</sup> T cells. In MHCII-restricted TCR-transgenic mice, the roles of PKC $\theta$  and the CBM are

more evident. *PKCθ*-deficient CD8<sup>+</sup> T cells, stimulated by their cognate Ag, display reduced proliferation, IL-2 expression and target lysis capacity *in vitro*. This phenotype is much more pronounced in *Bcl10*-deficient cells (Deenick et al., 2010b; Zeng et al., 2008). Interestingly, the survival and proliferation, but not the defect in cytotoxicity can be rescued by the addition of IL-2.

Downstream of the CBM complex, mice with T cell-specific ablation of *IKKβ* or *NEMO* display normal thymocyte development but reduced numbers of CD8<sup>+</sup> T cells in spleen and lymph nodes, possibly due to defective survival (Schmidt-Supprian et al., 2003). *IKKβ*-deficient CD8<sup>+</sup> T-cell survival, activation and proliferation upon polyclonal stimulation is largely maintained in *in vitro* assays (Schmidt-Supprian et al., 2003; Marc Schmidt-Supprian et al., 2004). This suggests either NF-κB-independent roles of the CBM complex, or compensatory mechanisms through other IKK members. Furthermore, administration of the pharmacological *IKKβ* inhibitor KINK-1, does not modify CD8<sup>+</sup> T-cell function *in vivo*, presumably because NFATc1 signalling acts as a compensatory mechanism (Heuser et al., 2017). Finally, expression of the IκB-SR (super-repressor) transgene in T cells, impairs CD8<sup>+</sup> T cell proliferation *in vitro* (Mora et al., 1999) and increases CD8<sup>+</sup> T cell apoptosis in response to TCR stimulation (Boothby et al., 1997). In line with this, dual co-stimulation through CD137 (4-1BB) and CD134 (OX-40), which leads to sustained phosphorylation of IκBα and strong NF-κB activation, induces powerful 'supereffector' CD8<sup>+</sup> T cells with enhanced survival, proliferation and cytotoxic function (Lee et al., 2008, 2007, 2004). Abolition of this phenotype through the use of the IKK inhibitor Bay11 demonstrated that NF-κB activity can be easily modulated to improve CD8<sup>+</sup> T-cell function *in vivo* (Lee et al., 2008).

Taken together, this suggests important functions for NF-κB in murine CD8<sup>+</sup> T cells that may in part occur, in contrast to CD4<sup>+</sup> T cells, independently of canonical PKCθ/CBM activation.

Regarding human CD8<sup>+</sup> T cells, the findings differ slightly. Patients completely deficient in *BCL10* have lower central memory CD8<sup>+</sup> TCM cells and decreased CD8<sup>+</sup> T-cell proliferation upon PHA or CD3/CD28 stimulation (Torres et al., 2014). Moreover, proliferation of human *CARMA1*-deficient CD8<sup>+</sup> T cells after CD3/CD28 stimulation is completely abolished (Stepensky et al., 2013). Finally, patients with *IKKβ* mutations display reduced CD8<sup>+</sup> T-cell proliferation and cytokine expression *in vitro* (Pannicke et al., 2013). In contrast, patients with *IKKβ* gain-of-function mutations, show a decrease in naïve CD8<sup>+</sup> T cells and an increase in CD8<sup>+</sup> T<sub>EM</sub> which are more proliferative, *in vitro* (Cardinez et al., 2018). This phenotype

can be recapitulated in mice harbouring the same mutation. This highlights major differences in the activation process and NF- $\kappa$ B-dependency in murine and human CD8<sup>+</sup> T cells.

## 5.2 NF- $\kappa$ B subunits in CD8<sup>+</sup> T cells

A series of studies have evaluated the precise roles of the individual NF- $\kappa$ B transcription factors from the classical pathway in CD8<sup>+</sup> T-cell biology. TCR-Transgenic *Nfkb1*<sup>-/-</sup> mice mount effective CTL primary and secondary responses, with normal proliferation and function (Deenick et al., 2010b). Similar results were obtained when using a polyclonal stimulation *in vitro* (Saibil et al., 2007). Interestingly, a unique population of conventional CD8<sup>+</sup> thymocytes with memory T cell-like properties is present in the periphery of *Nfkb1*-deficient mice (Gugasyan et al., 2012). In humans, contradictory results have been reported. Indeed, one study with patients lacking *NFKB1* shows decreased naïve and increased effector memory CD8 T cells, as well as an impaired proliferation in response to mitogens (Gonzalez-Granado et al., 2018), whereas another study shows no alteration in CD8<sup>+</sup> T-cell phenotype or proliferation (Tuijnburg et al., 2018).

To study the role of RelA, Mondor and colleagues generated a truncated RelA molecule lacking the two transactivation domains (TAD), normally present in RelA, but bearing the Rel homology domain (RHD) (Mondor et al., 2005). TAD-deficient RelA overexpression in CD8<sup>+</sup> T cells, leads to cell death both *in vitro* and *in vivo*, while initial T-cell activation is unaltered (Mondor et al., 2005). The TCR/IKK/RelA axis also sustains the expression on EOMES in memory CD8<sup>+</sup> T cells (Knudson et al., 2017). In addition, RelA is involved in the function of CD8<sup>+</sup> T cells *per se*. Indeed, upon stimulation, RelA is recruited to the enhancer region in the IFN $\gamma$  locus, whereas the absence of RelA greatly impairs IFN $\gamma$  production in CD8<sup>+</sup> T cells (Balasubramani et al., 2010). Interestingly, 'anergic' CD8<sup>+</sup> T cells display decreased RelA phosphorylation and acetylation at Ser311 and Lys310, respectively, associated with impaired gene expression (Clavijo and Frauwirth, 2012). Finally, a recent study using Genome-wide CRISPR Screens in primary human CD8<sup>+</sup> T cells revealed that RelA is strongly implicated in CD8<sup>+</sup> T-cell proliferation upon TCR stimulation (Shifrut et al., 2018).

Mouse CD8<sup>+</sup> T cells lacking c-Rel display a mild impairment in CD8<sup>+</sup> T-cell survival, which can be rescued by the addition of exogenous IL-2 (Saibil et al., 2007). Intriguingly, upon *in vivo* administration of their cognate Ag, TCR-transgenic *Rel*<sup>-/-</sup> T cells exhibit a transient activation and CTL function followed

by unresponsiveness (Deenick et al., 2010b). Therefore, c-Rel emerges as crucial for controlling T-cell activation versus anergy in CD8<sup>+</sup> T cells. Its absence may be functionally compensated by other subunits, as *Rel<sup>+/−</sup>* mice are able to clear viral infections (Harling-McNabb et al., 1999). However, in a patient with a *REL* loss-of-expression mutation, IFN $\gamma$  secretion was completely abolished upon polyclonal restimulation of PBMC, suggesting a role for c-Rel in the function of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Beaussant-Cohen et al., 2019).

Only sparse information is available concerning the precise role of non-canonical NF- $\kappa$ B signalling in CD8<sup>+</sup> T-cell biology. Mice with NIK deficiency in T cells have a significantly reduced frequency and number of memory CD8<sup>+</sup> T cells in the spleen (Li et al., 2016). Furthermore, patients with *NIK* loss-of-function mutation show impaired function of CD8<sup>+</sup> memory T cells (Willmann et al., 2014).

Collectively, current literature suggests that canonical NF- $\kappa$ B regulates CD8<sup>+</sup> T-cell homeostasis and function, whereas alternative NF- $\kappa$ B signalling mainly seems to be expendable.

## **6-NF- $\kappa$ B in other T-cell subsets**

### **6.1. Natural killer T cells**

NKT cells were originally identified as TCR-expressing, NK1.1 positive, CD4-CD8<sup>-</sup> double negative cells in the mouse thymus. They are characterised by their expression of an 'invariant' TCR (hence their common denomination of 'iNKT' cells), as a majority of NKT cells express an identical TCR $\alpha$  chain associated with a restricted set of TCR $\beta$  chains. This TCR recognizes self or foreign glycolipids (such as  $\alpha$ -Galactosylceramide,  $\alpha$ GalCer) presented by CD1d, an unconventional MHC molecule. Interestingly, NKT cells, as their conventional TCR $\alpha\beta$  counterparts, can specialise into IFN $\gamma$ , IL-4 or IL-17 producers, although this specification occurs directly in the thymus. Because of these diverse specialisations, NKT cells can exert protective or deleterious functions in many different immune responses, ranging from pathogen infection to autoimmunity and cancer (Bendelac et al., 2007). Of note, because the thymic development of NKT cells is tightly associated with their function, we will address both points in this section.

Canonical NF- $\kappa$ B has clearly been highlighted as critical for NKT-cell biology. *PKC $\theta$ <sup>-/-</sup>*, *IKK $\beta$*  cKO and *I $\kappa$ B $\alpha$ ( $\Delta$ N)-Tg* mice have strongly reduced NKT cell numbers in the thymus and spleen (M. Schmidt-



Supprian et al., 2004; Stanic et al., 2004a, 2004b). Surprisingly, NKT-cell thymic development seems to occur independently of the CBM complex, as normal proportions of peripheral NKT cells are found in CARMA1<sup>-/-</sup> (M. Schmidt-Supprian et al., 2004), Malt1<sup>-/-</sup> (Ruland et al., 2003) and Bcl10<sup>-/-</sup> (M. Schmidt-Supprian et al., 2004) mice. However, Bcl10 deficiency reduces NKT cells in spleen, liver, and bone marrow (BM), indicating that Bcl-10 may play a role in NKT-cell persistence (M. Schmidt-Supprian et al., 2004). In addition to its roles in development, the PKC $\theta$ /IKK/I $\kappa$ B axis is involved in mature NKT-cell activation, as *in vivo* administration of the specific IKK $\beta$  inhibitor PS-1145 reduces  $\alpha$ -GalCer-induced cytokine production (M. Schmidt-Supprian et al., 2004). Similarly, NKT cells from PKC $\theta$ <sup>-/-</sup> and I $\kappa$ B $\alpha$ ( $\Delta$ N)-Tg mice fail to produce IL-2, IL-4 or IFN $\gamma$  after  $\alpha$ GalCer administration, independently of their survival (Stanic et al., 2004b).

Interestingly, mice expressing a constitutively active IKK $\beta$  in T cells also display reduced numbers of NKT cells in the thymus, spleen, and liver, correlated with decreased ICOS expression and increased NKT cell death (Shen et al., 2011). As for conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells, this result highlights the importance of a balanced activation of the NF- $\kappa$ B signalling pathway in NKT cells biology.

A series of reports have addressed the roles of individual NF- $\kappa$ B subunits in NKT-cell biology. *Nfkb1*-deficiency markedly affects murine NKT-cell survival, resulting in a sharp reduction in total NKT cell number in a cell-intrinsic manner (Sivakumar et al., 2003). This impairment is either restricted to NK1.1<sup>+</sup> cells (Stanic et al., 2004a) or applies to all NKT cells (Stankovic et al., 2011), depending on the genetic background. NF- $\kappa$ B1 is also required for human NKT-cell development as *NFKB1* loss-of-function results in decreased NKT numbers in peripheral blood (Tuijnenburg et al., 2018). In mice with conditional RelA ablation in T cells, as well as in foetal liver chimeras, decreased NKT-cell numbers in the thymus and peripheral organs are detected (Stankovic et al., 2011; Vallabhapurapu et al., 2008). Mechanistically, RelA regulates NKT-cell expression of IL-15R $\alpha$  and  $\gamma$ C, which is needed for IL-15- and IL-7-dependent proliferation of CD44<sup>hi</sup>NK1.1<sup>-</sup> NKT-cell precursors (Vallabhapurapu et al., 2008). Finally, in mice, NKT-cell homeostasis does not require c-Rel (Stankovic et al., 2011). In addition, canonical NF- $\kappa$ B is critical for NKT-cell function, i.e. cytokine production. Following stimulation with  $\alpha$ GalCer or CD3/CD28, *Nfkb1*, *Rela* and *Rel*-deficient NKT cells exhibit impaired expression of IL-2, IL-4 and IFN $\gamma$  (Stanic et al., 2004b; Stankovic et al., 2011).

The numbers of NKT cells is dramatically reduced in *NIK*-mutant and *Relb*<sup>-/-</sup> mice (Elewaut et al., 2003; Sivakumar et al., 2003), while *Nfkb2* deficiency leads to a milder phenotype (Sivakumar et al., 2003). However, reciprocal BM transplantations between WT and *Relb*<sup>-/-</sup> mice revealed that RelB is needed in stromal components but not in hematopoietic cells for NKT-cell development. To date, no cell-autonomous role for the alternative NF-κB pathway in NKT cell biology has been reported.

Taken together, these studies reveal a complex requirement for canonical NF-κB subunits, in the development of NKT cells and in their production of cytokines.

## 6.2 Gamma delta T cells

γδ T cells represent a minor fraction of total T cells in lymphoid tissues but are highly abundant in mucosal tissues, such as the skin, where they are named dendritic epidermal T cells (DETCs). Similar to αβ T cells, they can secrete IFNγ, IL-4 and are major producers of IL-17, properties positioning them as first-line defence against pathogens and involving them in the pathogenesis of certain autoimmune diseases (Chien et al., 2014).

Normal γδ T-cell differentiation and function have been reported in *Nfkb1*-deficient mice (Powolny-Budnicka et al., 2011). In contrast, conditional ablation of *Rela* in total T cells impairs IL-17<sup>+</sup> γδ T (γδT17 cells) numbers (Powolny-Budnicka et al., 2011). This effect is not cell-intrinsic, but likely due to the low expression of lymphotoxin (LT) ligands by accessory thymocytes, leading to altered LTβR activation of γδ T cell thymic precursors. In humans, patients with either IκBα gain-of-function (Courtois et al., 2003) or IKKβ loss of expression (Pannicke et al., 2013) have no circulating γδ T cell. In addition, unlike mice, patients with *Nfkb1* deficiency have fewer γδ T cells (Gonzalez-Granado et al., 2018).

Though there is no difference in the number of γδ T cells in the thymus and peripheral organs of *Nfkb2*<sup>-/-</sup> or *Relb*<sup>-/-</sup> mice, they display a reduced production of IL-17A by γδ T cells upon PMA/ionomycin stimulation (Powolny-Budnicka et al., 2011). Mechanistically, and at odds with conventional TH17 cells, RelB seems to induce RORγt and RORα4 expression, enabling the differentiation of mature γδT17 from thymic precursors (Powolny-Budnicka et al., 2011). Consistent with a role of the alternative NF-κB signalling pathway in γδT17 cells, *NIK*-deficient CD27<sup>-</sup> γδ T cells altered IL-17 production upon PMA/ionomycin stimulation (Mair et al., 2015). In addition to γδT17, *NIK* deficiency also impacts skin-resident DETCs, but in a cell-extrinsic fashion (Mair et al., 2015).

Although the development of  $\gamma\delta$  T cells that produce IFN $\gamma$  relies on CD27 (Ribot et al., 2009), a member of the tumour necrosis factor receptor superfamily (TNFRSF) that activate both NF- $\kappa$ B signalling pathways (Ramakrishnan et al., 2004), the differentiation of CD27 $^+$   $\gamma\delta$  T cells does not require RelA or RelB (Powolny-Budnicka et al., 2011). Nevertheless, signalling *via* the CD70-CD27 axis in CD27 $^+$   $\gamma\delta$  T cells leads to p52 accumulation (Ramakrishnan et al., 2004). Thus, it remains to be determined whether the development of this  $\gamma\delta$  T-cell subset relies on other NF- $\kappa$ B transcription factors, such as NF- $\kappa$ B2.

## 7-NF- $\kappa$ B in pathological conditions: toward NF- $\kappa$ B-targeted therapies?

### 7.1 –Infectious pathogens

Patients with mutations in diverse components of NF- $\kappa$ B signalling pathways, are characterised by their susceptibility to infections. However, the contribution of NF- $\kappa$ B subunits to T-cell responses to pathogens, remains partially unclear and can sometimes be highly variable.

#### 7.1.1 Bacterial infections

In contrast to the well-described effect of bacteria and bacterial products on the activation of NF- $\kappa$ B in innate immune cells, little attention has been paid to the specific function of NF- $\kappa$ B in T cells during pathogenic bacterial infections. It was originally suggested that PKC $\theta$  was critical for the development of primary and secondary T-cell responses to *Listeria monocytogenes* (*L. monocytogenes*) (Sakowicz-Burkiewicz et al., 2008). However, these conclusions were infirmed in a later report (Valenzuela et al., 2009). Canonical NF- $\kappa$ B activation may still be important in this process, as improved NF- $\kappa$ B activity in A20 cKO mice, leads to improved control of acute *L. monocytogenes* infection, though secondary responses are altered (Just et al., 2016). In line with the latter, mice expressing a constitutively active IKK $\beta$  in T cells have impaired *in vivo* antigen-specific immune responses following *L. monocytogenes* infection, with altered T-cell expansion and excessive contraction (Krishna et al., 2012). T-cell autonomous alternative NF- $\kappa$ B activation also seems to be required for bacterial clearance. Indeed, a compromised immune response to *L. monocytogenes*, characterised by reduced number and IFN $\gamma$ -production by CD4 $^+$  and CD8 $^+$  effector T cells, was observed in mice with T-cell-specific *NIK* ablation (Li et al., 2016). This was confirmed in mice with a conditional deletion of *Relb* in T cells, which exhibit impaired response to *E. coli* infection, presumably owing to reduced IL-17 expression by  $\gamma\delta$  T cells

(Powolny-Budnicka et al., 2011). I $\kappa$ BNS plays an essential role in the activation, expansion, and TH1-cell differentiation of CD4<sup>+</sup> T cells but is dispensable for CD8<sup>+</sup> T-cell effector functions following *L. monocytogenes* infection (Frentzel et al., 2019). These studies seem to highlight a prominent role for the alternative pathway in T-cell responses to bacteria, but further studies are needed to evaluate the selective contribution of each subunit in this process.

### 7.1.2 Parasitic infections

The role of NF- $\kappa$ B in T-cell-mediated clearance of parasites, largely depends on the pathogen species. PKC $\theta$  is critical for the development of TH2 immune responses after infection of mice by the helminth *Nippostrongylus brasiliensis*, as illustrated by reduced IL-4-dependent IgE antibody production and IL-5-dependent blood and airway eosinophil infiltration (Marsland et al., 2004). However, PKC $\theta$  is dispensable for IFN $\gamma$  production and TH1-cell-mediated resistance against *Leishmania major* (*L. major*) (Marsland et al., 2004). Conversely, *Nfkb1*<sup>-/-</sup> (Artis et al., 2003), *Rel*<sup>-/-</sup> (Reinhard et al., 2011) and *Nfkb2*<sup>-/-</sup> deficient animals (Speirs et al., 2002) infected with *L. major* develop chronic non-healing lesions associated with persistent parasites. This is due to impaired T-cell proliferation and cytokine production, such as IFN $\gamma$  and IL-17, in a process that involves both T-cell-autonomous functions of NF- $\kappa$ B and cytokine production (in particular IL-12) by myeloid cells. Similarly, following *Toxoplasma gondii* (*T. gondii*) infection, I $\kappa$ B $\alpha$ ( $\Delta$ N)-Tg, *Nfkb1*<sup>-/-</sup>, *Nfkb2*<sup>-/-</sup> and *Rel*<sup>-/-</sup> mice develop toxoplasmic encephalitis (TE) and die during the chronic phase of the disease (Caamaño et al., 2000; Mason et al., 2004; Ziegler et al., 2020). These phenotypes are all associated with defects in the T-cell compartment. NF- $\kappa$ B1 is required in a T-cell intrinsic fashion for IFN $\gamma$  expression by CD8<sup>+</sup> T cells upon *T.gondii* infection, as revealed by cell transfer experiments (Ziegler et al., 2020). TE development in *Rel*<sup>-/-</sup> and *Nfkb2*<sup>-/-</sup> mice is associated with reduced numbers of T cells in the brain, decreased T-cell activation, proliferation and IFN $\gamma$  production (Caamaño et al., 2000; Mason et al., 2004). *Nfkb1* and *Nfkb2*, but not *Rel*-deficient animals, also fail to mount a proper TH2 immunity against the helminth *Trichuris muris* (Artis et al., 2002). Overall, these findings demonstrate a multi-layer implication of NF- $\kappa$ B subunits in T-cell immunity to parasites.

### 7.1.3 Viral infections

Patients harbouring mutations in the NF- $\kappa$ B pathway are often characterised by opportunistic viral infections, as well as Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV) reactivation, suggesting a function for NF- $\kappa$ B in T-cell immunity to viruses. However, findings in mouse models differ slightly. Indeed, *PKC $\theta$*  deficiency does not impair immunity to murine cytomegalovirus (MCMV) or lymphocytic choriomeningitis virus (LCMV) (Marsland et al., 2005; Valenzuela et al., 2009), with normal virus clearance and unimpaired CD8<sup>+</sup> T-cell priming and secondary responses. In contrast, ablation of *Bcl10* or *Malt1* impairs responses to LCMV and rabies (Ruland and Hartjes, 2019). Albeit, these defects may rely on NF- $\kappa$ B-independent functions, as conditional ablation of *Rela* in T cells does not impair antigen-specific CD8<sup>+</sup> T-cell differentiation or effector functions, as assessed by IFN $\gamma$  and Granzyme B expression upon LCMV infection (Vasanthakumar et al., 2017). Similarly, c-Rel is expendable during *Influenza* clearance, although the kinetics of CD8<sup>+</sup> responses are slightly delayed in KO mice (Harling-McNabb et al., 1999). Nevertheless, canonical NF- $\kappa$ B activation may be specifically required in the context of chronic viral infections. Upon chronic LCMV infection, canonical NF- $\kappa$ B is involved in the maintenance of the 'stem-like' phenotype of CD8<sup>+</sup> T cells, whereas it seems to be repressed in exhausted T cells (Jadhav et al., 2019). Moreover 'anergic T cells' display impaired NF- $\kappa$ B activation upon polyclonal restimulation (Clavijo and Frauwirth, 2012). These data are consistent with the role of RelA in the survival and identity of memory CD8<sup>+</sup> T cells (Knudson et al., 2017; Mondor et al., 2005). Aside from the canonical pathway, the NIK/NF- $\kappa$ B2 axis may play a more prominent role in the response to LCMV infection. *NIK*-mutant animals exhibit impaired memory T cells 1 to 2 months after infection whereas the initial CD8<sup>+</sup> T-cell response remains intact (Hamdan et al., 2020; Rowe et al., 2013). Similarly, p52 may be important for the clearance of the pathogen (Droebner et al., 2010).

Taken together, these data highlight the selective contributions of NF- $\kappa$ B subunits in the responses to different pathogens. These results may pave the way for the development of novel therapeutic approaches, for instance through the triggering of specific surface receptors of the TNFRSF family, to activate NF- $\kappa$ B CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells and promote pathogen clearance.

## **7.2 –Autoimmunity**

### *7.2.1 Systemic autoimmunity*

NF- $\kappa$ B has been extensively described as a pivotal regulator of T-cell-mediated autoimmunity. This was first demonstrated in mice harbouring a germline deletion of *Relb*, that die from multifocal autoimmune syndrome only a few weeks after birth (Weih et al., 1995). This disease characterised by a strong T-cell activation, actually relies on T-cell extrinsic mechanisms. Indeed, RelB is critical for the proper development of the thymic medulla, and the homeostasis and maturation of medullary thymic epithelial cells (mTECs) and medullary DCs (Burkly et al., 1995). Consequently, Relb-deficient animals exhibit a defect in the negative selection of autoreactive clones, leading to unleashed activation in the periphery.

Numerous studies then exposed NF- $\kappa$ B as a positive regulator of T-cell-mediated autoimmunity. For instance, deletion of the deubiquitinases *A20*, *Otulin* or *CYLD*, which negatively regulate canonical NF- $\kappa$ B signalling, lead to a large spectrum of autoimmune manifestations in mice, with aberrant T-cell activation as a hallmark (Damgaard et al., 2016; Giordano et al., 2014). However, in the case of *Otulin* deficiency, increased T-cell activation is not cell-autonomous but depends on the aberrant secretion of inflammatory cytokines by myeloid cells (Damgaard et al., 2016). In contrast, mature T-cell-restricted deletion of *A20* drives lung and liver inflammation, with increased expression of IFN $\gamma$  by CD8<sup>+</sup> T cells, and elevated circulating IL-17 and TNF (Giordano et al., 2014). Similarly, ablation of the NF- $\kappa$ B repressor *Foxj1* induces a TH1-cell-mediated autoimmunity (Lin et al., 2004). Consistently, constitutive activation of canonical NF- $\kappa$ B in CD4<sup>cre</sup>IKK $\beta$ <sup>CA</sup> mice, drives the spontaneous increase in IL-17 and IFN $\gamma$  expression by T cells and eventually non-lethal systemic autoimmunity (Krishna et al., 2012). Mirroring these observations, patients with loss-of-function mutations in *A20* or *Otulin* exhibit a wide range of autoimmune manifestations including enteritis and dermatitis, with elevated numbers of circulating TH9 and TH17 cells (Damgaard et al., 2016; Zhou et al., 2016).

Collectively, these observations highlight the central role of NF- $\kappa$ B in the autoimmune process, with the implication of both T-cell-autonomous and extrinsic mechanisms.

### *7.2.2 Tissue-specific autoimmunity: Focus on EAE and MS*

Aside from these multifocal autoimmune manifestations, T cells are also involved in the pathogenic process of tissue-specific diseases. Owing to the functions of NF- $\kappa$ B subunits in the development, maturation and identity of the different T-cell subsets described in the sections above, the implication of canonical and alternative pathways in these conditions is undeniable.

TH17 cells, through their expression of IL-17A/F and GM-CSF, are critical mediators of EAE symptoms (Codarri et al., 2011). High levels of these cytokines are detected in the blood and cerebrospinal fluid of patients with multiple sclerosis (Aram et al., 2019). Interestingly, in humans, GM-CSF producing T cells seem to be dominated by a TH1 phenotype, suggesting that the cytokine itself, rather than the global T-cell flavour, is the master determinant of the pathophysiological process (Rasouli et al., 2015). Accordingly, overexpression of GM-CSF in total T cells is sufficient to drive spontaneous neuroinflammation in mice (Spath et al., 2017). NF- $\kappa$ B appears to be at the centre of the pathogenesis of EAE and MS. Deficiency of *Carma1* or *Malt1* protects mice against EAE (Brüstle et al., 2012; Molinero et al., 2012) downstream of the CBM complex, and conditional ablation of IKK $\beta$  in T cells, or expression of the I $\kappa$ B $\alpha$ ( $\Delta$ N)-Tg, also abolish disease symptoms (Greve et al., 2007; Molinero et al., 2012). In line with these findings, germline deletion of *Nfkb1* and *Rel* leads to a similar phenotype (Hilliard et al., 2002). Aside from this critical function of canonical NF- $\kappa$ B members, the alternative pathway may also be implicated in EAE pathogenesis. Indeed, germline or conditional deletion of *NIK* in T cells provides an almost complete protection against the disease (Hofmann et al., 2011; Rizk et al., 2019). Similar results were obtained with mice expressing a non-cleavable form of p100 (Yu et al., 2014).

Mechanistically, the resistance of NF- $\kappa$ B-deficient animals can largely be attributed to the T-cell-autonomous function of NF- $\kappa$ B activation in the expression of pathogenic cytokines. Originally, NF- $\kappa$ B1 and c-Rel were shown to be required for the polarisation into TH1 cells in the context of EAE, by acting both on APC and T cells themselves (Hilliard et al., 2002). These studies have now been refined and it appears that canonical NF- $\kappa$ B, particularly c-Rel, is also critical for the expression of IL-17A and F (Chen et al., 2011), and more importantly, the secretion of GM-CSF by T cells (Gerondakis et al., 1996). Indeed, c-Rel may be involved in both chromatin remodelling of the *Csf2* promoter and in the expression of the gene (Brettingham-Moore et al., 2005). The cellular and molecular mechanisms underlying the function of the alternative pathway are a matter of greater debated. Notably, the resistance of *NIK*-mutant animals to EAE was first shown to be T-cell-extrinsic (Hofmann et al., 2011), but a later report showed that T-cell-restricted NIK expression was sufficient to confer protection, suggesting a T-cell autonomous function of the kinase (Li et al., 2016). In agreement with these results, a cell-autonomous role for p52 in GM-CSF expression by T cells was described. P52 can directly bind to the *Csf2* promoter and induce its expression in cell lines, independently of RelB (Yu et al., 2014). The role of RelB itself remains unclear, as *Relb*<sup>-/-</sup> T cells transferred to lymphopenic hosts, induce a severe EAE with elevated TH17

cytokines (Xiao et al., 2016). Interestingly, SMAC mimetics are able to abrogate TH17 polarisation and EAE by targeting canonical NF- $\kappa$ B while increasing the activation of the alternative pathway, thereby implying that activation of RelB and/or NF- $\kappa$ B2 is not sufficient to drive the pathogenic process (Rizk et al., 2019). Together, this shows that while canonical NF- $\kappa$ B is critical for Thelper function in EAE, the specific function of alternative NF- $\kappa$ B signalling remains to be clarified.

In line with these reports, emerging evidence suggests a function for NF- $\kappa$ B in the pathogenicity of T cells in patients with multiple sclerosis. As such, *NFKB1*, *NFKBIZ* and *RELA* loci were identified among the MS susceptibility variants (International Multiple Sclerosis Genetics Consortium (IMSGC) et al., 2013). More specific to T cells, a constitutive translocation of NF- $\kappa$ B RelA is present in circulating leukocytes from MS patients (Yan et al., 2018). In CD4<sup>+</sup> T cells isolated from donors carrying the rs228614-GG susceptibility allele, TNF stimulation drives aberrant activation of canonical NF- $\kappa$ B (Housley et al., 2015). Finally, transcriptome analyses of CD4<sup>+</sup> T cells show increased expression of NF- $\kappa$ B-dependent genes in the relapse phase compared to remission (Sato et al., 2008). The exact function of NF- $\kappa$ B subunits in the pathogenicity of human T cells remains to be determined, but these findings argue in favour of a conserved role for canonical NF- $\kappa$ B between humans and mice during neuro-inflammation.

Of note, this critical role of NF- $\kappa$ B in T-cell-mediated autoimmunity, is further illustrated in other models of organ-specific autoimmune diseases. For instance, genetic alterations of the NF- $\kappa$ B pathway in lymphocytes have been emphasized in type 1 diabetes or Crohn's disease (reviewed in Dale et al., 2006). In agreement with this, deletion of either *PKC $\theta$* , *IKK $\alpha$*  or *IKK $\beta$*  in T cells, abolishes their ability to induce colitis when transferred to lymphopenic recipients (Anderson et al., 2006; Chen et al., 2015; Marc Schmidt-Supprian et al., 2004).

### *7.2.3 The other side of the mirror: NF- $\kappa$ B in Treg cells is required to prevent autoimmunity*

Even though extensive literature highlights the pathogenic role of NF- $\kappa$ B activation in the autoimmune process, it is important to keep in mind that the same pathway is also critical for the development, homeostasis and function of Treg cells. As described in section 4.5, conditional deletion of canonical NF- $\kappa$ B or its upstream regulators in Treg cells, generally leads to systemic autoimmunity. Moreover, such alterations render them unable to suppress autoimmune colitis. In addition, germline deletion of



*Rel* in non-obese diabetic (NOD) mice, accelerates diabetes onset owing to defective Treg numbers in the pancreas (Ramakrishnan et al., 2016). Similarly, ablation of *Pdk1* in T cells drives spontaneous colitis mediated by  $\gamma\delta$  T cells, due to impairment Treg development (Park et al., 2010). Interestingly, in humans, a number of mutations of *NFKB1* or *NFKB2*, usually leading to combined variable immunodeficiency, are also associated with autoimmune or inflammatory syndromes with reduced proportions of peripheral Treg cells (Kaustio et al., 2017; Klemann et al., 2019; Vasanthakumar et al., 2017), suggesting that a primary function of NF- $\kappa$ B is to systematically maintain immune tolerance. These reports underline the duality of NF- $\kappa$ B signalling in disease. Additional work is now required to decipher the selective contribution of each NF- $\kappa$ B subunit in Treg cells during tissue-specific autoimmunity.

#### 7.2.4 Therapeutic applications

As a consequence of this research on NF- $\kappa$ B signalling in autoimmunity, different approaches have been setup to decrease its activity in pre-clinical models. Literally each step of NF- $\kappa$ B activation, from antigen-receptor triggering to posttranslational modifications, has been the subject of targeted therapies for tissue-specific autoimmunity. These strategies have largely been described previously (Herrington et al., 2016).

However, only a limited number of studies have directly assessed the effect of NF- $\kappa$ B inhibitors on TH1 and TH17 polarisation. Among these, one recurrent strategy is the use of NEMO-binding domain (NBD) peptides that prevent the formation of the IKK complex. NBD peptides were first demonstrated to reduce the severity of collagen-induced arthritis, a mouse model of rheumatoid arthritis in which TH17 play a deleterious role (Jimi et al., 2004). Similarly, prolonged NBD administration significantly reduces EAE severity and impairs peripheral T-cell phenotype (Dasgupta et al., 2004). Accordingly, NBD peptides decrease TH17 polarisation *in vitro* (Brüstle et al., 2012). Other IKK inhibitors, such as curcumin, PS-1145 or 6-amino-4-(4-phenoxyphenylethylamino)quinazoline, were shown to reduce EAE severity by decreasing TH17 cells *in vivo* (Greve et al., 2007; Molinero et al., 2012; Xie et al., 2009).

These studies, together with the genetic evidence supporting a pathogenic role for NF- $\kappa$ B activation in autoimmunity, pave to way to NF- $\kappa$ B-targeted therapies for treating patients with autoimmunity. Tremendous efforts have been made aimed at targeting the different steps of canonical NF- $\kappa$ B

activation, such as IKK/I $\kappa$ B proteins or the ubiquitin-proteasome system. However, these strategies essentially modulate the canonical NF- $\kappa$ B pathway as a whole, which is likely to induce undesired side effects (Baud and Karin, 2009). Consistently, an attractive approach would be to specifically target unique NF- $\kappa$ B subunits, for instance using decoy oligonucleotides. Such therapies were shown to reduce the severity of colitis or rheumatoid arthritis, when administered systemically or locally in preclinical models (Fichtner-Feigl et al., 2005; Tomita et al., 1999). Similarly, the administration of pentoxifylline reduces EAE symptoms and TH17 differentiation *in vivo*, by targeting c-Rel while leaving other subunits unaltered (Brettingham-Moore et al., 2005; Qiu et al., 2020). The next step will likely be to design small molecule inhibitors able to specifically target one NF- $\kappa$ B subunit, to ensure a tissue or Tconv (but not Treg)-restricted effect.

## 7.3 NF- $\kappa$ B in T-cell immunity to cancer

### 7.3.1 *The multifaceted roles of NF- $\kappa$ B in cancer immunity*

The multiple roles of NF- $\kappa$ B in the homeostasis and effector function of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggest an implication of these transcription factors in the protective function of 'effector' T cells in tumour immunity. Indeed, immunosuppression in tumours and tumour ascites is associated with decreased RelA translocation, and peripheral anergic CD8<sup>+</sup> T cells, which resemble exhausted T cells in tumours, exhibit impaired NF- $\kappa$ B p65 activation at steady-state and upon polyclonal stimulation (Clavijo and Frauwirth, 2012). In agreement with this possible role of NF- $\kappa$ B in T-cell response to cancer, conditional ablation of IKK $\beta$  in T cells, leads to increased tumour growth in a highly immunogenic model of transplantable melanoma (Caamaño et al., 2000). Conversely, constitutive activation of IKK $\beta$  drives spontaneous rejection of tumours (Evaristo et al., 2016). In the same line, ablation of A20 in TCR-transgenic T cells strongly improves their anti-tumour potential (Giordano et al., 2014). Hence, canonical NF- $\kappa$ B appears to play a critical role in T cells during cancer. However, whether this effect is due to the influence of NF- $\kappa$ B in CD4<sup>+</sup> and or CD8<sup>+</sup> T cells remains unclear. In addition to 'normal' T-cell responses, NF- $\kappa$ B may also be involved in the reinvigoration of T-cell immunity upon PD-1 immune checkpoint blockade. First, it was demonstrated that signals through CD28 in CD8<sup>+</sup> T cells, which trigger canonical NF- $\kappa$ B among other pathways, are essential for tumour clearance in mice treated with anti-PD-1 Abs (Kamphorst et al., 2017). Second, the requirement for IFN $\gamma$ , the expression of which is NF- $\kappa$ B-dependent, for the efficacy of anti-CTLA4+anti-PD1 in mouse melanomas, suggests an involvement of

the canonical NF- $\kappa$ B pathway (Shi et al., 2016). Finally, transcriptomic analyses show increased activation of NF- $\kappa$ B target genes upon checkpoint-blockade therapies in mouse and human cancers (Wei et al., 2017). More directly, engagement of the PD-1 receptor is thought to inhibit the TCR “signalosome”, arguing in favour of an effect on NF- $\kappa$ B activation (Arasanz et al., 2017), as previously shown in human dendritic cells from tumour biopsies (Karyampudi et al., 2016). PD-1 blockade on activated human T cells leads to increased PDK1 phosphorylation and subsequent NF- $\kappa$ B activation (Fenwick et al., 2019). Future studies are necessary to refine the specific functions of NF- $\kappa$ B subunits in the helper and cytotoxic functions of T cells in cancer immunity and immunotherapy.

At odds with this apparent protective function of NF- $\kappa$ B in tumour immunity, canonical NF- $\kappa$ B plays a well-established role in orchestrating Treg cell function in the tumour microenvironment, thereby suggesting a multifaceted role of this signalling pathway in cancer. As described in section 4.5, Treg-restricted ablation of members of the CBM complex, as well as c-Rel, inhibits their accumulation in tumours and the establishment of the aTreg phenotype. Specifically, *Carma1*- and *Rel*- deficient Treg cells express aberrant levels of IFN $\gamma$  while losing their aTreg core signature. This mechanism is likely responsible for the reduced tumour burden in mutant mice. To date, no function of other NF- $\kappa$ B subunits in Treg cells during cancer was demonstrated, thereby suggesting a subunit-specific role for c-Rel in this context.

### 7.3.2 NF- $\kappa$ B-targeted cancer immunotherapies: inhibition or activation? A matter of subunit.

These dual functions of canonical NF- $\kappa$ B in tumour immunity and tolerance raise a major question for cancer immunotherapy: should we inhibit or rather stimulate NF- $\kappa$ B activity *in vivo*?

A series of investigations unveil NF- $\kappa$ B inhibition as a promising strategy to impair Treg cell function in cancer, and therefore enhance anti-tumour immunity and decrease tumour burden. Originally, the IKK inhibitor curcumin was highlighted for its anti-tumour effect, alone or in combination with anti-PD-1/ anti-PD-L1 therapy. More recently, it was shown that *in vivo* inhibition of Malt1 using Mepazine, of IKK $\beta$  using KINK-1, or of c-Rel using pentoxifylline or R96A, significantly reduces the growth of transplanted melanoma or breast tumours, especially when combined to anti-PD-1 and/or chemotherapy (Di Pilato et al., 2019; Gao et al., 2009; Grinberg-Bleyer et al., 2017; Heuser et al., 2017; Li et al., 2020). The CBM/IKK/c-Rel axis is therefore an attractive target for cancer therapy. This follows three decades of

work establishing NF- $\kappa$ B as critical in the transformation, proliferation and metastasis potential of tumour cells themselves.

However, another strategy may consist in re-activating NF- $\kappa$ B in exhausted effector T cells to potentiate their anti-tumour function. In the last few years, a series of novel immunotherapies have emerged that all consist in the stimulation of surface receptors of the CD28 and TNFRSF families, such as 4-1BB, GITR, or OX-40. These receptors can be expressed both by activated effector T cells, and by Treg cells. Agonistic Abs against these molecules potently stimulate effector CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells *in vivo*, leading to tumour rejection, which suggests that the triggering of effector T cells is the dominant mechanism (Lee et al., 2004; Xiao et al., 2015). A common feature of all these receptors is the fact that they all induce or sustain NF- $\kappa$ B activation in complement to TCR stimulation (Ward-Kavanagh et al., 2016). Similarly, ICOS agonists, that are now reaching the stage of clinical application, may activate NF- $\kappa$ B upon PI3K/PDK1 recruitment (Han et al., 2012). Therefore, NF- $\kappa$ B is likely mandatory for T-cell reactivation upon TNFRSF engagement, but whether a specific pathway or subunit is involved in tumour clearance remains unknown. One could speculate that these novel therapies may avoid activating c-Rel because of its prominent immunosuppressive role. Conversely, because RelA, for instance, is expendable for Treg function in cancer (Grinberg-Bleyer et al., 2017), it may be an appropriate target downstream of stimulatory surface receptors.

Altogether, the discoveries of the past years on the role of NF- $\kappa$ B in effector and regulatory T cells, highlight the modulation of NF- $\kappa$ B signalling as a promising immunotherapy strategy for cancer.

## 8. Concluding remarks

In this review we have largely highlighted the complexity of NF- $\kappa$ B transcription factors in the regulation of T-cell identity and function. Owing to the development of high-throughput gene expression assays and novel gene edition strategies, it is now clear that NF- $\kappa$ B can no longer be defined as a single entity. Hence, we must delineate the selective contribution of each of the 5 NF- $\kappa$ B subunits in order to fully understand the different layers of regulation of T-cell identity. {INSERT FIGURE 5 HERE}

It may seem overambitious to attribute a role for 5 different proteins to the dozens of different T-cell flavours in different pathological settings. However, this seems to be a necessary step if we are to develop targeted therapies to either enhance or dampen immune responses in a specific fashion.

Thanks to high-throughput sequencing technologies, novel mouse models and patients carrying mutations in the NF- $\kappa$ B pathways, we have in hand the strategies to overcome this complexity (Figure 5). At the era of immunotherapies, NF- $\kappa$ B might be the key to fully unleash or control the power of T cells in cancer, autoimmunity and infections.

## Acknowledgements

This work was supported by grants from ATIP-Avenir, the Association pour la Recherche sur le Cancer (ARC) Foundation, the Bristol-Meyer Squibbs (BMS) Foundation and the Association pour la Recherche sur la Sclérose en Plaques (ARSEP) Foundation. We are grateful to Brigitte Manship and Guilhem Lalle for critical reading of the manuscript.

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## Figure Legends

### Figure 1. NF- $\kappa$ B signalling in T cells

This figure depicts the most common pathways involved in NF- $\kappa$ B activation in T cells. Other pathways can be triggered through the engagement of additional receptors, e.g. TLRs. Of note, a number of studies highlight the formation of heterodimers combining subunits of the canonical and alternative pathways; they are not shown here as the mechanisms leading to their formation remains unclear.

### Figure 2: Cell-autonomous functions of NF- $\kappa$ B in Thelper polarisation

This figure depicts the cell-intrinsic, known roles of members of the NF- $\kappa$ B pathway. Green annotations denote positive regulators, whereas red proteins are inhibitors. Protein names are placed next to the TH subset when the role of NF- $\kappa$ B was attributed to the whole lineage, without distinction between the master transcription factor and the effector cytokines. Question marks denote indirect evidence or controversial roles for the proteins.

### Figure 3: NF- $\kappa$ B subunits in Treg cell development and homeostasis

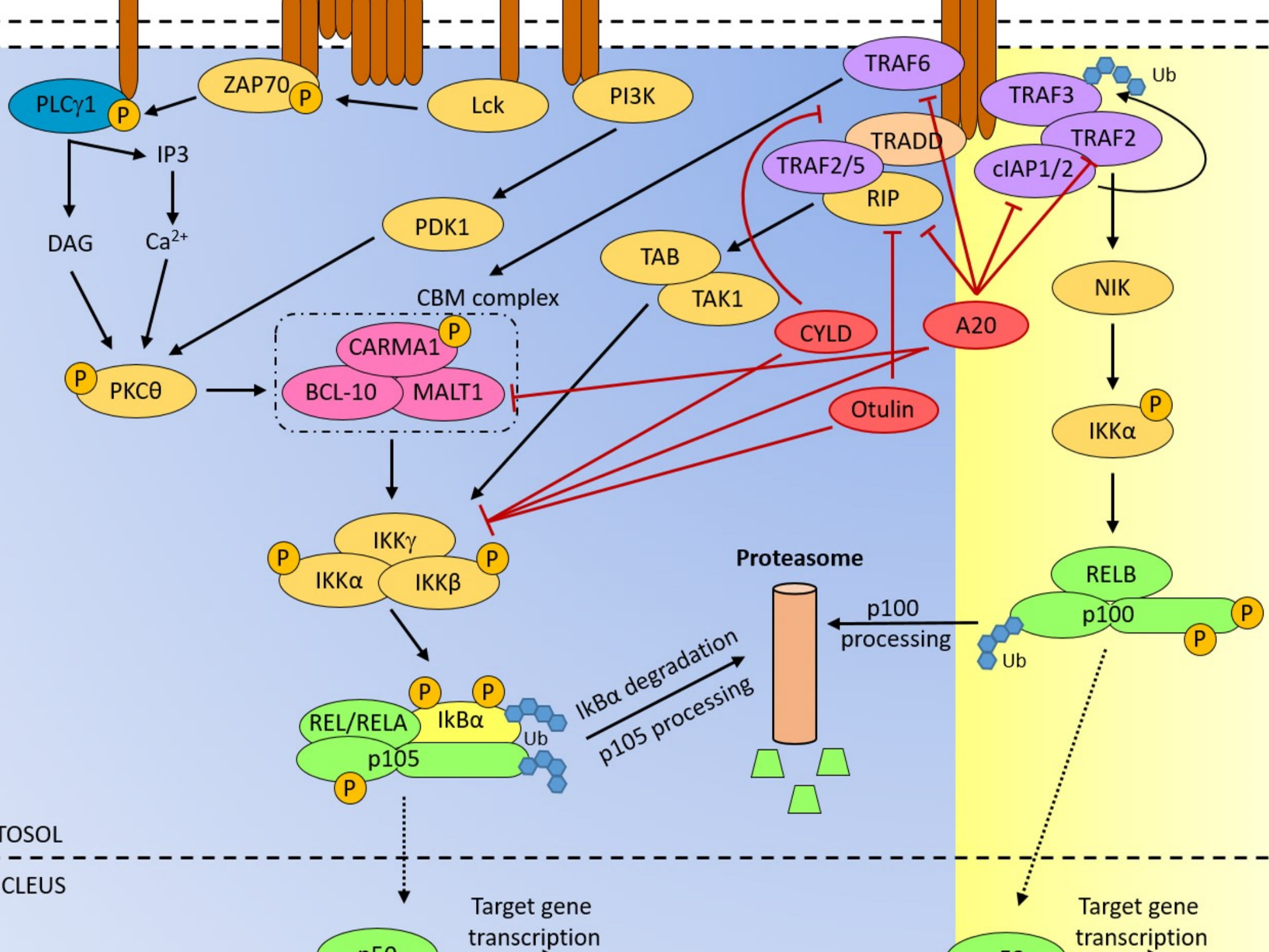
This figure highlights the cell-autonomous function of NF- $\kappa$ B subunits in Treg cells. Additional evidence demonstrates the implication of signalling intermediates downstream of TCR, CD28 and TNFRSF engagement, e.g. PDK1, the CBM and IKK complexes. Question marks denote unknown mechanisms of regulation. Protein names written in red indicate deleterious functions in Treg cell homeostasis or in disease outcome.

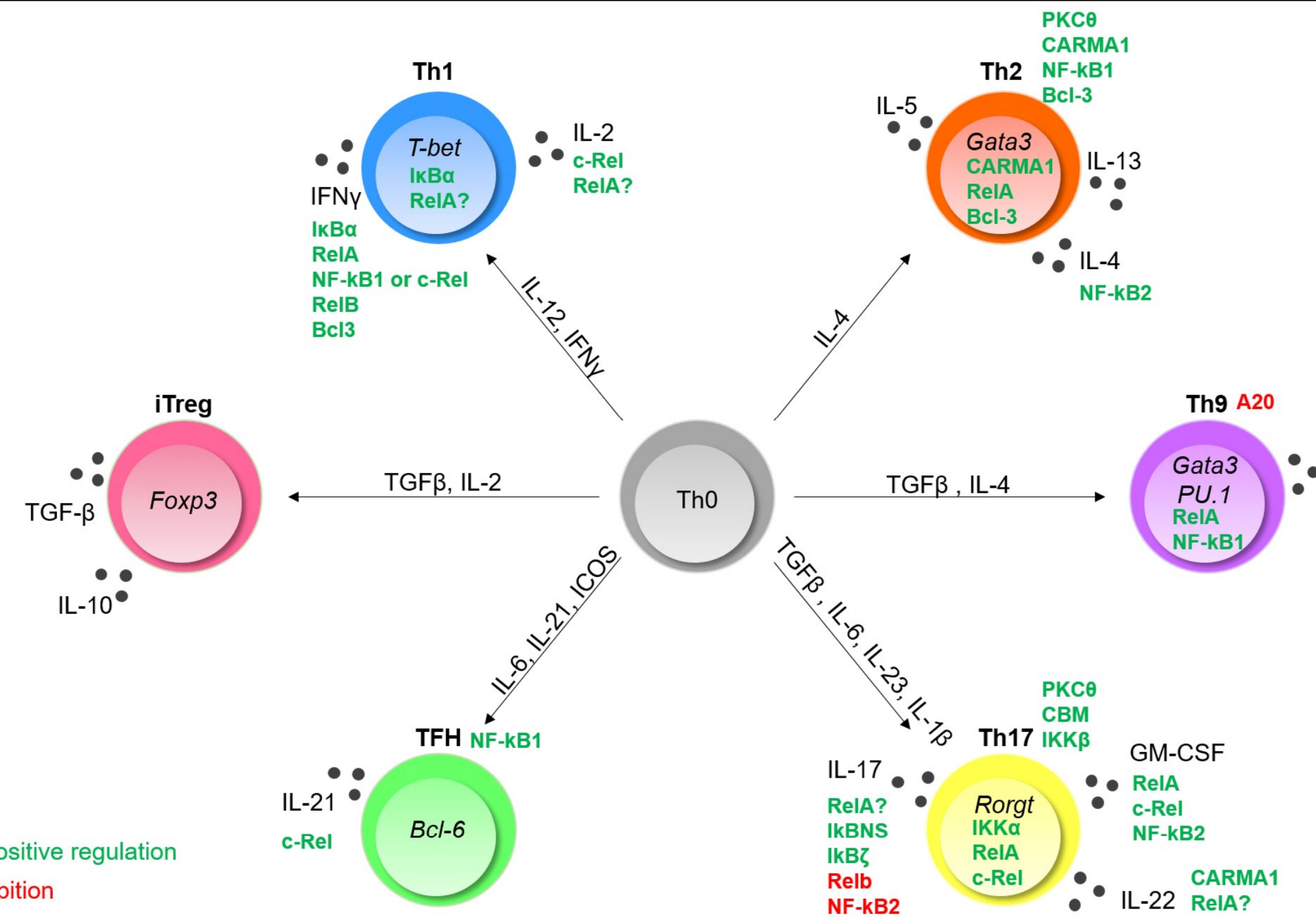
### Figure 4. The discrete roles of the PKC $\theta$ /CBM/IKK/NF- $\kappa$ B axis in mature CD8<sup>+</sup> T cells

The figure highlights the commonalities and differences between human (H, in blue) and mouse (M, in green) cell biology. TCR-Tg: evidence obtained using TCR-transgenic mouse models.

### Figure 5. Global strategy to delineate the selective contributions of NF- $\kappa$ B subunits and highlight novel therapeutic targets





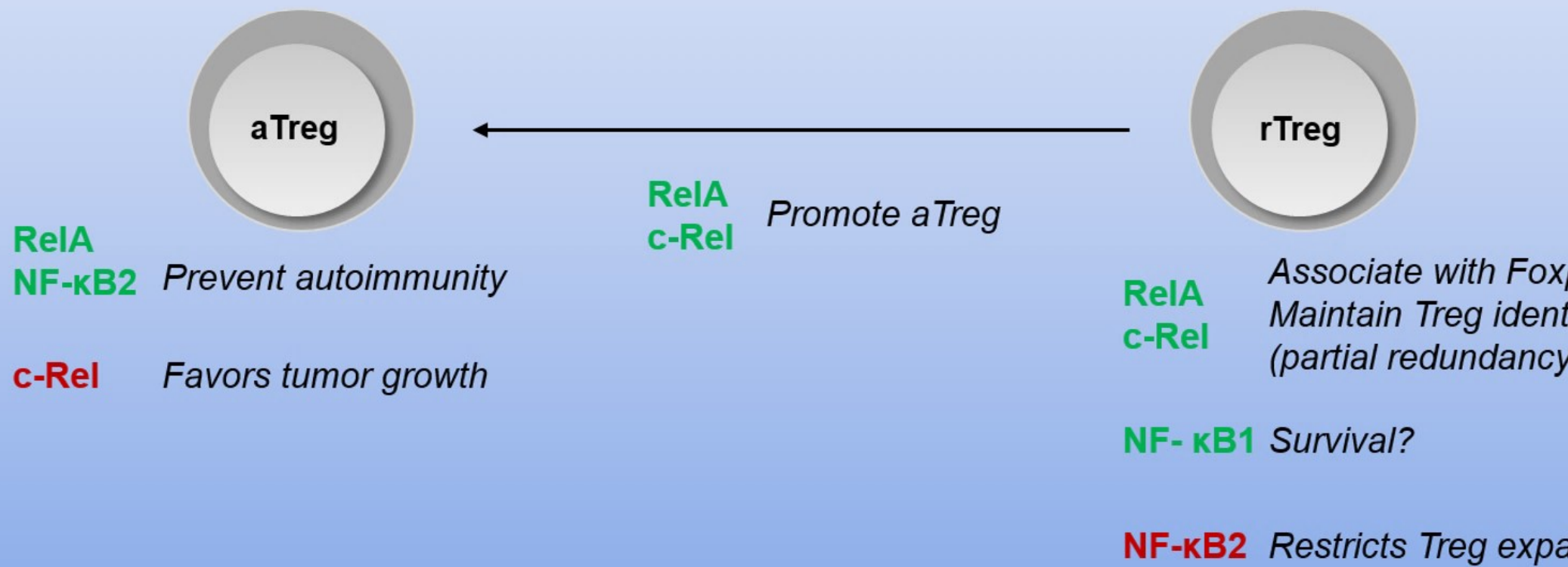


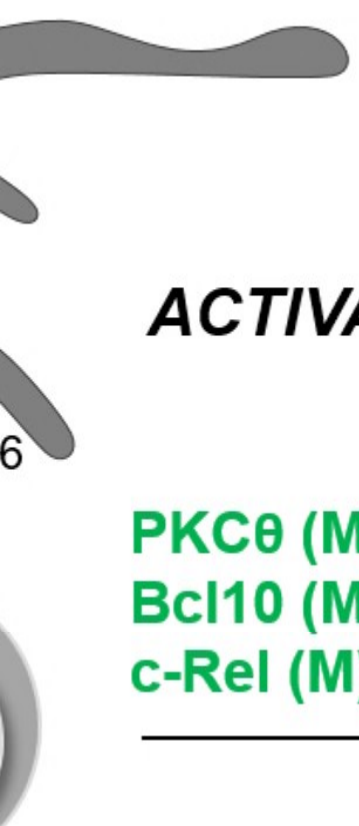


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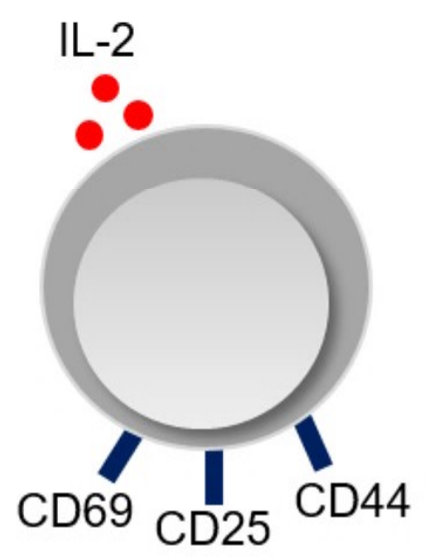
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**ACTIVATION**

PKC $\theta$  (M-TCR-Tg)  
Bcl10 (M-TCR-Tg)  
c-Rel (M)



**PROLIFERATION**

CBM (M,H)  
IKK $\beta$ (H)  
RelA (M)  
NF-kB1? (H)



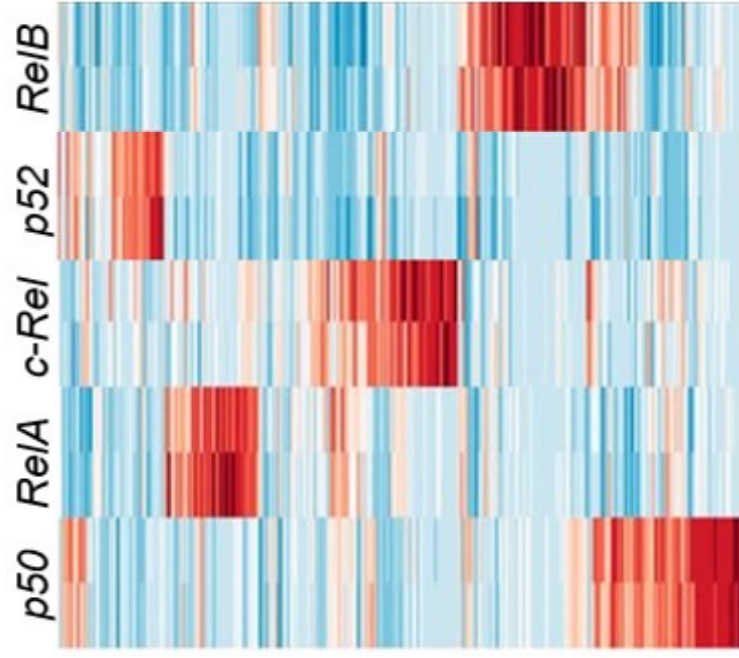
**DIFFERENTIAL**

PKC $\theta$  (M-TCR-Tg)  
IKK $\beta$ (H)  
RelA (M)

BCL10 (H)  
RelA (M)

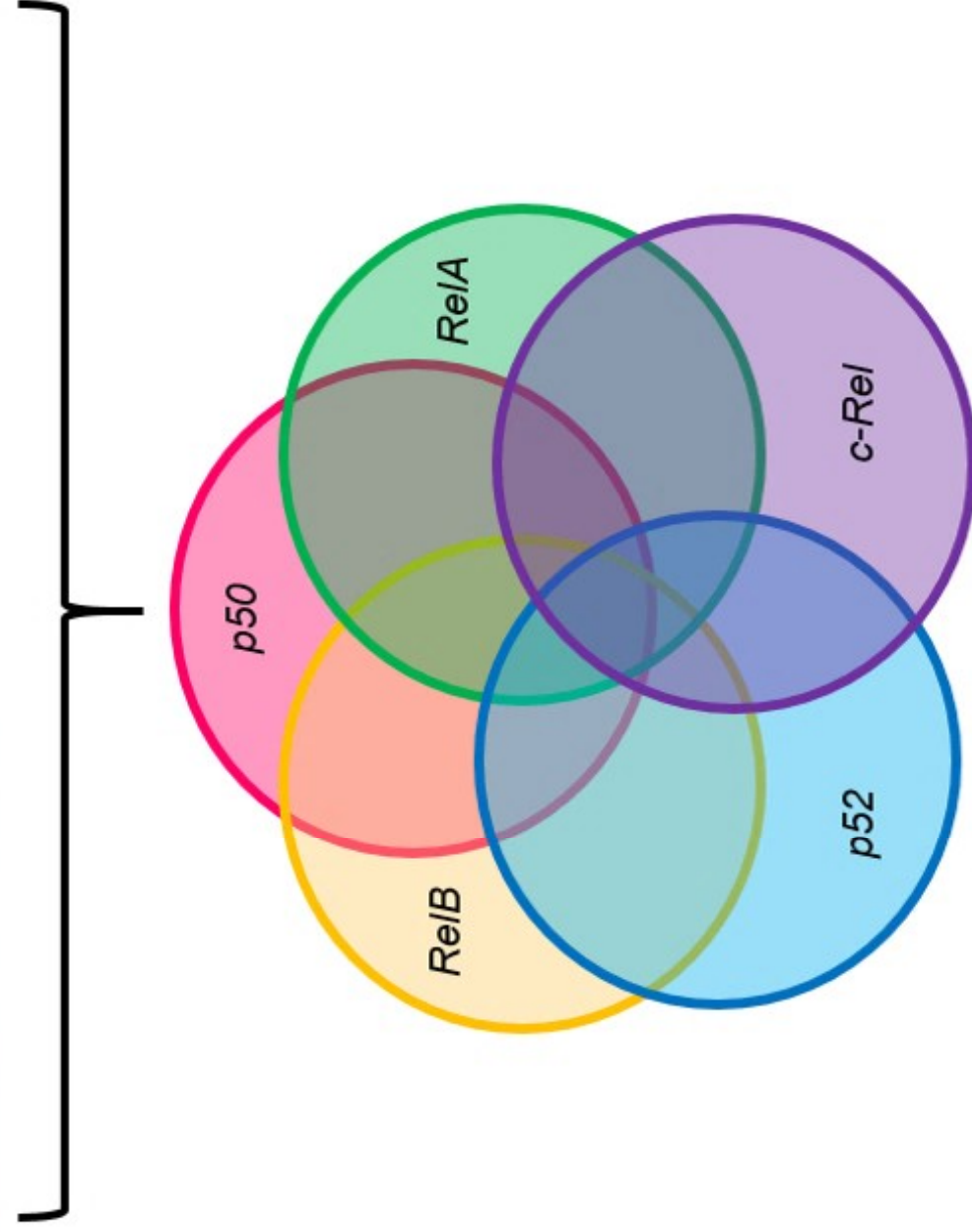
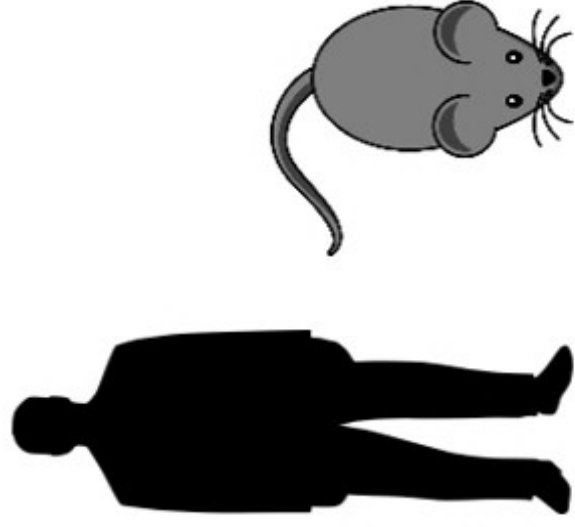


High throughput analyses  
RNAseq, CHIPseq



*In vivo* observations

Patients      Mouse models



Unique and shared functions in disease



Therapeutic intervention