A role for NF-κB-inducing kinase in regulatory T cell development, tissue maintenance and function

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„Nichts in der Geschichte des Lebens ist beständiger als der Wandel.“

Charles Darwin

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Abbreviations

A	Ampere
ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
Aire	Autoimmune regulator
Aly	Alymphoplasia
AP	Alkaline phosphatase
APC	Antigen-presenting cell
Areg	Amphiregulin
ATP	Adenosine triphosphate
BATF	Basic leucine zipper transcription factor, ATF-like
Bcl	B cell lymphoma
Bp	Base pair
BSA	Bovine serum albumin
CCL	C-C motif chemokine
CCR	C-C motif chemokine receptor
cIAP	Cellular inhibitor of apoptosis
CD	Cluster of differentiation
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor-1
CNS	Conserved non-coding sequence
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Ctrl	Control
CXCR3	C-X-C motif chemokine receptor 3
DC	Dendritic cell

dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DN	Double-negative
DNA	Deoxyribonucleic acid
DP	Double-positive
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FA	Formaldehyde
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FIC	$Foxp3^{Ires\text{-}Cre}$
FoxN1	Forkhead box protein N1
Foxp3	Forkhead box protein P3
g	Gramme
g	Acceleration
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft-versus-host disease
h	Hour
HBSS	Hanks' Balanced Salt Solution
H&E	Haematoxylin & eosin
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IDO	Indoleamine-pyrrole 2,3-dioxygenase
ID3	DNA-binding protein inhibitor 3
IEC	Intestinal epithelial cell
IFN-γ	Interferon-γ
IL	Interleukin
iLN	Inguinal lymph nodes

IL-2R	Interleukin-2 receptor
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iTreg	Induced Treg
Kb	Kilobase
$KHCO_3$	Potassium bicarbonate
KO	Knockout
LAG-3	Lymphocyte-activation gene-3
LAP	Latency associated peptide
LCFA	Long chain fatty acid
LPS	Lipopolysaccharide
M	Molar
MACS	Magnetic cell separation
MAPK	Mitogen-activated protein kinase
MAP3K14	Mitogen-activated protein kinase kinase kinase 14
MFI	Median fluorescence intensity
mL	Millilitre
mLN	Mesenteric lymph nodes
mM	Millimolar
mg	Milligramme
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
NaCl	Sodium chloride
NaN_3	Sodium azide
NCR	Non-catalytic region
NFIL3	Nuclear factor, interleukin-3-regulated
NH_4Cl	Ammonium Chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NOD	Non-obese diabetic
NRD	Negative regulatory domain

OD	Optical density
OTUD7B	OTU domain-containing protein 7B
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen Strep	Penicillin Streptomycin
PP	Peyer's patches
PMA	Phorbol-12-myristat-13-acetat
PPARγ	Peroxisome proliferator-activated receptor γ
pTreg	Peripherally-induced Treg
RA	Retinoic acid
Rag	Recombination activating gene
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RORγt	Retinoic-acid-receptor-related orphan receptor γt
Rpm	Rounds per minute
RT	Room temperature
SA	Streptavidin
SCFA	Short chain fatty acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIRPα	Signal regulatory protein α
SLE	Systemic lupus erythematosus
STAT5	Signal transducer and activator of transcription 5
TAE	Tris acetate EDTA
$T_{Ann.}$	Annealing temperature
TARC	Translational animal research centre
TBS	Tris-buffered saline
TBS-T	TBS containing Tween20
Tconv	Conventional T cell
TCR	T cell receptor
Teff	Effector T cell

TGF-β	Transforming growth factor β
Th	T helper
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF3	TNF receptor-associated factor
Treg cell	Regulatory T cell
TregP	Treg cell precursor
Tris	Tris(hydroxymethyl)aminomethane
tTreg	Thymus-derived Treg
TSDR	Treg-specific demethylated region
U	Units
V	Volt
VCAM	Vascular cell adhesion molecule
VCT	CellTrace violet
v/v	Volume per volume
WT	Wild type
w/v	Weight per volume
µL	Microlitre
°C	Temperature in degree Celsius

1 Introduction

1.1 Treg cell discovery and function

Back in 1982 Shimon Sakaguchi reported a new subtype of T cells, which he referred to as regulatory T cells (Treg cells), that were able to prevent oophoritis in mice after thymectomy (Sakaguchi, Takahashi and Nishizuka, 1982). A few years later, in rats, it was shown that this suppressive cluster of differentiation 4^+ ($CD4^+$) subtype expresses surface marker CD25, the interleukin (IL) 2 receptor (IL-2R) α chain (Hall *et al.*, 1990). Thereafter, Sakaguchi showed that deletion of this $CD4^+$ $CD25^+$ T cell subtype led to severe autoimmunity in mice (Sakaguchi *et al.*, 1995). The cell type claimed further significance when a few back-to-back publications showed the presence of $CD4^+$ $CD25^+$ Treg cells in humans now opening the horizon for the use of Treg cells as a therapeutic target (Baecher-Allan *et al.*, 2001; Dieckmann *et al.*, 2001; Ng *et al.*, 2001). With the discovery of forkhead box P3 (Foxp3) and its association to Treg cells as their bona fide transcription factor, Treg cells could now be distinguished from activated $CD4^+$ effector T cells (Teff), that also upregulate CD25 upon activation, representing their own distinct $CD4^+$ T cell subpopulation (Schubert *et al.*, 2001; Fontenot, Gavin and Rudensky, 2003; Hori, Nomura and Sakaguchi, 2003). Foxp3 is essential for the regulation of many target genes crucial for Treg cell suppressive capacity such as CD25 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Wing and Sakaguchi, 2012). IL-2 is crucial for $Foxp3^+$ Treg cell homeostasis (Fontenot *et al.*, 2005). Yet, in humans not just Treg cells but also $CD4^+$ Teff cells express both CD25 and Foxp3 upon activation (Kmieciak *et al.*, 2009). Nevertheless, human patients harbouring Foxp3 mutations and, hence, loss of Foxp3 function develop severe multi-organ autoimmunity known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), a lethal condition if left untreated (Bennett *et al.*, 2001; Bacchetta *et al.*, 2006).

Therefore, through exerting their suppressive mechanisms, $Foxp3^+$ Treg cells play a fundamental role in the maintenance of immune homeostasis and the prevention of autoimmunity (Kim, Rasmussen and Rudensky, 2007). $Foxp3^+$ Treg cells belong to the adaptive immune cell compartment, such as their $CD4^+$ T cell precursors, and need to be activated through antigen-specific T cell receptor (TCR) signalling in order to be suppressive, but the cells to be suppressed do not necessarily need to recognise the same antigen epitopes as their Treg cell counterparts (Sakaguchi *et al.*, 2013). $Foxp3^+$ Treg cells possess a vast array of mechanisms in order to prevent inflammation (**Figure 1**) (Vignali, Collison and Workman, 2008; Shevach, 2018).

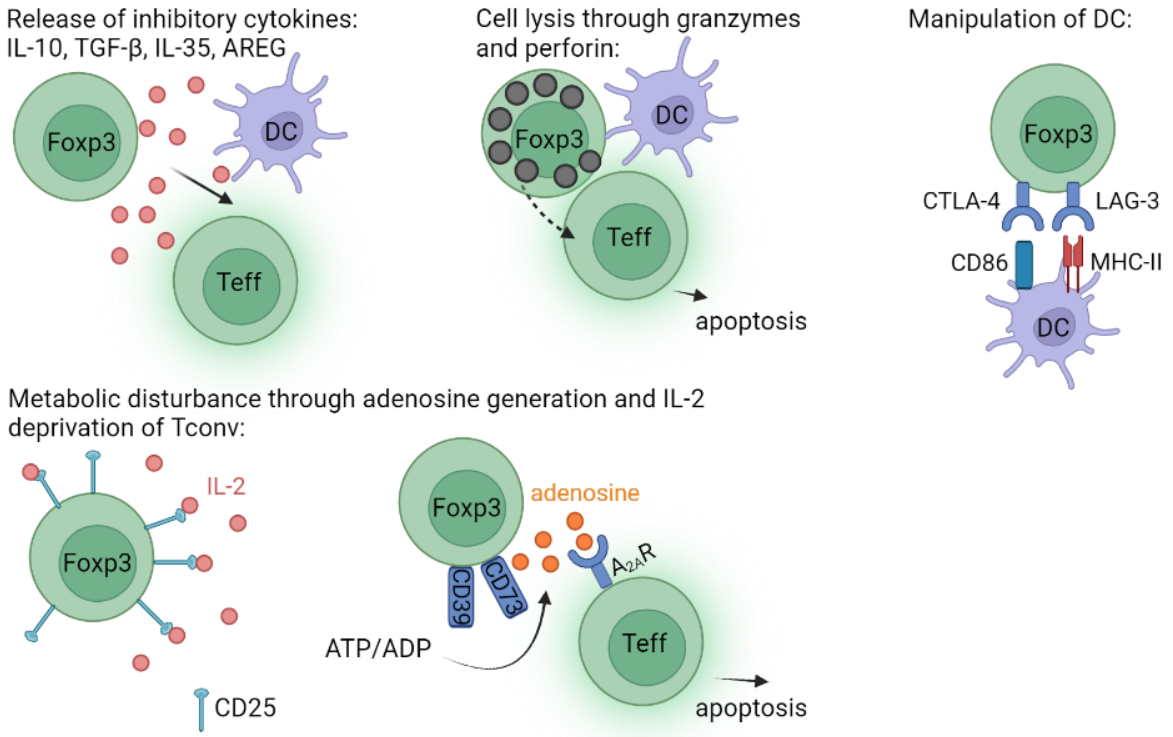

Figure 1: Mechanisms of Treg cell suppression. Treg cells dampen the immune response by regulating Teff cells and APCs such as DCs. Treg-mediated suppression is realised by secretion of inhibitory cytokines IL-10, TGF-β, IL-35 and AREG or through granzymes and perforin, by which perforin permeabilises the Teff cell membrane for granzymes to enter and induce apoptosis. Additionally, Foxp3$^+$ Treg cells express CTLA-4 and LAG-3, which block co-stimulation and maturation of APCs, respectively, that in turn results in ablation of further T cell activation. Furthermore, Foxp3$^+$ Treg cells express high levels of CD25, the alpha chain of the IL-2 receptor, through which they deprive conventional T cells of IL-2 which they in turn need for proliferation. Lastly, surface receptors CD39 and CD73 expressed on Treg cells collectively generate adenosine from adenosine triphosphate (ATP)/ adenosine diphosphate (ADP) which hinders DC maturation and dampens T cell activation (adapted from Shevach, 2018).

One mechanism of suppression is the secretion of anti-inflammatory cytokines such as IL-10, transforming growth factor-β (TGF-β), membrane-bound TGF-β, also called latency associated peptide (LAP) as well as IL-35 that suppress Teff cells and antigen-presenting cells (APC) (Powrie *et al.*, 1996; Asseman *et al.*, 1999; Belkaid *et al.*, 2002; Collison *et al.*, 2007; Gandhi *et al.*, 2010; Cuende *et al.*, 2015).

Treg cells can induce apoptosis in Teff cells and APCs through multiple ways, one being the release of either granzyme A or granzyme B as well as perforin (Grossman *et al.*, 2004; Lu *et al.*, 2005; Cao *et al.*, 2007). While Teff cells need IL-2 to proliferate, Treg cells can block IL-2 messenger ribonucleic acid (mRNA) production in Teff cells (Thornton and Shevach, 1998). Importantly, Treg cells express higher levels of CD25 than Teff cells meaning they can take up more IL-2 from the surroundings, hence, depriving Teff cells of IL-2 rendering them apoptotic (Pandiyan *et al.*, 2007). Another way to induce apoptosis in target cells is through the release of galectin-9 which acts through its receptor T cell immunoglobulin and mucin-domain containing-3 (TIM-3) (Wang *et al.*, 2009; Liberal *et al.*, 2012).

In addition to dampening the immune response through the release of soluble mediators, Treg cells can manipulate APCs via direct cell-cell contact through surface receptors (Tang *et al.*, 2006). One of those surface receptors constitutively expressed by Treg cells is CTLA-4, that binds to CD80/CD86 on dendritic cells (DC), which use CD80/CD86 to provide co-stimulatory signals to conventional T cells (Tconv) (Takahashi *et al.*, 2000; Wing *et al.*, 2008). The interaction through CTLA-4 with CD80/CD86 leads to downregulation of the latter as well as to a decrease of CD80 mRNA levels (Cederbom, Hall and Ivars, 2000; Onishi *et al.*, 2008). Moreover, it has been reported that Treg cells utilise CTLA-4 to capture CD80/CD86 off the surface of APCs through trans-endocytosis and subsequent intracellular degradation (Qureshi *et al.*, 2011). Lastly, binding of CTLA-4 to CD80/CD86 induces the production and release of indoleamine-pyrrole 2,3-dioxygenase (IDO) by DCs that in turn induces apoptosis Teff cells (Grohmann *et al.*, 2002).

Besides, through ectonucleotidases CD39 and CD73, Treg cells produce anti-inflammatory adenosine that inhibits T cell activation and DC maturation (Lappas, Rieger and Linden, 2005; Borsellino *et al.*, 2007; Deaglio *et al.*, 2007; Fletcher *et al.*, 2009).

In summary, it becomes evident that the presence of Treg cells, hence their maintenance and stability, is essential in preventing autoimmunity or looking at the clinical setting, preventing graft rejection (Shevach, 2018). Maintenance and stability, amongst others, can be regulated on a transcriptional level through conserved non-coding sequences (CNS), CNS0, CNS1, CNS2 and CNS3, by binding of CNS-specific transcription factors such as canonical nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) transcription factor c-Rel, that can for instance initiate Foxp3 transcription (Fu *et al.*, 2012; Lee and Lee, 2018). In order for transcription factors to bind for instance to CNS2, that is required for the transcription of genes needed for Treg cell stability, the Foxp3 locus, in particular CNS2 needs to be demethylated and accessible ensuring open chromatin regions (Lee and Lee, 2018). However, in the cancer setting for instance, the presence of Treg cells is rather undesired since they suppress anti-tumour responses (Shevach, 2018). Nevertheless, Treg cell stability depends on the environment (inflammatory, non-inflammatory) as well as whether a Treg cell developed in the thymus (tTreg cell), in the periphery (pTreg cell) or *in vitro* (iTreg cell) from naïve $CD4^+$ T cells with tTreg cells showing increased stability over iTreg cells (Floess *et al.*, 2007; Polansky *et al.*, 2008; Zhou *et al.*, 2009).

To further introduce developmentally different Treg cell subsets, the next chapter will elaborate on Treg cell development.

1.2 Treg cell development

The expansion of human Treg cells already finds its relevance in the clinical setting such as for prevention of graft-versus-host disease (GvHD), while the generation of iTreg cells for clinical purposes is being considered as well (Brunstein *et al.*, 2016; Alvarez-Salazar *et al.*, 2020). *In vitro*, the stimulation of naïve $CD4^+$ T cells with anti-CD3, anti-CD28 generally activates T cells but in order to generate and expand iTreg cells, additionally, TGF-β as well as IL-2 need to be added, as they induce Foxp3 expression through their transcription factors Smad7 and signal transducer and activator of transcription 5 (STAT5), respectively (Zheng *et al.*, 2002; Chen *et al.*, 2003; Wan and Flavell, 2005; Burchill *et al.*, 2007; Pandiyan *et al.*, 2007; Tone *et al.*, 2008).

In contrast, *in vivo*, T cell precursors are generated in the bone marrow but migrate to the thymus where they develop into different T cell subsets (Rothenberg, Moore and Yui, 2008). Thymus-entering T cell precursors are double-negative (DN) for CD4 and CD8 but while they undergo thymic maturation, they start expressing and, through the recombination activating gene (Rag), rearranging their TCR as well as begin transitioning into the double-positive (DP) stage, where they upregulate both CD4 and CD8 (Germain, 2002). During positive selection, DP thymocytes with functionally rearranged TCRs recognise antigens presented by cortical thymic epithelial cells (cTEC) and develop into either CD8 single-positive (CD8SP) cells by interacting with major histocompatibility complex I (MHC-I) molecules on cTEC or develop into CD4SP cells if they recognise MHC-II molecules on cTEC instead (Famili, Wiekmeijer and Staal, 2017). Subsequent negative selection removes self-reactive T cells that show a very high affinity to autoantigens, whereas T cells which are not activated by autoantigens are released into the periphery as naïve $CD4^+$ T cells (Germain, 2002). T cell precursors that show intermediate affinity of their TCR to autoantigens are destined to become Treg cells (**Figure 2**) (Owen, Sjaastad and Farrar, 2019). Thereby, autoantigens are presented by medullary thymic epithelial cells (mTEC), which are regulated by transcription factor autoimmune regulator (Aire), or presented by bone marrow-derived APCs such as B cells and DCs (Oh and Shin, 2015; Yamano *et al.*, 2015; Kieback *et al.*, 2016).

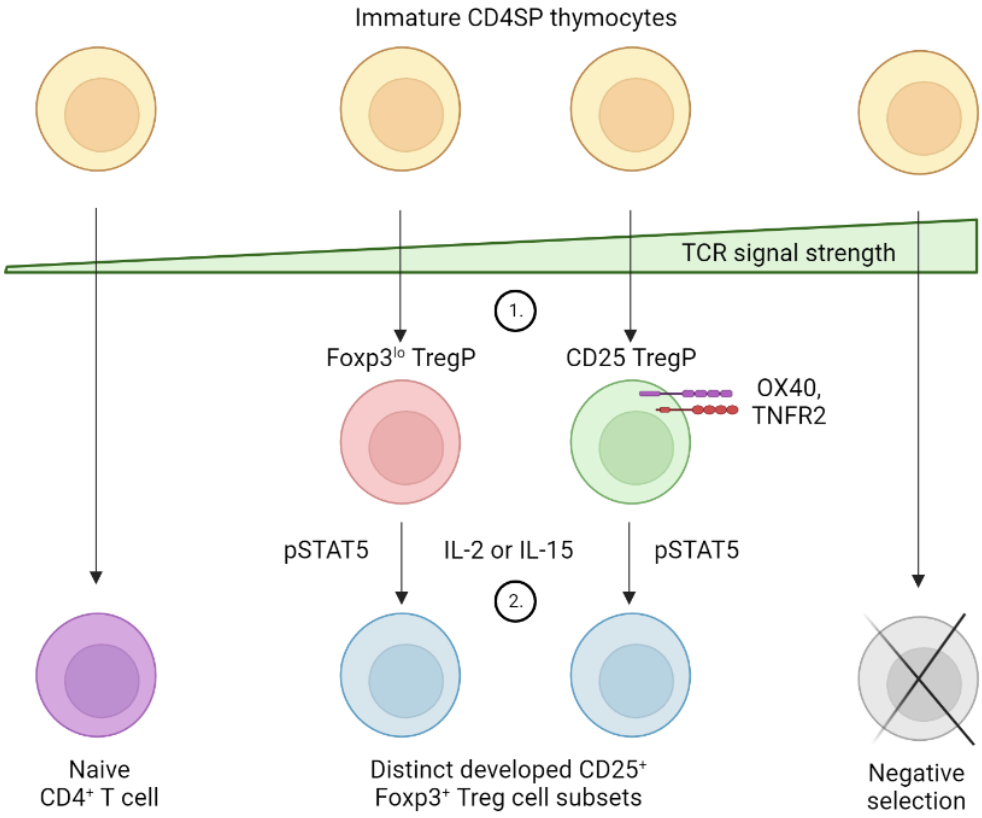

Figure 2: Thymic Treg cell development. Autoantigens are presented to immature CD4SP T cells, whose subsequent developmental fate is determined by the signal strength of their respective TCR. CD4SP thymocytes whose TCR show weak affinity to autoantigens are released into the periphery as naïve $CD4^+$ T cells, whereas thymocytes that have a high affinity to autoantigens undergo apoptosis. 1: Thymocytes with intermediate TCR signals enter the Treg cell lineage, where lower TCR signals give rise to the $Foxp3^{lo}$ TregP population and stronger TCR signals induce the upregulation of CD25 generating the CD25 TregP subset. Thereby, TCR signal strength correlates with the upregulation and hence surface expression levels of TNFR superfamily receptors OX40, TNFR2 and GITR (Mahmud et al., 2014). 2: $Foxp3^{lo}$ TregP as well as CD25 TregP subsequently signal through IL-2 or IL-15 that through STAT5 induces Foxp3 transcription. This results in two distinct mature Foxp3 and CD25 co-expressing Treg cell subsets that are released into the circulation as tTreg cells (adapted from Owen, Sjaastad and Farrar, 2019).

In a first step during thymic Treg cell development, intermediately strong TCR signals correlate with the upregulation of CD25 as well as tumour necrosis factor receptor (TNFR) superfamily receptors glucocorticoid-induced TNFR-related protein (GITR) (CD357), tumour necrosis factor receptor 2 (TNFR2) (CD120b) and OX40 (CD134) on CD25 Treg cell precursors (TregP) (Mahmud *et al.*, 2014). Slightly lower TCR signals induce Foxp3 expression in a second precursor subset, namely $Foxp3^{lo}$ TregP (Owen *et al.*, 2019). In CD25 TregP as well as $Foxp3^{lo}$ TregP, Foxp3 expression is upregulated in a TCR-independent mechanism through STAT5 upon encounter of cytokines IL-2 or IL-15 (Burchill *et al.*, 2008; Lio and Hsieh, 2008; Owen, Sjaastad and Farrar, 2019). Eventually, two functionally distinct $CD25^+$ $Foxp3^+$ Treg cell populations are released into the periphery as tTreg cells, where tTreg cells arising from CD25 TregP were able to prevent experimental autoimmune encephalomyelitis (EAE) whereas tTreg cells from $Foxp3^{lo}$ TregP did not (Owen *et al.*, 2019). Of note, it has been shown that tTreg cells can also recirculate

back into the thymus and can be distinguished from developing thymic Treg cells by the use of the markers CD73, IL-1R2 and chemokine receptor (CCR) 6 (Owen *et al.*, 2019; Nikolouli *et al.*, 2021).

Interestingly, a recent publication by the Becher lab suggested that tTreg cells aid in regulating thymic development through the elimination of autoreactive T cells in addition to negative selection (Haftmann *et al.*, 2021). Strikingly, Mahmud and colleagues showed that collective deletion of GITR, OX40 and TNFR2 abrogated thymic Treg cell development highlighting the importance of TNFR superfamily receptors in Treg cell biology (Mahmud *et al.*, 2014). Furthermore, both TregP subsets have distinct TCR repertoires, mice lacking CNS3 in Foxp3^{+} Treg cells and the *nfkb1* gene lack the Foxp3^{lo} TregP subset, while the CD25 TregP subset is more prone to apoptosis (Owen *et al.*, 2019). However, to date, it remains elusive why Foxp3^{lo} TregP are responsive to IL-2 signalling when they do not express the receptor for it. Owen *et al.* suggest that GITR expression enhances TCR signalling in Foxp3^{lo} TregP rendering them more sensitive to IL-2 (Owen *et al.*, 2019). Wu *et al.* found that CD25 is a target gene of Foxp3 (Wu *et al.*, 2006). However, consequently, this then raises the question of how Foxp3^{lo} TregP cannot express CD25. Do they potentially upregulate CD25 only after FACS phenotyping, since they might have slower kinetics due to lower TCR signal strength? This would be in line with the Foxp3^{lo} TregP subset being more mature than CD25 TregP (Owen *et al.*, 2019). Noteworthy, *in vivo*, IL-2 might be trans-presented to IL-2Rβ (CD122) and IL-2Rγ (CD132) chain on Foxp3^{lo} TregP via IL2Rα chain CD25 on T helper cells (Ikemizu, Chirifu and Davis, 2012).

Nevertheless, naïve CD4^{+} T cells that leave the thymus entering into the circulation can develop into Foxp3^{+} Treg cells in the periphery, pTreg cells, upon antigen encounter in addition to IL-2 and TGF-β (Kretschmer *et al.*, 2005). While pTreg cells are believed to protect from antigens entering externally through mucosal surfaces, tTreg cells are thought to protect against autoimmune diseases (Shevach and Thornton, 2014; van der Veeken *et al.*, 2022). Yet, to date the use of Helios as a marker for tTreg cells to distinguish tTreg from pTreg cells in the periphery, although widely used, does not remain undisputed (Himmel *et al.*, 2013; Szurek *et al.*, 2015; Elkord, 2016). However, in a recent publication by the lab of Alexander Rudensky intestinal pTreg cells developed from naïve CD4^{+} T cells have been shown to express transcription factor RAR-related orphan receptor gamma t (RORγt) and not Helios (van der Veeken *et al.*, 2022).

Despite the role of tTreg and pTreg cells in lymphoid organs, their function in distant peripheral tissues is much less studied. Thus, the next chapter will introduce the function and biology of Treg cells within peripheral tissues, in particular in the colon and the liver.

1.3 Tissue Treg cells

As elaborated in the previous chapter, tTreg cells exit the thymus and circulate between the blood and secondary lymphoid organs. However, there is a need for Treg cells to enter peripheral tissues, such as the gut and the liver, to prevent excessive immune activation, to promote tolerance as well as tissue homeostasis, regeneration and repair making them yet again an attractive therapeutic target (Burzyn *et al.*, 2013; Kuswanto *et al.*, 2016; Estrada Brull, Panetti and Joller, 2022).

The field of tissue Treg cells is still a relatively novel and an emerging one. Quite recently, in 2009, Markus Feuerer and colleagues were the first to discover that Treg cells mediated tissue homeostasis in adipose tissue (Feuerer *et al.*, 2009). It has been shown that already during development, neonatal Treg cells are deposited into peripheral tissues where they contribute to tissue regulation (Boothby *et al.*, 2021). While naïve Treg cells migrate between secondary lymphoid organs, Treg cells that migrate into tissues are more of a effector Treg cell type and can express the integrin CD103 (Huehn *et al.*, 2004; Guo *et al.*, 2008; Smigiel *et al.*, 2014). It is presumed that tTreg cells are activated in secondary lymphoid organs by specific antigens prior to migrating into peripheral tissues (C. Li *et al.*, 2018; Sivasami and Li, 2020). Indeed, intestinal DCs were reported to migrate into secondary lymphoid organs presenting gut-derived antigens to Treg cells initiating their priming and hence migration (Stagg, Kamm and Knight, 2002; Iwata *et al.*, 2004; Mora and von Andrian, 2006). Precursor tissue Treg cells still present in secondary lymphoid organs depend on transcription factors basic leucine zipper transcription factor, ATF-like (BATF) and nuclear factor, interleukin 3 regulated (NFIL) to further take on their tissue phenotype (Delacher *et al.*, 2020). Eventually, loss of transcription factor inhibitor of deoxyribonucleic acid (DNA)-binding 3 (ID3) leads to the acquisition of the tissue Treg phenotype (Sullivan, Höllbacher and Campbell, 2019). Interestingly, once Treg cells have reached the tissue, as it has been shown for the intestine, they have been found to be able to also traffic back into secondary lymphoid organs (Morton *et al.*, 2014).

Within the colon, immune cells are mainly localised in the mucosal lamina propria (Panduro, Benoist and Mathis, 2016). Colonic Treg cells originate either from the thymus (tTreg cells) or are induced locally on site (pTreg cells) (Cebula *et al.*, 2013). While pTreg cells express transcription factor RORγt, tTreg cells are positive for transcription factors Helios and GATA-3 (Ohnmacht *et al.*, 2015; Panduro, Benoist and Mathis, 2016; van der Veeken *et al.*, 2022). The RORγt-negative, Helios-positive population maintains its Foxp3 expression in part by GATA-3, which also induces expression of ST2, the receptor for IL-33, and gene transcription of amphiregulin (Areg) that is downstream of ST2 (Schiering *et al.*, 2014). Proliferation of intestinal Treg cells is mainly independent of IL-2 (Korn *et al.*, 2014; Smigiel *et al.*, 2014). The tTreg compartment depends on IL-33 for expansion (Schiering *et al.*, 2014). IL-33 is secreted by epithelial cells and can induce Areg production by Treg cells that directly acts on barrier tissues

promoting tissue regeneration (Pichery *et al.*, 2012; Zaiss *et al.*, 2013; Arpaia *et al.*, 2015; Harb *et al.*, 2021). However, it is not known whether colonic ST2$^+$ Treg cells produce Areg. Nevertheless, the tTreg subset seems to require GATA-3 in order to be maintained under inflammatory conditions to protect from colitis (Wohlfert *et al.*, 2011). Helios$^+$ Treg cells have been found to be more of an effector Treg cell subtype with higher percentages of CD103$^+$, OX40$^+$, TNFR2$^+$ and Ki67$^+$ amongst them in comparison to Helios$^-$ Treg cells. The authors further reported that Foxp3 expression was more stable in Helios$^+$ Treg cells and can be, hence, advertised as a marker for Treg cell stability (Thornton *et al.*, 2019). Helios binds to the IL-2 promoter and represses IL-2 transcription in Treg cells (Hagihara *et al.*, 2019). Interestingly, colonic Treg cells developing from transferred naïve CD4$^+$ T cells can also upregulate Helios but this is seen only in transferred infant Tconv cells while adult Tconv cells tend to upregulate RORγt in the colon (Pratama *et al.*, 2020).

Yet, the majority of Treg cells in the gut is the RORγt-expressing pTreg cell subset which is induced from naïve CD4$^+$ T cells on site through the microbiota (Atarashi *et al.*, 2011; Cebula *et al.*, 2013; Ohnmacht *et al.*, 2015; Sefik *et al.*, 2015). The microbiota produces short-chain fatty acids (SCFA) like butyrate or other food-derived acids (Arpaia *et al.*, 2013; Hao *et al.*, 2021; Pompura *et al.*, 2021). SCFA stimulate TGF-β production by intestinal epithelial cells (IEC) that in turn induces Foxp3$^+$ Treg cells from naïve CD4$^+$ T cells (Chen *et al.*, 2003; Atarashi *et al.*, 2013). In addition, intestinal DCs produce TGF-β and the vitamin A-derivate retinoic acid (RA) which then leads again to Treg cell formation (Benson *et al.*, 2007; Coombes *et al.*, 2007; Sun *et al.*, 2007).

Interestingly, long chain fatty acid (LCFA) diet resulted in the development of EAE, which could be rescued by a treatment with SCFA (Haghikia *et al.*, 2015). RORγt$^+$ Treg cells are stable and suppressive during intestinal inflammation (Yang *et al.*, 2016). Indeed, RORγt$^+$ Treg cells have been shown to control intestinal T helper (Th) 1 and Th17 inflammation while tTreg cells were suggested to participate in repair processes (Atarashi *et al.*, 2011; Geuking *et al.*, 2011; Hegazy and Powrie, 2015; Ohnmacht *et al.*, 2015; Sefik *et al.*, 2015; van der Veeken *et al.*, 2022). Yet, whether pTreg cells additionally contribute to tissue regeneration still needs to be investigated.

In any case, colonic Treg cells are a critical player in maintaining immune tolerance to the commensal microbiota through the production of IL-10 (Asseman *et al.*, 1999). IL-10 is one of the key cytokines reassuring intestinal tissue homeostasis, exemplified by IL-10 deficiency in Foxp3$^+$ Treg cells that results in spontaneous colitis in mice (Rubtsov *et al.*, 2008).

The liver is constantly in contact with colon-derived microbes through the intestinal venous blood circulation (Balmer *et al.*, 2014). Similar to the colon where immune cells are continuously exposed to foreign microbial antigens, Treg cells in the liver are required to maintain homeostasis and tolerance as

well (Crispe *et al.*, 2006). Soon after birth, the liver of neonates is seeded with Treg cells whose frequency was shown to increase within the first two weeks and that was dependent on the intestinal microbiota (Maria, English and Gorham, 2014; Li *et al.*, 2020). While the majority of hepatic Treg cells are positive for Helios and can be assumed to have migrated there from the periphery as tTreg cells, DCs can induce Treg cells on site through the presentation of intestinal antigens to naïve $CD4^+$ T cells (Kamanaka *et al.*, 2011; Li *et al.*, 2020). Furthermore, hepatocytes have been shown to contribute to the generation of Treg cells in liver (Burghardt *et al.*, 2014). Additionally, blocking of TGF-β resulted in a decrease of frequency of hepatic Treg cells in neonates (Maria, English and Gorham, 2014). As a homing receptor for Treg cells to the liver, common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) with the help of intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) has been suggested (Shetty *et al.*, 2011). CCR4 enables hepatic Treg cells to migrate along the C-C motif chemokine ligand (CCL) 22 chemokine gradient towards intrahepatic DCs, whereas CXC motif chemokine receptor (CXCR) 3 allows Treg cells to home to the inflamed liver (Oo *et al.*, 2010; Erhardt *et al.*, 2011). Importantly, hepatic Treg cells exert regulatory functions preventing hepatitis through the production of IL-10 (Erhardt *et al.*, 2011; Kamanaka *et al.*, 2011). Furthermore, they have been shown to express functional surface markers CTLA-4, CD39 and lymphocyte activation gene-3 (LAG-3) (Chen *et al.*, 2016; Atif, Warner and Oo, 2018; Oo *et al.*, 2019). Hepatic stellate cells induced aryl hydrocarbon receptor (AhR) signalling in hepatic Treg cells upon lipopolysaccharide (LPS) treatment (S. Kumar *et al.*, 2017). Gene expression analysis revealed that hepatic Treg cells express genes for peroxisome proliferator-activated receptor γ (PPARγ), T-bet and Areg and mediate self-tolerance by regulating Th1 responses (Li *et al.*, 2020). Generally, liver Treg cells show more of an effector memory phenotype and have been described to be more apoptotic and proliferative in comparison to splenic Treg cells (Chen *et al.*, 2016; Li *et al.*, 2020). The liver is believed to be a rather harsh environment for Treg cells and while IL-2 levels in the liver have been reported to be low, it was shown that hepatic Treg cells expand in response to IL-33 as was seen in the colon (Chen *et al.*, 2016; Delacher *et al.*, 2017).

Yet, although a lot of progress in describing tissue Treg cells has been made in recent years, much more research needs to be conducted deciphering several aspects of their tissue biology such as how they are being maintained in a rather harsh environment. One such signalling pathway that has been associated with mediating Teff cell maintenance is the non-canonical NF-κB pathway.

1.4 The non-canonical NF-κB signalling pathway

This chapter will describe the induction of the non-canonical NF-κB signalling pathway, while following chapters will further elaborate on the biological functions that each of its intracellular members exerts on Treg cells.

In general, NF-κB signalling comprises of two distinct intracellular pathways, the canonical (classical) as well as the non-canonical (alternative) NF-κB pathway, that regulate a vast number of diverse target genes depending on cell type and stimulus (Vallabhapurapu and Karin, 2009). Canonical NF-κB signalling is mediated by transcription factors p50, RelA (p65) as well as c-Rel and has been shown to be critical for Treg cell activation, function, stability and last but not least thymic Treg cell development (Grinberg-Bleyer *et al.*, 2017; Oh *et al.*, 2017; Ronin *et al.*, 2019; Hövelmeyer, Schmidt-Supprian and Ohnmacht, 2022). On the contrary, non-canonical NF-κB activated by binding of TNFR superfamily members induces transcription factors p52 and RelB but is much less studied in regards to Treg cell biology in contrast to its classical counterpart (Claudio *et al.*, 2002; Coope *et al.*, 2002; Dejardin *et al.*, 2002; Kayagaki *et al.*, 2002). Surface receptors of the TNFR superfamily that activate non-canonical NF-κB signalling in Treg cells are described in more detail in chapter 1.7. Under steady-state conditions, NF-κB-inducing kinase (NIK) is constantly degraded by the proteasome through tumour necrosis factor (TNF) receptor-associated factor (TRAF) 3-mediated ubiquitination (**Figure 3**) (Sun and Ley, 2008). However, upon TNFR superfamily receptor ligation, the TRAF3 complex including TRAF2 and cellular inhibitor of apoptosis protein (cIAP) is orchestrated away from NIK to the intracellular TNFR superfamily receptor domain, which mediates the degradation of TRAF3 via ubiquitination and leads to subsequent stabilisation of NIK (Zarnegar *et al.*, 2008; Vallabhapurapu *et al.*, 2008). Thereafter, NIK as the key enzyme in non-canonical NF-κB signalling phosphorylates and activates inhibitor of nuclear factor kappa-B kinase alpha (IKKα) that in turn phosphorylates p100 and induces its processing into p52 (Senftleben *et al.*, 2001; Xiao, Fong and Sun, 2004). Eventually, this leads to the translocation of the p52:RelB heterodimer into the nucleus and the transcription of non-canonical NF-κB target genes (Bonizzi *et al.*, 2004).

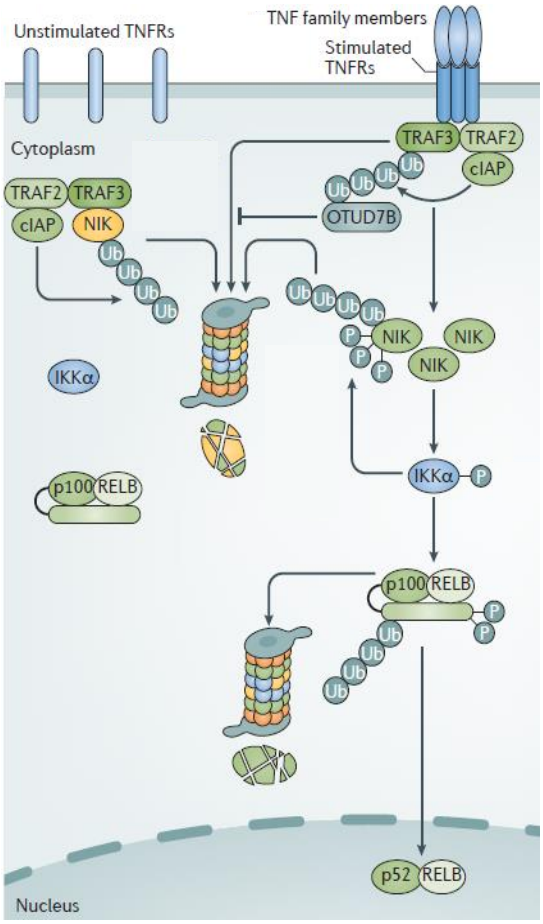

Figure 3: The non-canonical NF-κB signalling pathway. Under homeostatic conditions NIK is continuously subjected to proteasomal degradation by K48 linkage of ubiquitin chains through cIAP. Of note, TRAF3 binds to NIK and needs TRAF2 as an adaptor protein to bind to the E3 ligase cIAP. Upon ligation of surface receptors of the TNFR superfamily (on Treg cells: TNFR2, OX40, CD137, CD30 and CD27) to their respective ligands, NIK is stabilised since the translocation of the TRAF2-TRAF3-cIAP complex to the receptor leads to ubiquitination of TRAF3 by cIAP and subsequent degradation. Some receptors recruit a deubiquitinase named OUT domain-containing protein 7B (OTUD7B) that deubiquinates TRAF3 but this has not been reported for TNFR superfamily receptors on Treg cells. However, exemplary, NIK phosphorylates IKKα that consequently phosphorylates p100, which is then ubiquitinated and processed into p52. Now that p100 is processed, it cannot keep RelB in the cytoplasm and, hence, the p52:RelB dimer eventually translocates into the nucleus (adapted from Sun, 2017).

Interestingly, molecular signalling components of canonical and non-canonical NF-κB pathways are structurally very conserved and hence very similar to each other, which can result in crosstalk between the two NF-κB pathways (Sun, 2012). In T cells, p52 can form heterodimers with c-Rel that leads to production of granulocyte-macrophage colony-stimulating factor (GM-CSF) in encephalitogenic cells (Yu *et al.*, 2014). In *nfkb2*-deficient mice, RelB forms heterodimers with canonical p50 (Dhar *et al.*, 2019). Moreover, constitutive overexpression of NIK leads to induction of both canonical and non-canonical NF-kB pathways in TRAF3-deficient mice through activation of the IKK complex and in addition p100 processing (Zarnegar *et al.*, 2008). Besides, p100 is known to keep canonical Rel proteins in the cytoplasm dampening canonical NF-κB signalling (Zarnegar *et al.*, 2008). Additionally, TNFR superfamily receptors recruit specific TRAF proteins and can, depending on which TRAF proteins are recruited, activate both the canonical as well as the non-canonical NF-κB pathway (Wajant, Henkler and Scheurich, 2001; Murray *et al.*, 2011).

Taken together, the non-canonical NF-κB signalling pathway is an induced, complex network delicately regulated. The following chapter further elucidates the role of intracellular non-canonical NF-κB signalling molecules, in particular NIK as the major player of non-canonical NF-κB signalling, on Treg cell biology.

1.5 Status quo of NIK signalling in Foxp3$^+$ Treg cells

The few publications on the role of NIK in Foxp3$^+$ Treg cells will be described below as well as contradictory and conclusive findings highlighted.

The *NIK* gene is located on chromosome 11 in mice encoding a serine/threonine protein kinase that binds to IKKα via its non-catalytic region (NCR) at its C-terminal end (**Figure 4**) (Pflug and Sitcheran, 2020).

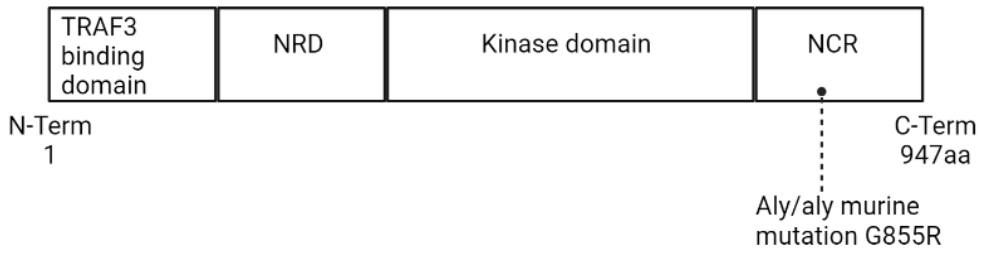

Figure 4: The structure of NIK. Murine NIK, also known as Mitogen-Activated Protein Kinase Kinase Kinase 14, (MAP3K14) contains a TRAF3 binding site at its N-Terminus and a non-catalytic region (NCR) at its C-Terminus. In addition, the kinase features a negative regulatory domain (NRD) and a kinase domain. The naturally occurring mutation of NIK in *aly/aly* mice localises at its NCR region (adapted from Pflug and Sitcheran, 2020).

To investigate the immunological relevance of NIK, certain mouse models have been utilised. Alymphoplasia (*aly*) mice harbour a naturally occurring point mutation in the *NIK* gene that prevents the binding of NIK to downstream molecule IKK-α and consequently abrogates non-canonical NF-κB signalling (Matsushima *et al.*, 2001). These mice are devoid of lymph nodes and Peyer's patches, show defects in splenic lymphoid follicle structures and thymic structures concerning cTEC and mTEC and are susceptible to viral infections due to hampered B and T cell responses (Miyawaki *et al.*, 1994). Nevertheless, *aly/aly* mice do not succumb to pre-mature death and can survive up to one year (Miyawaki *et al.*, 1994). Highlighting the role of NIK as a clinically relevant molecule, *aly/aly* mice are used as a mouse model for Sjögren's syndrome based on T cell infiltration and T cell-induced infiltration of mononuclear cells into amongst others salivary glands and kidneys (Furukawa *et al.*, 1996). Of note, on a cellular level, T cells from *aly/aly* mice are able to proliferate in response to autoantigens and do not show migratory defects (Furukawa *et al.*, 1996). Looking at Treg cells, *aly/aly* mice possess reduced numbers of thymic and splenic Treg cells due to their disturbed thymic structure (Kajiura *et al.*, 2004).

A similar mouse model to *aly/aly* mice are genetically-modified complete NIK knockout (KO) (NIK$^{-/-}$) mice, that have been utilised to study the role of NIK in CD4$^+$ T cells, wherein CD4$^+$ T cells from NIK$^{-/-}$ mice failed to induce EAE, a mouse model of multiple sclerosis (MS), in Rag2-deficient animals as NIK seems to be required for the differentiation of Th17 but not iTreg cells (Jin *et al.*, 2009).

Furthermore, the generation of $NIK^{fl/fl}$ mice allowed for conditional deletion of NIK in different immune cell subsets using Cre lines for more precise analysis excluding cell-extrinsic effects (Lacher *et al.*, 2018). Conditional deletion of NIK in all αβ T cells using the CD4-Cre ($NIK^{\Delta T}$ mice) confirmed that NIK is required for the generation of Teff cells and the induction of EAE (Li *et al.*, 2016; Lacher *et al.*, 2018). However, Hofmann *et al.* claim that not T cells but DCs require NIK for the induction of EAE (Hofmann *et al.*, 2011). Nevertheless, NIK-deficient $CD4^+$ T cells failed to induce GvHD, highlighting once again the importance of NIK for effector functions of conventional $CD4^+$ T cells, while NIK was dispensable for TCR-mediated activation of $CD4^+$ Tconv cells (Murray *et al.*, 2011). In contrast, Lacher and colleagues found that $CD4^+$ T cells from $NIK^{\Delta T}$ mice show reductions in the phosphorylation of signalling molecules downstream of TCR signalling (Lacher *et al.*, 2018). Yet, using $NIK^{\Delta T}$ mice, it was confirmed that T cell migration is not affected by NIK deficiency (Lacher *et al.*, 2018).

Interestingly, by deleting NIK using the forkhead box protein N1 (Foxn1)-Cre it has been found that NIK is required in thymic mTEC for the education of $Foxp3^+$ Treg cells, that were otherwise poorly suppressive and decreased in numbers in the thymus, periphery and tissues (Haftmann *et al.*, 2021). Strikingly, deletion of NIK using the CD4-Cre resulted in reduced frequencies of thymic and peripheral $Foxp3^+$ Treg cells in steady state, while the deletion of NIK using the Foxp3-Cre ($NIK^{\Delta Treg}$) did not affect the frequencies of peripheral and thymic $Foxp3^+$ Treg cells (Lacher *et al.*, 2018). This finding indicates an implication of NIK in thymic Treg cell development and argues against a role for NIK in Treg cell maintenance in peripheral lymphoid organs. On the contrary, bone marrow chimera experiments using WT and $NIK^{-/-}$ bone marrow suggested that NIK is not implicated in thymic Treg cell development but in peripheral Treg cell maintenance (Murray, 2013). Nonetheless, NIK-deficient $Foxp3^+$ Treg cells showed similar expression levels of important Treg cell effector molecules such as CD25, CTLA-4 and CD44 in comparison to WT Treg cells along with normal *in vitro* suppressive capacity (Murray, 2013). Constitutive expression of NIK using the CD4-Cre led to severe inflammation that resulted in premature death of mice around the age of 30 days (Murray *et al.*, 2011). Furthermore, T cell-specific overexpression of NIK resulted in increased numbers of peripheral $Foxp3^+$ Treg cells that were characterised by impaired suppressive capacity and decreased Foxp3 levels in splenic Treg cells (Murray *et al.*, 2011). The question of whether the expansion of $Foxp3^+$ Treg cells was Treg cell-intrinsic could not be resolved by Polesso *et al.* who conditionally overexpressed NIK in $Foxp3^+$ Treg cells (Polesso *et al.*, 2017). Anyway, constitutive expression of NIK in $Foxp3^+$ Treg cells resulted in the development of lung inflammation in aged mice from 6 to 8 months as well as in impaired *in vitro* suppressive capacity (Polesso *et al.*, 2017).

Taken together, to date publications about the role of NIK in $Foxp3^+$ Treg cells are very limited leaving unanswered questions concerning *in vivo* suppressive function, maintenance in peripheral lymphoid

organs and peripheral tissues as well as whether NIK is implicated in thymic Treg cell development or not.

Next, Treg cell-specific functions of further non-canonical NF-κB intracellular signalling molecules will be highlighted in the following section.

1.6 The role of intracellular non-canonical NF-κB signalling molecules in Treg cells

This section will summarise the signalling effects of other intracellular non-canonical molecules TRAF3, IKKα, p100/p52 and RelB on Treg cells. First, conditional deletion of TRAF3 using the CD4-Cre resulted in an increase of Foxp3$^+$ Treg cells in primary and secondary lymphoid organs but did neither affect proliferation nor expression of Foxp3 or CTLA-4 on Treg cells. Interestingly, the authors further saw that thymic TregP transition into mature Foxp3$^+$ Treg cells was enhanced when TRAF3 was deleted in CD4$^+$ T cells (Yi *et al.*, 2014).

Chen *et al.* reported that IKKα deficiency in all αβ T cells (CD4-Cre) results in a reduction of Foxp3$^+$ Treg cells in the thymus, spleen and lymph nodes. Additionally, this study further revealed that IKKα-deleted Treg cells show compromised *in vivo* suppressive capacity using the model of T cell transfer colitis and failed to repopulate lymphopenic hosts due to hampered proliferative capacity (Chen *et al.*, 2015). In contrast, *Ren et al.* found that IKKα-deficient T cells do not have a defect in proliferation but that IKKα instead exerts anti-apoptotic functions in double-positive thymocytes from mice that lack IKKα in T cells (Ren *et al.*, 2002).

RelB deletion specifically in αβ T cells resulted in slightly increased Treg cell numbers in lymph nodes but not the spleen (Grinberg-Bleyer *et al.*, 2018). Surprisingly, in another study, RelB deficiency specifically in CD4$^+$ T cells as well as Foxp3$^+$ Treg cells did neither affect the immune homeostasis of these mice nor the numbers of Foxp3$^+$ Treg cells, which suggests that Treg cell maintenance and suppressive function are not affected by cell-specific RelB deletion in steady state. However, in this study, the authors further showed that complete RelB deletion led to severe autoimmunity along with increased numbers of well suppressive Treg cells but this effect came from the requirement of RelB in non-haematopoietic cells to regulate autoimmunity (Li *et al.*, 2018). Indeed, mice harbouring dysfunctional RelB showed signs of autoimmune inflammation but normal T cell development (Weih *et al.*, 1995). Yet, RelB-mutant mice on a Rag-deficient background did not develop severe autoimmunity, which suggests that T cells indeed require RelB to exert their effector functions (Weih *et al.*, 1996).

Nevertheless, Grinberg-Bleyer *et al.* found that the deletion of the *nfkb2* gene (p100 and p52) using the CD4-Cre as well as the Foxp3-Cre resulted in an increase of Foxp3$^+$ Treg cells in the spleen and lymph nodes. These Foxp3$^+$ Treg cells lacking the *nfkb2* gene were characterised by decreased Foxp3 median

fluorescence intensity (MFI) but increased proliferation and were not suppressive *in vivo*, which was reflected in the development of inflammation in the *nfkb2* Foxp3-Cre strain upon ageing. Further, the absence of NF-κB2 resulted in an accumulation of RelB in the nucleus and in RelB-NF-κB2 double KO mice using the Foxp3-Cre, inflammation in aged mice was ablated and Treg cell numbers went back to normal, except for a small decrease of Treg cells in the spleen (Grinberg-Bleyer *et al.*, 2018). In line with these findings, Dhar *et al*. found increased numbers of effector Treg cells in the periphery of *nfkb2*-deficient mice that were yet suppressive *in vitro* and showed increased proliferation. They further showed that RelB deficiency did not affect Foxp3$^+$ Treg cell numbers. Interestingly, the authors saw that *nfkb2* deletion led to an accumulation of heterodimers consisting of RelB and canonical p50 in the nuclei of Treg cells, which suggests that the effect we see in *nfkb2*-deficient strains stems from an activation of the canonical NF-κB pathway (Dhar *et al.*, 2019).

However, deletion of the *nfkb2* gene affects both p100 and p52 expression since the *nfkb2* gene encodes for both (De Silva *et al.*, 2016; Grinberg-Bleyer *et al.*, 2018). To avoid this issue, it is recommended that NF-κB2$_{Lym1}$ mice are studied, since these mice harbour a mutation in the *nfkb2* gene which consequently leads to defective p100 processing and, hence, to disturbed T cell development (Tucker *et al.*, 2007; Sun, 2017). In fact, NF-κB2$_{Lym1}$ mutation in transferred T cells rendered them incapable of inducing EAE in Rag-deficient animals in contrast to RelB-deficient T cells which did induce EAE (Yu *et al.*, 2014). However, the NF-κB2$_{Lym1}$ mouse model yet needs to be utilised in order to study Foxp3$^+$ Treg cells.

In addition to the above described intracellular non-canonical NF-κB signalling molecules and their role in Treg cell biology, the following chapter will further elucidate on what has been reported on receptor signalling that, at least in part, induces non-canonical NF-κB signalling in Treg cells.

1.7 TNFR superfamily-induced signalling in Treg cells

Non-canonical NF-κB signalling and NIK accumulation, as NIK being the key player of non-canonical NF-κB signalling, are induced upon ligation of surface receptors belonging to the TNFR superfamily, such as TNFR2, OX40, CD137, CD27 and CD30 (Wajant, Henkler and Scheurich, 2001; Rauert *et al.*, 2010; Sun, 2017). While the role of non-canonical NF-κB signalling is well defined for amongst others B cells, DCs and thymic epithelial cells, its function is less studied in T cells, where it is known to be critical for Teff cell maintenance and function (**Figure 5**).

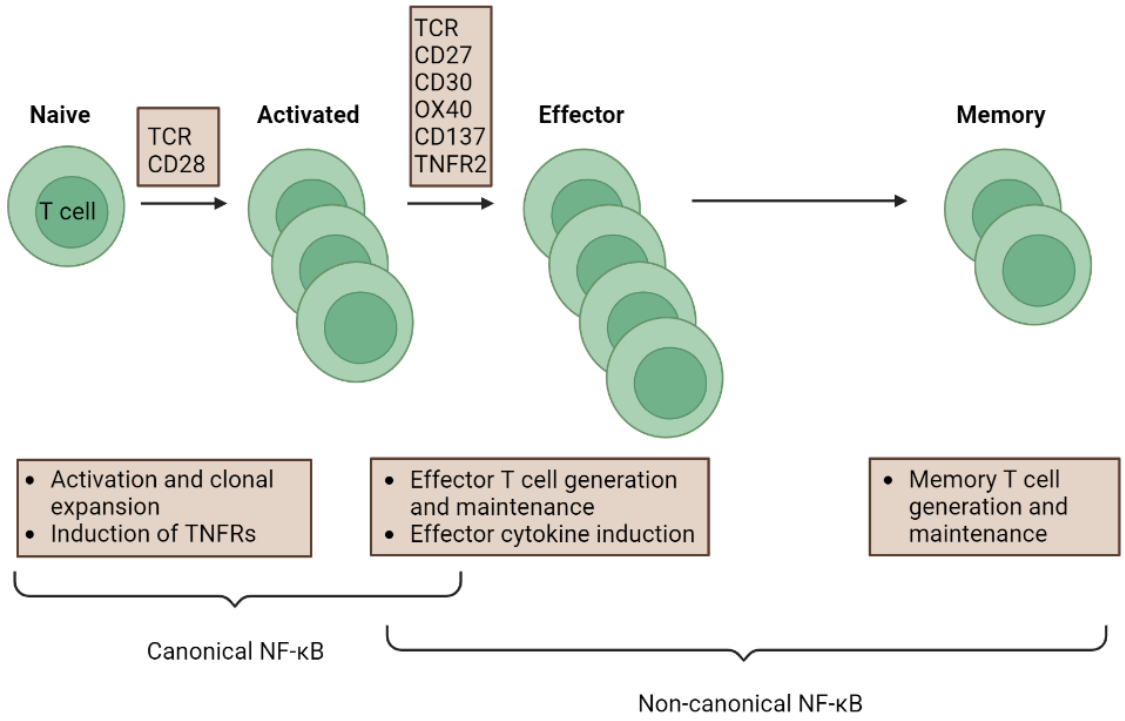

Figure 5: Canonical and non-canonical NF-κB in T cell function. While the canonical NF-κB pathway is required for initial T cell activation of naïve T cells mediated through TCR signalling, Teff cell maintenance and function are regulated through the non-canonical NF-κB counterpart mediated by TNFR superfamily receptors CD27, CD30, OX40, CD137 and TNFR2. TNFR superfamily members are being upregulated in response to TCR signalling, which is in part mediated by canonical NF-κB signalling (adapted from Sun, 2017).

To date, the biological relevance of the non-canonical NF-κB signalling pathway is even less studied in Treg cells, for that reason the biological implications of TNFR superfamily member signalling on Treg cells will be summarised below.

TNFR2

The cytokine TNF has pro- or anti-inflammatory functions depending on the type of receptor, TNFR1 or TNFR2, through which it signals (Yang *et al.*, 2018). Originally, TNF was recognised as a pro-inflammatory cytokine and it came to surprise that therapies with anti-TNF (Infliximab) and soluble TNF receptor (Etanercept) induced systemic lupus erythematosus (SLE) in patients suffering from rheumatoid arthritis (Shakoor *et al.*, 2002). Nowadays, we know that pro-inflammatory properties of TNF are mediated through TNFR1, that is expressed by most cell types, and that anti-inflammatory properties are mediated by TNFR2 (Yang *et al.*, 2018).

In thymocytes soluble TNF signals through TNFR2, in which it along with suboptimal concentrations of IL-2 induces proliferation *in vitro* (Grell *et al.*, 1998). Xin Chen and colleagues have found that TNFR2 is

expressed at much higher levels on resting Treg cells than on conventional $CD4^+$ T cells, while TNFR1 is not expressed by Treg cells (Chen *et al.*, 2007). Furthermore, synergistic treatment of Treg cells with IL-2 and TNF enhanced their p-STAT5 expression and their *in vitro* suppressive capacity in contrast to stimulation with IL-2 alone (Chen *et al.*, 2007). Importantly, the expression level of TNFR2 on Treg cells correlates with their suppressive capacity (Chen *et al.*, 2008). Also in humans, $Foxp3^+$ Treg cells expressed high levels of TNFR2 and the TNF - TNFR2 axis mediated Treg cell expansion and suppression, while the combination of TNF and IL-2 induced maximal expansion and suppression of $CD4^+$ $CD25^+$ $TNFR2^+$ Treg cells (Chen *et al.*, 2010; Okubo *et al.*, 2013). Furthermore, membrane-bound TNFR2 agonist in combination with IL-2 expands murine $Foxp3^+$ Treg cells (Padutsch *et al.*, 2019).

Indeed, the TNF - TNFR2 axis plays a crucial role in Treg cell function and therefore the prevention of autoimmune diseases such as EAE (Atretkhany *et al.*, 2018). Additionally, the stimulation of TNFR2 on Treg cells reduces the withdrawal threshold and therefore diminishes neuropathic pain in mice (Fischer *et al.*, 2019). Furthermore, TNFR2 agonist selectively expands tTreg cells and drastically increases the survival of GvHD in mice (Chopra *et al.*, 2016). TNFR2-deficient mice show a reduction of thymic and splenic $Foxp3^+$ Treg cells and the lack of expansion of TNFR2-deficient Treg cells in response to TNF has been proposed to be the reason for not being able to suppress an adoptive T cell transfer colitis (Chen *et al.*, 2013). Indeed, the TNF - TNFR2 axis is important in tTreg cells, but not in iTreg cells, for the suppression of T cell-mediated colitis (Housley *et al.*, 2011). Of note, pre-activation of TNFR2-deficient tTreg cells with TGF-β restores their suppressive capacity and results in the prevention of an adoptive T cell transfer colitis (Housley *et al.*, 2011). Moreover, Chen *et al.* found that synergistic TNF and IL6 stimulation enhances the $Foxp3^+$ Treg cell percentage *in vitro* in comparison to TNF or IL-6 stimulation alone (Chen *et al.*, 2013). Membrane-bound TNFR2 agonist expands $CD4^+$ $Foxp3^+$ Treg cells, increases TNFR2 expression levels on $Foxp3^+$ Treg cells and alleviates collagen-induced arthritis in mice by reducing inflammation (Fischer *et al.*, 2018; Lamontain *et al.*, 2019). However, no increase in proliferation upon treatment with membrane-bound TNFR2 agonist has been observed (Fischer *et al.*, 2018).

Two groups showed that antigen-specific $CD4^+$ T cells boost Treg cell expansion through TNF secretion and therefore drastically decreased diabetes incidence (Grinberg-Bleyer *et al.*, 2010; Leclerc *et al.*, 2016). Furthermore, it was suggested that murine as well as human Treg cells shed soluble TNFR2 that exerts protective functions *in vitro* (van Mierlo *et al.*, 2008). Of note, *in vitro* studies suggest that human DCs use the TNF - TNFR2 axis to induce suppressive $Foxp3^+$ Treg cells (Kleijwegt *et al.*, 2010).

Not many publications have investigated the molecular mechanisms of the TNF – TNFR2 axis. By applying p38 mitogen-activated protein kinases (MAPK) inhibitors Sulfasalazine and Bay, that are IKK-specific inhibitors, it was found that p38 mediates the upregulation of TNFR2 and Foxp3 expression

levels on Treg cells upon TNF stimulation along with proliferation-induced expansion of murine Treg cells upon LPS stimulation (He *et al.*, 2018). In human Treg cells, TNF treatment induced non-canonical NF-κB signalling increasing RelB, NIK, p-p100 and p52 expression levels, while, interestingly, TNF treatment alone did not induce Ki67-assessed proliferation in contrast to synergistic treatment with TNF and IL-2 (Wang *et al.*, 2018). Furthermore, TNF induced the canonical NF-κB transcription programme in human Treg cells that resulted in the upregulation of TNFR superfamily members OX40 and CD137, anti-apoptotic genes and phosphorylation of p65 but, nevertheless, increased expression of p52, RelB and B cell lymphoma (Bcl) 3 (Nagar *et al.*, 2010).

In summary, the aforementioned publications highlight the importance of TNFR2 signalling on Treg cell expansion and function making the TNF - TNFR2 axis therapeutically attractive (Moatti and Cohen, 2021).

OX40

OX40, a member of the TNFR superfamily, binds to its ligand OX40L that is amongst others expressed on APCs such as B cells, DCs and macrophages (Croft *et al.*, 2009). OX40 has been found to be upregulated on activated $CD4^+$ Tconv cells, where it mediates the upregulation of Bcl-xL and Bcl-2 preventing apoptosis and characteristically for TNFR superfamily members induces canonical as well as non-canonical NF-κB signalling (Kawamata *et al.*, 1998; Rogers *et al.*, 2001). Later, OX40 expression was detected on thymic and induced $Foxp3^+$ Treg cells, where initially it was reported that OX40 engagement led to increased proliferation of Treg cells but to decreased suppressive capacity *in vitro* (Takeda *et al.*, 2004; Vu *et al.*, 2007). Anyway, $Foxp3^+$ Treg cells are reduced in the colon of $OX40^{-/-}$ mice (Griseri *et al.*, 2010) and fail to repopulate lymphopenic hosts (Piconese *et al.*, 2010).

To date, unlike for other TNFR superfamily members, there is no consensus about the effect of OX40 signalling on Treg cell suppression and proliferation. Several groups reported that OX40 signalling is not implicated in Treg cell proliferation (Valzasina *et al.*, 2005; Griseri *et al.*, 2010; Bell *et al.*, 2016; Lubrano di Ricco *et al.*, 2020) while others found that Treg cell-specific OX40 signalling results in increased proliferation *in vitro* and *in vivo* (Ruby *et al.*, 2009; Bresson *et al.*, 2011; Alharshawi *et al.*, 2017; Kumar *et al.*, 2017; Polesso *et al.*, 2019). Moreover, it has been shown that OX40 expands Treg cells (Baeyens *et al.*, 2015) through the engagement of CD40L on APCs (Marinelarena *et al.*, 2018; Deng *et al.*, 2020). One study reported that OX40-deficient Treg cells are more apoptotic (Griseri *et al.*, 2010).

In regards to suppressive capacity, Treg cells require OX40 in order to suppress T cell-mediated colitis (Griseri *et al.*, 2010; Piconese *et al.*, 2010) and increased numbers of OX40-expressing Treg cells from colorectal cancer patients correlate with better survival of patients (Lam *et al.*, 2021). Additionally,

treatment with OX40 agonists augment type I diabetes (Bresson *et al.*, 2011; Kumar *et al.*, 2017), suppresses antigen-specific T cells in pemphigus (Iriki *et al.*, 2021) and ameliorates EAE if injected before onset of EAE (Ruby *et al.*, 2009). On the contrary, anti-OX40 treatment of Treg cells abrogated Treg cell suppressive function and could not prevent GvHD (Valzasina *et al.*, 2005) and has been reported to be a favourable option in cancer therapy (Piconese, Valzasina and Colombo, 2008; Kitamura *et al.*, 2009; Curti *et al.*, 2013; Bell *et al.*, 2016; Hinterbrandner *et al.*, 2021). Noteworthy, injection with anti-OX40 boosts the activation of Tconv cells to an extent where Treg cells are not capable of suppressing Tconv cells anymore as shown in the context of EAE (Ruby *et al.*, 2009). Of note, OX40 signalling abrogated IL-10 production by Tr1 cells, that are regulatory cells secreting IL-10 that do not constitutively express Foxp3 (Ito *et al.*, 2006). Interestingly, activation of Treg cells via CD40L on APCs ablated the conversion of naïve $CD4^+$ T cells into Treg cell *in vitro* (So and Croft, 2007; Vu *et al.*, 2007).

Mechanistically, it has been shown that triggering OX40 on Treg cells induces canonical as well as non-canonical NF-κB signalling (Song, So and Croft, 2008; Murray *et al.*, 2011) and that OX40 inhibits Foxp3 expression through chromatin closure of the Foxp3 locus and metabolic inhibition of Foxp3 transcription (Zhang *et al.*, 2018).

CD137

CD137 is expressed on activated T cells as well as on resting Treg cells and binds to its receptor CD137L that is expressed, amongst others, on B cells and APCs (McHugh *et al.*, 2002; Shao and Schwarz, 2011). CD137 is upstream of canonical as well as non-canonical NF-κB signalling and in Treg cells it is a downstream target of transcription factor Foxp3, which is why it is already expressed in resting Treg cells (Marson *et al.*, 2007; Zapata *et al.*, 2018). Of note, human $CD137^+$ $Foxp3^+$ Treg cells have a highly demethylated Treg-specific demethylated region (TSDR) indicating stable Foxp3 expression (Schoenbrunn *et al.*, 2012; Nowak *et al.*, 2018). Synergistic stimulation with TNF and IL-2 further induces the expression of CD137 on Treg cells and their expansion (Hamano *et al.*, 2011). TNF stimulation and CD137 agonist treatment boosted Treg cell proliferation and Foxp3 expression in comparison to IL-2 stimulus alone (Zheng, Wang and Chen, 2004; Hamano *et al.*, 2011). In contrast, another group reported that CD137 and CD137L were neither required for $Foxp3^+$ Treg cell maintenance nor Foxp3 stability (Foda *et al.*, 2020). However, interestingly, mice lacking CD137 specifically in Treg cells showed normal numbers of Treg cells in peripheral lymphoid organs but reduced frequencies of Treg cells within tumours, in turn highlighting a role for CD137 in maintaining adequate Treg cell numbers under inflammatory conditions and in peripheral tissues (Buchan *et al.*, 2018).

Besides, many publications highlighted the importance of CD137 signalling for *in vitro* and *in vivo* suppression (Liu *et al.*, 2008; Kachapati *et al.*, 2013; Foda *et al.*, 2020; Swatler *et al.*, 2022). Interestingly, $CD137^+$ $Foxp3^+$ Treg cells are a source of soluble CD137 that ameliorates the onset of diabetes in non-obese diabetic (NOD) mice (Kachapati *et al.*, 2012, 2013). Moreover, one study suggests that $CD137^+$ Treg cells modulate APCs via trogocytosing their surface CD137L (Luu *et al.*, 2021).

While many studies showed positive effects of CD137 signalling on Treg cells, it has been reported that CD137 agonist treatment resulted in tumour rejection in mice along with comparable percentages of Treg cells in the spleen but a massive increase in $CD8^+$ T cell proliferation (Smith *et al.*, 2011). Indeed, CD137 agonist induced proliferation to a much higher extent in activated $CD8^+$ than $CD4^+$ T cells (Shuford *et al.*, 1997). Conclusively, CD137 agonist treatment did not ablate *ex vivo* Treg suppressive capacity but activated $CD8^+$ T cells to an extent where Treg cells were not able to suppress them any longer (Robertson *et al.*, 2008; Smith *et al.*, 2011). Nevertheless, many studies using anti-CD137 agonists provided insights into the effect of CD137 signalling on Treg cell biology in line with what was described above. Treatment of mice with an anti-CD137 agonist resulted in increased suppressive capacity, proliferation, granzyme B and TNF production of $Foxp3^+$ Treg cells (Zhang *et al.*, 2007; Akhmetzyanova *et al.*, 2016). Consequently, CD137 agonist treatment and its Treg cell-boosting effects might be considered as a treatment option for patients with autoimmune disorders since it was shown to ameliorate psoriasis, experimental allergic conjunctivitis, colitis, increased allogenic islet survival as well as to completely suppress the development of EAE (Lee *et al.*, 2005; Elpek *et al.*, 2007; Sumi *et al.*, 2009; Kim *et al.*, 2011; Yoo *et al.*, 2016).

CD27

In 1987 it was discovered that human T cells express CD27 and that its expression was upregulated upon stimulation (van Lier *et al.*, 1987). CD27 signalling is mediated via canonical and non-canonical NF-κB signalling (Akiba *et al.*, 1998). To date, the only ligand documented for CD27 is CD70 that is transiently expressed on murine T cells, B cells, DCs and mTECs (Tesselaar *et al.*, 2003). In human, CD27 distinguishes Teff cells from Treg cells, among which Foxp3 was expressed with a 10-fold increase in the $CD4^+$ $CD25^+$ $CD27^+$ compartment in contrast to the $CD4^+$ $CD25^+$ $CD27^-$ compartment (Koenen, Fasse and Joosten, 2005; Ruprecht *et al.*, 2005; Duggleby *et al.*, 2007). Additionally, $CD27^+$ Treg cells are found at sites of inflammation, such as the inflamed lung, where its expression was mainly restricted to Treg cells (Mack *et al.*, 2009). Expectedly, $CD27^+$ Treg cells have been reported to be suppressive *in vitro* and *in vivo* (Ruprecht *et al.*, 2005; Coenen *et al.*, 2006; Duggleby *et al.*, 2007; Arroyo Hornero *et al.*, 2020). One mechanism of suppression specific to $CD27^+$ Treg cells has been found to be the downregulation of CD70

expression via CD27 (Dhainaut *et al.*, 2015). Several studies suggest that CD27 is important for Treg cell function given that decreased percentages of $CD27^+$ Treg cells correlated with the more aggressive polyarticular form of juvenile idiopathic arthritis as well as with active SLE (Ruprecht *et al.*, 2005; Schmidt *et al.*, 2017). In the murine counterpart, CD27-deficient Treg cells increased the survival of tumour-burdened mice although there was no obvious difference in proliferation between WT and CD27-deficient Treg cells (Muth *et al.*, 2022). Another study found that tumour formation was delayed in $CD27^{-/-}$ mice potentially due to an increased CD8 response and decreased percentages of CD27-deficient $CD4^+$ $CD25^+$ Treg cells that were characterised by enhanced apoptosis (Claus *et al.*, 2012). Similarly, it was shown that $CD27^{-/-}$ mice displayed reduced numbers of Treg cells in the spleen and the thymus and that binding of CD27 to CD70 on APCs increased Treg cell survival by inhibiting mitochondrial apoptosis (Coquet *et al.*, 2013). CD27-deficient mice displayed earlier onset of atherosclerosis along with a systemic reduction of $Foxp3^+$ Treg cells that were more apoptotic (Winkels *et al.*, 2017). While these studies suggest a role for CD27 in Treg cell survival and maintenance, another study reported that CD27 was not required for Treg cell accumulation but that inhibiting CD27 signalling resulted in decreased percentages of IL17A-producing and RORγt-expressing Treg cells in culture claiming a role for CD27 in Treg cell differentiation (Remedios *et al.*, 2018).

CD30

CD30 was originally detected as a surface marker on Hodgkin's lymphoma cells and can also be shed as a soluble form *in vitro* and *in vivo* (Josimovic-Alasevic *et al.*, 1989). Later it was shown that CD30 is expressed on T cells upon activation inducing canonical and non-canonical NF-κB signalling (Bowen *et al.*, 1996; Aizawa *et al.*, 1997). CD30 binds to CD30L (CD153) that is expressed on T cells, B cells, granulocytes, macrophages, monocytes and mTECs (Horie and Watanabe, 1998). Interestingly, T cells derived from $CD30^{-/-}$ mice proliferate normally *in vitro* while $CD30^{-/-}$ mice have a defect in thymic negative selection (Amakawa *et al.*, 1996). Human $CD30^+$ $Foxp3^+$ Treg cells are characterised by an increased Foxp3 MFI over $CD30^-$ $Foxp3^+$ Treg cells and have been described as suppressive *in vitro* (de Kleer *et al.*, 2010). $CD30^+$ $CD4^+$ T cells from synovial fluid produced high amounts of IL-10 and CD30 expression on T cells correlated with IL-4 production so that CD30 is suggested to play a regulatory role in rheumatoid synovitis (Gerli *et al.*, 2000). In mice, Treg cells upregulated expression of CD30 upon allogenic immunisation, $CD30^+$ Treg cells were suppressive *in vitro* and *in vivo.* CD30 was furthermore required to control allograft rejection mediated by effector memory $CD8^+$ T cells (Dai *et al.*, 2004). While there is no consent in regards as to whether CD30 expression correlates with $Foxp3^+$ Treg cell infiltration and increased survival in post-transplant lymphoproliferative disorders (Hartley *et al.*, 2017; Kinch *et al.*, 2020), it has been reported that there is an increased expression of CD30 on circulating $Foxp3^+$ Treg

cells in colorectal cancer patients in comparison to healthy controls. The authors further found that the presence of $CD30^+$ $OX40^+$ Treg cells correlated with improved survival of colorectal cancer patients (Lam *et al.*, 2021).

In summary, the big majority of publications acknowledges the role of TNFR superfamily members in Treg cell maintenance and function.

1.8 Objectives

Canonical NF-κB signalling has been shown to be crucial for thymic Treg cell development. However, it is not known whether NIK as the key player of the non-canonical NF-κB counterpart is implicated in thymic Treg cell development. Members of the TNFR superfamily such as TNFR2 and OX40, which signal at least in part through non-canonical NF-κB, have been found to be expressed on thymic CD25 TregP being crucial for their expansion. However, one report suggests that NIK is not implicated in thymic Treg cell development. Yet, another study reports a decrease of $Foxp3^+$ Treg cells in secondary lymphoid organs in mice lacking NIK in αβ T cells using the CD4-Cre. This decrease was abolished when deleting NIK in $Foxp3^+$ Treg cells using the Foxp3-Cre, which suggests that NIK is indeed implicated in thymic Treg cell development. For this reason, by utilising conditional NIK KO mice (CD4-Cre and Foxp3-Cre) and TNFR2-deficient mice as well as novel flow cytometric gating strategies differentiating thymus-developing from recirculating T lymphocytes based on the expression of surface marker CD73, it was investigated whether NIK is required for the expansion of thymic TregP.

Furthermore, one report suggested that NIK is cell-intrinsically required for Treg cell maintenance in secondary lymphoid organs such as the spleen and lymph nodes. Yet, a previous publication from our laboratory showed that conditional deletion of NIK in $Foxp3^+$ Treg cells resulted in equal percentages of Treg cells in primary and secondary lymphoid organs concluding exactly the opposite. Through flow cytometric analysis of mice lacking NIK in αβ T cell as well as $Foxp3^+$ Treg cells, we re-examined whether a reduction of Treg cells in secondary lymphoid organs is indeed cell-intrinsic. In any case, previous findings show that NIK is required for the maintenance of Teff cells. Treg cells are *per se* more activated than Tconv cells. Moreover, tTreg cells that migrate into tissues are characterised by an effector memory phenotype in comparison to those residing in secondary lymphoid organs. Yet, there are no reports that have investigated whether Treg cells require NIK for tissue homeostasis and for Treg cell maintenance in peripheral tissues in steady state. To investigate whether Treg cell-derived NIK is crucial for peripheral tissue homeostasis and maintenance, the colon and the liver of mice deficient for NIK in $Foxp3^+$ Treg cells were analysed by flow cytometry and histology.

Last, it was reported that NIK-deficient Tconv cells fail to induce EAE in mice, which suggests a role for NIK in T cell function *in vivo*. Furthermore, NIK-deficient Treg cells were reported to be suppressive *in vitro*. However, for instance IKKα- and TNFR2-deficient Treg cells failed to prevent T cell transfer colitis in Rag-deficient animals. Thus, by using the model of T cell transfer colitis and respective flow cytometric as well as histological analysis, we investigated whether NIK is required for Treg cell suppressive function *in vivo*.

2 Materials and methods

2.1 Chemicals, reagents and solutions

The below listed chemicals, reagents and solutions from respective suppliers were used in order to perform the methods used in the scope of thesis (Table 1).

Table 1: Suppliers for chemicals, reagents and solutions.

Name	Supplier
Acetic acid	VWR International S.A.S.
Agarose	Biozym Scientific GmbH
Ammonium chloride (NH_4Cl)	Sigma-Aldrich Corp.
Bovine serum albumin (BSA)	Sigma-Aldrich Corp.
Brefeldin A	AppliChem GmbH
Collagenase D	Roche Holding AG
Chloroform	Carl Roth GmbH
Dithiothreitol (DTT)	AppliChem GmbH
DNase I	Sigma-Aldrich Corp.
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Corp.
Ethanol	AppliChem GmbH
Fetal calf serum (FCS)	Boehringer Mannheim GmbH
Formaldehyde (4 % ROTI Histofix)	Carl Roth GmbH
Glycine	Carl Roth GmbH
HEPES buffer	Gibco/ Life Technologies Inc.
Hanks' Balanced Salt Solution (HBSS) (-/-)	Gibco/ Life Technologies Inc.
Heparin sodium	Leo Pharma AS
Ionomycin	Sigma-Aldrich Corp.
Isopropanol	Hedinger GmbH
Liberase TL, Research Grade	Roche Holding AG
MEM Non-Essential Amino Acids	Gibco/ Life Technologies Inc.
MOPS	Carl Roth GmbH
Penicillin Streptomycin (Pen Strep)	Gibco/ Life Technologies Inc.
Percoll	Sigma-Aldrich Corp.
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich Corp.
Phosphate-buffered saline (PBS)	Sigma-Aldrich Corp.
Potassium bicarbonate ($KHCO_3$)	Carl Roth GmbH

Proteinase K	7Bioscience GmbH
RPMI 1640 Medium (with L-Glutamine)	Gibco/ Life Technologies Inc.
Skim milk powder	Sigma Aldrich Corp.
Sodium azide (NaN_3)	AppliChem GmbH
Sodium chloride (NaCl)	Carl Roth GmbH
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis GmbH
Sodium Pyruvate	Gibco/ Life Technologies Inc.
Tris	Carl Roth GmbH
Tris/HCl	Sigma Aldrich Corp.
TRIzol	Ambion /Life Technologies Inc.
Trypan Blue Stain (0.4 %)	Gibco/ Life Technologies Inc.
Tween20	AppliChem GmbH
2-β-mercaptoethanol	Fluka Chemie GmbH

2.2 Buffers and media

Home-made buffers and media and their composition used in the context of this thesis are listed below (Table 2).

Table 2: Composition of buffers and media.

Name	Composition
Tail lysis buffer	420 mL dH_2O 100 mM Tris/HCl 5 mM EDTA 0.2 % SDS 200 mM NaCl
50X TAE buffer	1000 mL dH_2O 2 M Tris 5.71 % (v/v) acetic acid 50 mM EDTA pH 8.3
10X ACK lysis buffer	200 mL dH_2O 1.55 M NH_4Cl 100 mM $KHCO_3$ 12.73 mM EDTA

	pH 7.4
FACS staining buffer	400 mL PBS 1 % BSA 0.1 % NaN_3
FACS acquisition buffer	400 mL PBS 1 % BSA 0.1 % NaN_3 20 mM EDTA
MACS buffer	500 mL PBS 2 mM EDTA 0.5 % BSA
20X MOPS buffer	1000 mL dH_2O 1 M Tris 1 M MOPS 20.5 mM EDTA 69.3 mM SDS
10X transfer buffer	1000 mL dH_2O 250.1 mM Tris 1.9 M Glycine
10X TBS	800 mL dH_2O 250 mM Tris 1.7 M NaCl pH 7.6
T-cell medium	500 mL RPMI 1640 (with L-Glutamine) 1 mM sodium pyruvate 10 mM HEPES buffer 1 % (v/v) Mem non-essential amino acids 10 % (v/v) heat-inactivated FCS 100 units/mL penicillin 100 µg/mL streptomycin 50 µM 2-β-mercaptoethanol
3 % medium	500 mL RPMI 1640 (with L-Glutamine) 3 % (v/v) heat-inactivated FCS 20 mM HEPES buffer 100 units/mL penicillin

	100 µg/mL streptomycin
0 % medium	500 mL RPMI 1640 (with L-Glutamine) 25 mM HEPES buffer 100 units/mL penicillin 100 µg/mL streptomycin 1 mM sodium pyruvate 25 µM 2-β-mercaptoethanol
Shake medium	500 mL RPMI 1640 (with L-Glutamine) 20 mM HEPES buffer 100 units/mL penicillin 100 µg/mL streptomycin 2 mM EDTA

2.3 Mice

2.3.1 Mouse models

Mice were housed in specific pathogen-free conditions in the official animal facilities of the Translational Animal Research Center (TARC) at the University Medical Center Mainz. All strains were of C57BL/6 background. $NIK^{fl/fl}$ mice were generated for us by TaconicArtemis GmbH and crossed with either CD4-Cre (Lee *et al.*, 2001) or Foxp3-Cre (Wing *et al.*, 2008) strains in order to conditionally delete NIK signalling in all αβ T cells or $Foxp3^+$ Treg cells, respectively. Deletion of exons 4, 5 and 6 of the NIK gene in $NIK^{fl/fl}$ mice resulted in deletion of parts of the kinase domain rendering the kinase dysfunctional. $NIK^{Tg/Tg}$ mice that still the TRAF3 binding site (Sasaki *et al.*, 2008) were crossed with the Foxp3-Cre (Wing *et al.*, 2008) strain to specifically overexpress NIK in $Foxp3^+$ Treg cells. $TNFR2^{-/-}$ mice (Erickson *et al.*, 1994) are characterised by global deletion of TNFR2. Animal experiments were officially approved by Landesuntersuchungsamt of Rheinland-Pfalz and conducted in accordance with TARC guidelines.

2.3.2 Generation of single cell suspensions

This section describes the generation of single-cell suspensions from secondary lymphoid organs (spleen, Peyer's patches (PP), inguinal lymph nodes (iLN), mesenteric lymph nodes (mLN)), primary lymphoid organs (thymus) and peripheral tissues (colon, liver) as well as the preparation of serum from blood.

Secondary lymphoid organs: In order to obtain single cell suspensions, secondary lymphoid organs were mashed through a 40 µm cell strainer (Sarstedt AG & Co.KG and Greiner Bio-One GmbH) in ice-cold PBS

containing 2 % of heat-inactivated FCS. Spleens were, subsequently, treated with 1X ACK lysis buffer for 3 minutes at RT to lyse erythrocytes. Cells were kept at 4 °C until further processing.

Thymus: Thymi were kept in ice-cold PBS containing 2% of heat-inactivated FCS at 4 °C until further processing. Thymi were either mashed using the same protocol as for secondary lymphoid organs for T-cell analysis or digested in HBSS (-/-) containing 0.125 % (w/v) Collagenase D and 0.1 % (w/v) DNase I for 30 minutes at 37 °C for thymic dendritic cell and thymic epithelial cell analysis.

Colon: Colons were washed with ice-cold PBS to clean off faeces. To remove intraepithelial lymphocytes, they were incubated in 20 mL of 3 % medium containing 5 mM DTT and 5 mM EDTA for 20 minutes at 37 °C on a rotator followed by further mechanical disruption in shake medium (shaking 1 minute, twice) at RT. Subsequent enzymatic digestion for 27 minutes at 37 °C on a rotator in 6 mL of 0 % medium per sample containing 100 µg/mL of Liberase TL and 0.83 mg/mL of DNase I yielded a lamina propria single cell suspension which was then filtered first through a 70 µm and then a 40 µm cell strainer (both Sarstedt AG & Co.KG and Greiner Bio-One GmbH) in ice-cold 3 % media and ultimately kept in 0 % media containing 10 % of heat-inactivated FCS at 4°C until further processing.

Liver: The gall bladder was removed and liver tissue was mashed through a 100 µm cell strainer (Greiner) in RPMI 1640 medium (with L-glutamine) containing 25 mM HEPES buffer and 10 % (v/v) heat-inactivated FCS (pH 7.4) to obtain a single-cell suspension. Lymphocytes were then isolated by gradient centrifugation in 10 mL HBSS (-/-) consisting of 100 U/mL heparin sodium and 37.5 % (v/v) percoll (centrifugation 30 minutes at RT, 900 g, no brake). The single cell suspension was subjected to 1X ACK treatment for 3 minutes to lyse erythrocytes and eventually resuspended in ice-cold PBS containing 2 % (v/v) heat-inactivated FCS and kept at 4 °C until further processing.

Serum: Venous blood from the right ventricle was collected into a micro tube containing clot activators (Micro tube 1.1 mL Z-Gel, Sarstedt AG & Co.KG) yielding serum after centrifugation. Serum was, then, stored at -20 °C until further processing.

Cell numbers of single cell suspensions were determined using Luna II cell counter (Logos Biosystems Inc.) through dead cell exclusion with Trypan blue.

2.4 Molecular biology

2.4.1 Genotyping

The genotyping procedure encompasses isolation of genomic DNA from mouse biopsies, amplification of the gene of interest through polymerase chain reaction (PCR) and, eventually, the separation of gene fragments by size through gel electrophoresis.

Isolation of genomic DNA: Toe biopsies from mice were obtained between day 3 to 5 after birth and lysed in tail lysis buffer containing 400 mg/mL proteinase K at 56 °C overnight. DNA was precipitated using isopropanol and pelleted by centrifugation (RT, 20 minutes, 13500 rpm). Pellets were dried for 2 hours at 37 °C and resuspended in 500 µL dH_2O.

PCR: In order to determine mouse genotypes detecting floxed alleles and transgenes, PCR was performed in Thermocyclers T3000 and TRIO Kombi (both Analytik Jena GmbH). Therefore, approximately 30 ng/µL of genomic DNA as well as the following PCR primers (Table 3) were added to REDTaq® PCR Reaction Mix (Sigma-Aldrich Corp.) and reactions run at indicated annealing temperatures.

Table 3: Primers used for PCR and their direction (forward or reverse), primer sequence, primer concentration (conc.) in pmol per reaction and respective annealing temperature ($T_{Ann.}$).

Primer	Primer sequence (5' – 3')	Primer conc. (pmol)	$T_{Ann.}$ (°C)
NIK 1 - forward	TAT GAA CTG CTC CCG TTT CG	0.5	60
NIK 2 - reverse	CCT GTG CAT CAC AGA GTA TAC TAG C	0.5	60
NIK 4 - reverse	TTC CTG TGA ACT CAA ACA CTC CC	0.5	60
FIC wt - forward	TGT GTG ATA GTG CCC GTG GTT C	0.5	58
FIC wt - reverse	TTC GCA AGA AGA GGA GCC AAC G	0.5	58
FIC tg - forward	CTG CTT CCT TCA CGA CAT TCA AC	0.5	58
FIC tg - reverse	AAG TGC TTT GTG CGA GTG GAG AGC	0.5	58
CD4-Cre - forward	GCA CTG ATT TCG ACC AGG TT	0.5	58
CD4-Cre - reverse	CCC GGC AAA ACA GGT AGT TA	0.5	58
NIK Rosa26 - forward	AAA GTC GCT CTG AGTTGT TAT C	1	68
NIK Rosa26 - reverse	GAT ATG AAG TAC TGG GCT CTT	0.5	68
NIK Rosa26 Neo - reverse	GCA TCG CCT TCT ATC GCC T	0.5	68
Jax - forward	CAA ATG TTG CTT GTC TGG TG	0.5	60
Jax - reverse	GTC AGT CGA GTG CAC AGT TT	0.5	60
Rosa26 - forward	AAA GTC GCT CTG AGT TGT TAT	1	60
Rosa26 - reverse	GGA GCG GGA GAA ATG GAT ATG	0.5	60
Rosa26 Neo - reverse	CAT CAA GGA AAC CCT GGA CTA CTG	0.5	60

Gel electrophoresis: Gene fragments were separated by size (130 V, 20 minutes) through gel electrophoresis. 1.5 % (w/v) agarose gels were prepared using 1X TAE buffer and Midori Green (Sigma-

Aldrich Corp.). Gene fragment sizes were determined using 100 bp or 1 kb gene ladders (Thermo Fisher Scientific Inc.).

2.4.2 RNA quantification

In order to measure gene expression through mRNA levels, RNA was extracted from single-cell suspensions or the thymus, followed by cDNA synthesis and DNA amplification by real-time PCR.

RNA isolation: RNA from single cell suspensions was isolated using ReliaPrep RNA Cell Miniprep System (Promega Corp.) according to manufacturer's instructions.

RNA from the thymus was obtained by incubation with 800 µL TRIzol (Ambion /Life Technologies Inc.) at RT for 5 minutes followed by mechanical disruption (bead beating tubes and Fast Prep-24 mechanical homogeniser; both MP Biomedicals Inc.). The sample was centrifuged at 12,000 g for 10 minutes at 4 °C and, subsequently, the supernatant incubated with 200 µL of chloroform for 3 minutes at RT inverting the sample several times. After centrifugation at 12,000 g for 15 minutes at 4°C, 400 µL of isopropanol was added to the supernatant and then vortexed. The sample was centrifuged at 12,000 g for 10 minutes at 4 °C and the pellet was washed twice with 500 µL ethanol. Pelleted RNA was air dried for 30 – 60 minutes and eventually resuspended in 100 µL of dH_2O and incubated at 55 °C for 10 minutes.

RNA concentration was determined on 'Infinite M200 Pro' plate reader (Tecan Group AG) using i-control software (Tecan Group AG). The plate reader measures the optical density whereof a ratio of OD_{260}/OD_{280} of 2 indicates pure RNA.

cDNA synthesis

cDNA was synthesized from RNA using 'QuantiTect Reverse Transcription Kit' (Qiagen N.V.) according to manufacturer's protocol at 42 °C for 32 minutes followed by 3 minutes at 95 °C.

Real-Time PCR

cDNA was amplified and detected using 'StepOnePlus Real-Time PCR System' (Applied Biosystems/ Life Technologies Inc.) along with 'GoTaq RT-qPCR' kit (Promega Corp.) according to manufacturer's instructions. Relative gene expression levels were obtained by normalisation to housekeeping gene β-actin and to wild-type controls ($2^{-\Delta\Delta Ct}$).

2.5 Cell biology

2.5.1 Flow cytometry

Cell numbers of single-cell suspensions of respective organs, of which the isolation is described in chapter 2.3.2, were determined and cell numbers adjusted to a particular concentration for each sample. If cytokine stainings were performed cells were, first, reactivated in T-cell medium containing 50 ng/mL PMA, 500 ng/mL Ionomycin and 1 µg/mL Brefeldin A for 4 hours at 37 °C. Antibodies and dyes that were used are listed below (Table 4). Single cell suspensions were incubated with Fc receptor block in PBS for 10 minutes at 4 °C to avoid unspecific binding of flow cytometry antibodies to Fc regions. Extracellular and viability stainings were performed in FACS staining buffer for 20 minutes at 4 °C. Samples for which biotinylated antibodies were used were incubated with fluorophore-coupled streptavidin (SA) in FACS staining buffer in a second step again for 20 minutes at 4 °C. In order to maintain RFP reporter signals cells were fixed in 2 % FA for 1 h at 4 °C. Otherwise single cell suspensions were fixed using 'Foxp3 Transcription Factor Staining Buffer Set' (eBioscience Inc.) for 1 h at 4 °C. Intracellular stainings targeting transcription factors and cytokines were performed in permeabilisation buffer from this kit over night at 4 °C. Finally, depending on the cell count, cells were resuspended in FACS acquisition buffer. Samples were acquired at FACS Canto II (BD Biosciences GmbH) and analysed using the software FlowJo v10.

Table 4: Fluorophore-coupled antibodies and dyes, their respective clones, dilutions and suppliers.

Antigen or dye and fluorophore	Clone	Dilution	Supplier
Fc receptor block	2.4G2	1:100	Bio X Cell Inc.
Viability Dye APC-Cy7	-	1:1000	Invitrogen AG
CD3ε PerCP	145-2C11	1:100	BD Biosciences GmbH
CD4 Brilliant Violet 510	RM4-5	1:300	BioLegend GmbH
CD4 PerCP	GK1.5	1:1000	BioLegend GmbH
CD8α PE-Cy7	53-6.7	1:1000	BioLegend GmbH
CD11b PE-Cy7	M1/70	1:1000	BioLegend GmbH
CD11b Brilliant Violet 510	M1/70	1:1000	BioLegend GmbH
CD11c APC	HL3	1:800	BD Biosciences GmbH
CD11c PE-Cy7	N419	1:2000	eBioscience Inc.
CD19 PE-Cy7	6D5	1:200	BioLegend GmbH
CD25 FITC	7D4	1:200	BD Biosciences GmbH
CD44 FITC	IM7	1:1000	BioLegend GmbH
CD45 Brilliant Violet 510	30-F11	1:200	BioLegend GmbH

CD45.1 FITC	A20	1:1000	BioLegend GmbH
CD45.2 V500	104	1:200	BD Biosciences GmbH
CD62L APC	MEL-14	1:1000	BioLegend GmbH
CD73 PerCP/Cy5.5	TY/11.8	1:400	BioLegend GmbH
CD80 APC	16-10A1	1:200	BioLegend GmbH
CD86 PE	GL1	1:600	eBioscience Inc.
CD90.2 PerCP	53-2.1	1:1000	BioLegend GmbH
CD103 PerCP/Cy5.5	2 E7	1:100	BioLegend GmbH
CTLA-4 PE	UC10-4F10-11	1:200	BD Biosciences GmbH
EpCAM PE-Cy7	G8.8	1:800	eBioscience Inc.
Foxp3 APC	FJK-16s	1:400	eBioscience Inc.
Foxp3 eFluor 450	FJK-16s	1:100	eBioscience Inc.
Helios Alexa Fluor 488	22F6	1:100	BioLegend GmbH
Ki67 APC	16A8	1:200	BioLegend GmbH
Ly51 PE	BP-1	1:200	BD Biosciences GmbH
MHC-II FITC	M5/114.15.2	1:1000	BioLegend GmbH
OX40 PE	OX-86	1:200	BioLegend GmbH
TCR-β Biotin	H57-597	1:200	BD Biosciences GmbH
TCR-β APC	H57-597	1:1000	BioLegend GmbH
TNFR2 PE	TR75-89	1:200	BioLegend GmbH
RORγt Brilliant Violet 421	Q31-378	1:800	BD Biosciences GmbH
IFNγ PE-Cy7	XMG1.2	1:1000	eBioscience Inc.
IL-17A eFluor 450	eBio17B17	1:300	eBioscience Inc.
Ly6C V450	AL-21	1:300	BD Biosciences GmbH
Ly6G FITC	1A8	1:100	BioLegend GmbH

2.5.2 Magnetic cell separation

For magnetic cell separation (MACS), single cell suspensions obtained from spleens and lymph nodes as previously described in chapter 2.3.2, were counted and washed in ice-cold MACS buffer. Total $CD4^+$ T cells were isolated using 'CD4+ T Cell Isolation Kit', naïve $CD4^+$ T cells were isolated utilising the 'Naïve $CD4^+$ T Cell Isolation Kit' and Treg cells were purified using the '$CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit' (all Miltenyi Biotec B.V. & CO. KG) according to manufacturer's protocol. In principle, cells were incubated with magnetically-labelled antibodies and separated from a heterogeneous cell suspension using magnetic columns.

2.6 *In vitro* experiments

2.6.1 Treg suppression assay

Single cell suspensions of spleens and lymph nodes were obtained, as described in chapter 2.3.2, of which Treg cells were isolated using MACS, see above chapter 2.5.2. MACS purification of $CD4^+$ $CD25^+$ Treg cells also yields $CD4^+$ $CD25^-$ Tconv cells. To assess cell proliferation, these $CD4^+$ Tconv cells were labelled with a fluorescent dye, CellTrace Violet (VCT; Invitrogen AG) according to manufacturer's instructions except for the following changes: labelling was performed in PBS (10^7 cells/mL) containing 3 µM VCT for 10 minutes at RT in the dark. Next, $5x10^4$ VCT-labelled $CD4^+$ Tconv cells were plated along with Treg cells in ratios of 1:1, 1:2, 1:4, 1:8, 1:16 and without Treg cells. Cells were stimulated for 3.5 days at 37 °C with 0.5 µg/mL of soluble anti-CD3 and 6 ng/mL of soluble anti-CD28. Finally, proliferation of $CD4^+$ Tconv cells was assessed by flow cytometric analysis. Antibodies and their respective clones as well as suppliers are described below (**Table 5**).

2.6.2 T cell cultures

Splenocytes were obtained as described in chapter 2.3.2. $CD4^+$ T cells and Treg cells were isolated using MACS, chapter 2.5.2. For *in vitro* stimulation, either splenocytes, $CD4^+$ T cells or Treg cells were stimulated for 4 days at 37 °C with soluble TNF (R&D Systems Inc), IL-2 (PromoCell GmbH), anti-CD3 or anti-CD28 in indicated concentrations in T-cell medium unless specified otherwise. *In vitro* cultures were assessed by flow cytometry.

2.6.3 NIK stabilisation

Splenic Treg cells or $CD4^+$ T cells were isolated using MACS, see chapter 2.5.2, and stimulated with 10 µM of smac mimetic AT-406 (Xevinapant; Selleck Chemicals LLC) in T cell medium for 48 hours along with 2 µg/mL anti-CD3 and 6 ng/mL anti-CD28 ($CD4^+$ T cells) or AT-406 for 4 h (Treg cells) at 37 °C. AT-406 was kindly provided by the laboratory of Professor Vasileios Bekiaris from DTU Denmark.

Table 5: Antibodies used for *in vitro* stimulation.

Antibody	Clone	Supplier
Anti-CD3	17A2	Bio X Cell Inc.
Anti-CD28	37-51	Bio X Cell Inc.

2.7 *In vivo* experiments

2.7.1 Adoptive T cell transfer colitis

By using MACS purification, described in chapter 2.5.2, $5x10^5$ wild-type and NIK-deficient Treg cells as well as CD45.1 (Ly5.1) naïve $CD4^+$ T cells were isolated from spleens of their respective hosts. Naïve $CD4^+$ T cells were intraperitoneally injected in 200 µL of PBS into T- and B-cell-deficient $Rag1^{-/-}$ mice alone or co-injected with either wild-type or NIK-deficient Treg cells. Mini endoscopy (Karl Storz SE & Co. KG) on $Rag1^{-/-}$ mice was performed at the start and at the end of the experiment to assess stool consistency, granularity, fibrin, vascularity and translucency. Mice were weighed at least twice per week according to animal welfare regulations. At the end of the experiment spleen, mLN and colon were isolated for histological as well as flow cytometric analysis. H&E sections of proximal colons were scored using a colitis scoring system adapted from Kihara *et al.*, 2003. Inflammation severity: 0 = none, 1 = mild, 2 = moderate, 3 = severe; inflammation extent: 0 = none, 1 = mucosa, 2 = submucosa, 3 = transmural; crypt damage: 0 = none, 1 = basal with 1/3 damage, 2 = basal with 2/3 damage, 3 = crypt loss but surface epithelium present, 4 = crypt loss and surface epithelium lost.

2.7.2 Treg cell co-injection into $Rag1^{-/-}$ recipients

Essentially, this experiment is a modified version of the model of adoptive T cell transfer colitis, described in the above chapter 2.7.1. In principle, by using MACS purification, see section 2.5.2, $2.5x10^5$ CD90.1 (Thy1.1) wild-type and $2.5x10^5$ CD90.2 (Thy1.2; or CD90.1 CD90.2-double-positive) NIK-deficient Treg cells as well as $5x10^5$ CD45.1 (Ly5.1) naïve $CD4^+$ T cells were isolated from spleens and lymph nodes of their respective hosts and in 200 µL of PBS intraperitoneally co-injected into $Rag1^{-/-}$ mice. According to animal welfare regulations, spleen, mLN as well as the colon were isolated before onset of colitis and investigated by flow cytometry.

2.8 Histology: Haematoxylin and eosin stainings

Tissues were fixed in 4 % formaldehyde for 2 days at RT before they were embedded into paraffin. Haematoxylin and eosin (H&E) stainings from paraffin sections in addition to embedding of tissues into paraffin were performed in collaboration with Claudia Braun (histology core facility of University Medical Center of Johannes-Gutenberg University Mainz). Principally, haematoxylin stains nuclei in dark violet, whereas eosin stains cytoplasmic and extracellular matrix structures in pink. According to the core facility, sections were dried at 60 °C for 30 minutes and then stained as follows: 5 minutes in xylol, 4 minutes in 100 % ethanol twice, 4 minutes in 96 % ethanol twice, 4 minutes in 70 % ethanol, 4 minutes in dH_2O, 10 minutes in haematoxylin, 10 minutes in tap water, 30 seconds in eosin, rinsed with tap

water, 4 minutes in 70 % ethanol, 4 minutes in 96 % ethanol, 4 minutes in 100 % ethanol twice and 5 minutes in xylol before mounting with Entellan (Merck KGaA) was performed.

2.9 Biochemistry

2.9.1 ELISA

In order to determine serum IgA levels, ELISA was performed. In a first step 96-well flat-bottom plates (Greiner Bio-One GmbH) were coated with 10 µg/mL anti-IgA (#1040-01, Southern Biotech Inc.) and subsequently blocked with PBS containing 5 % BSA (w/v). A 1:5 serial dilution of the standard (10 µg/mL; #0106-01, Southern Biotech Inc.) and sera (1:50 starting dilution) was performed before the biotin-conjugated IgA detection antibody in a 1:1000 dilution (#1040-08, Southern Biotech Inc.) was added. The detection antibody was then coupled to a streptavidin-AP conjugate (dilution 1:3000, Roche Diagnostics GmbH) that converted the PNPP, disodium salt substrate (dilution 1:5, Santa Cruz Biotechnology Inc.). The signal was eventually measured at the 'Infinite M200 Pro' plate reader (Tecan Group AG) at 570 nm.

2.9.2 Western blot

Single-cell suspensions were obtained as described in chapter 2.3.2 and pelleted before proteins were extracted by incubation with 50 µL (per 5 mio cells) 1X RIPA buffer (#9806, Cell Signaling Technology, Inc) containing protease inhibitor (1X, Roche Diagnostics GmbH) and phosphatase inhibitor (1X, Roche Diagnostics GmbH) for 20 minutes at 4 °C. Total protein concentrations were quantified by photometric measurements on the BioPhotometer plus (Eppendorf SE) using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc) according to manufacturer's instructions. 30 µg of protein were incubated with 20 % (v/v) NuPAGE LDS Sample Buffer 4X (Invitrogen AG) and 34.5 mM DTT for 10 minutes at 70°C before they were loaded onto a 4-12 % Bis-Tris gel (Invitrogen AG) and separated by molecular mass via gel electrophoresis using 1X MOPS buffer. The gel was run for around 2 hours at 120 V (first 20 minutes at 70 V) until proteins were well separated. In order to transfer proteins from the gel onto a polyvinylidene difluoride membrane (Immobilion-P Membran, Sigma-Aldrich Corp.), the membrane was activated with methanol first. Semi-dry blotting was performed in a TRANS-BLOT SD blotter (Bio-Rad Laboratories Inc.) using 1X transfer buffer containing 15 % methanol (v/v) for 1 hour at 0.2 A and maximum 25 V. For antibody staining, the membrane was blocked with 1X TBS containing Tween20 (dilution 1:1000; TBS-T, Sigma-Aldrich Corp.) and 5 % skim milk powder (w/v, Sigma-Aldrich Corp.) for 1 hour at RT followed by overnight probing with monoclonal NF-κB2 p100/p52 (dilution 1:1000, Cell Signaling Technology Inc, #4882). After washing 10-15 minutes 3 times with 1X TBS-T at RT the membrane was incubated for 1

hour at RT with a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (dilution 1:5000, Santa Cruz Biotechnology Inc., #sc-2054) antibody followed again by 3 washing steps using TBS-T. Eventually, ECL Detection Reagents (Cytiva) were used according to manufacturer's instructions and HRP-mediated stainings visualised on a ChemiDoc XRS$^+$ (Bio-Rad Laboratories Inc). Blots were further analysed using Image Lab software 6.0.1 (Bio-Rad Laboratories Inc).

2.10 Statistical analysis

Whether samples were normally distributed or not was determined by performing a Shapiro-Wilk test. Subsequently, for normally distributed samples an unpaired two-sided Student's t-test was used to assess the comparison between data. On the contrary, if no Gaussian distribution could be assumed a Mann-Whitney test was performed. If not stated otherwise data are represented as mean ± SD. P values ≤ 0.05 were considered significant: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001. Statistical analysis was carried out using GraphPad Prism version 7.04.

3 Results

As previously described, the role of NF-κB-inducing kinase (NIK) in thymic regulatory T cell (Treg cell) development and Treg cell maintenance in lymphoid organs remains controversial. Questions concerning the requirement for NIK in peripheral tissue Treg cell maintenance and Treg cell-mediated peripheral tissue homeostasis have not been addressed yet. To date, there are no reports investigating whether NIK is essential for Treg cells to be suppressive *in vivo*. To address these open questions, pointed out in more detail in chapter 1.8, the below sections will first describe results regarding the involvement of NIK in thymic Treg cell development, followed by Treg cell maintenance in lymphoid organs and peripheral tissues as well as the role of NIK in Treg cell-mediated peripheral tissue homeostasis and suppression of effector cells during colitis.

3.1 Requirement for NIK in the maintenance of thymic Treg cell precursors

To delete NIK in thymic Treg cell precursors (TregP), $NIK^{\Delta T}$ mice, in which NIK is deleted in all αβ T cells including $CD8^+$ and $CD4^+$ cells, were utilised. First, it was confirmed whether the deletion of NIK in $CD4^+$ T cells did indeed occur. For that splenic $CD4^+$ T cells from $NIK^{\Delta T}$ and control mice were MACS purified and stimulated with either anti-CD3, anti-CD28 or anti-CD3, anti-CD28 and the IAP antagonist AT406, a smac mimetic, that stabilises NIK and therefore induces non-canonical NF-κB signalling (Rizk *et al.*, 2019). As described previously in chapter 1.4, NIK is the key player of non-canonical NF-κB signalling that ultimately, upon activation, leads to the processing of p100 to p52. As anticipated, the stimulation with anti-CD3 and anti-CD28 did not induce non-canonical NF-κB signalling neither in WT nor in NIK-deficient $CD4^+$ T cells. Expectedly, the addition of AT406, however, did induce the processing of p100 into p52 in WT $CD4^+$ T cells but not in NIK-deficient $CD4^+$ T cells (**Figure 6**). Hence, these data confirm that NIK signalling was abrogated in $CD4^+$ T cells from $NIK^{\Delta T}$ mice.

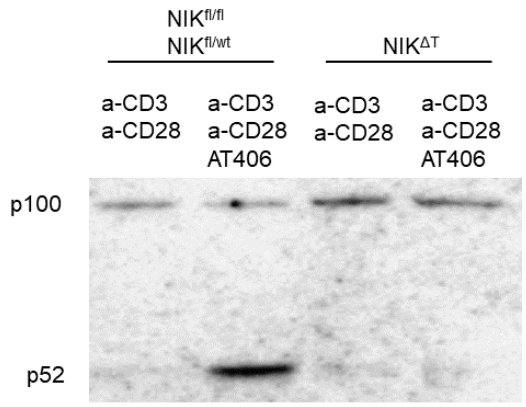

Figure 6: Abrogated non-canonical NF-κB signalling in CD4⁺ T cells from mice with conditional deletion of NIK in αβ T cells. Single cell suspensions from spleens of 12-week-old NIK-deficient mice and control animals were generated and $CD4^+$ T cells isolated by CD4 MACS purification. $CD4^+$ T cells were stimulated with 2µg/mL anti-CD3, 6ng/mL anti-CD28 and either with or without 10µM AT406 for 2 days at 37 °C. Whole-cell extracts were subjected to Western blot analysis that revealed the processing of p100 into p52 in WT $CD4^+$ T cells. Shown is one out of two technical replicates that were pooled from n = 4 (WT = 4, KO = 4) biological replicates.

As previously described, Lacher *et al*., observed a potential role for NIK in thymic Treg cell development (Lacher *et al.*, 2018). Consequently, we analysed thymic Treg cells as well as thymic TregP of $NIK^{\Delta T}$ and control mice by flow cytometry. First, recirculating $CD73^+$ $Foxp3^+$ Treg cells were found to be reduced in frequencies (**Figure 7A, B**) and in total cell numbers in $NIK^{\Delta T}$ mice (**Figure 7C**). Interestingly, there was a reduction of $Foxp3^{lo}$ TregP and mature Treg cells (mTreg) but not CD25 TregP in terms of frequencies (**Figure 7D, E**) and cell numbers (**Figure 7F**). Looking more closely on CD25 TregP, strikingly, a reduction of $TNFR2^+$ CD25 TregP could be detected percentage-wise (**Figure 7G, H**) and in cell numbers in $NIK^{\Delta T}$ mice (**Figure 7I**). Additionally, NIK-deficient $OX40^+$ CD25 TregP were also reduced in frequencies (**Figure 7J, K**) and cell numbers (**Figure 7L**). In summary, the deletion of NIK in $CD4^+$ T cells leads to a reduction of thymic TregP as well as mTreg and recirculating Treg cells.

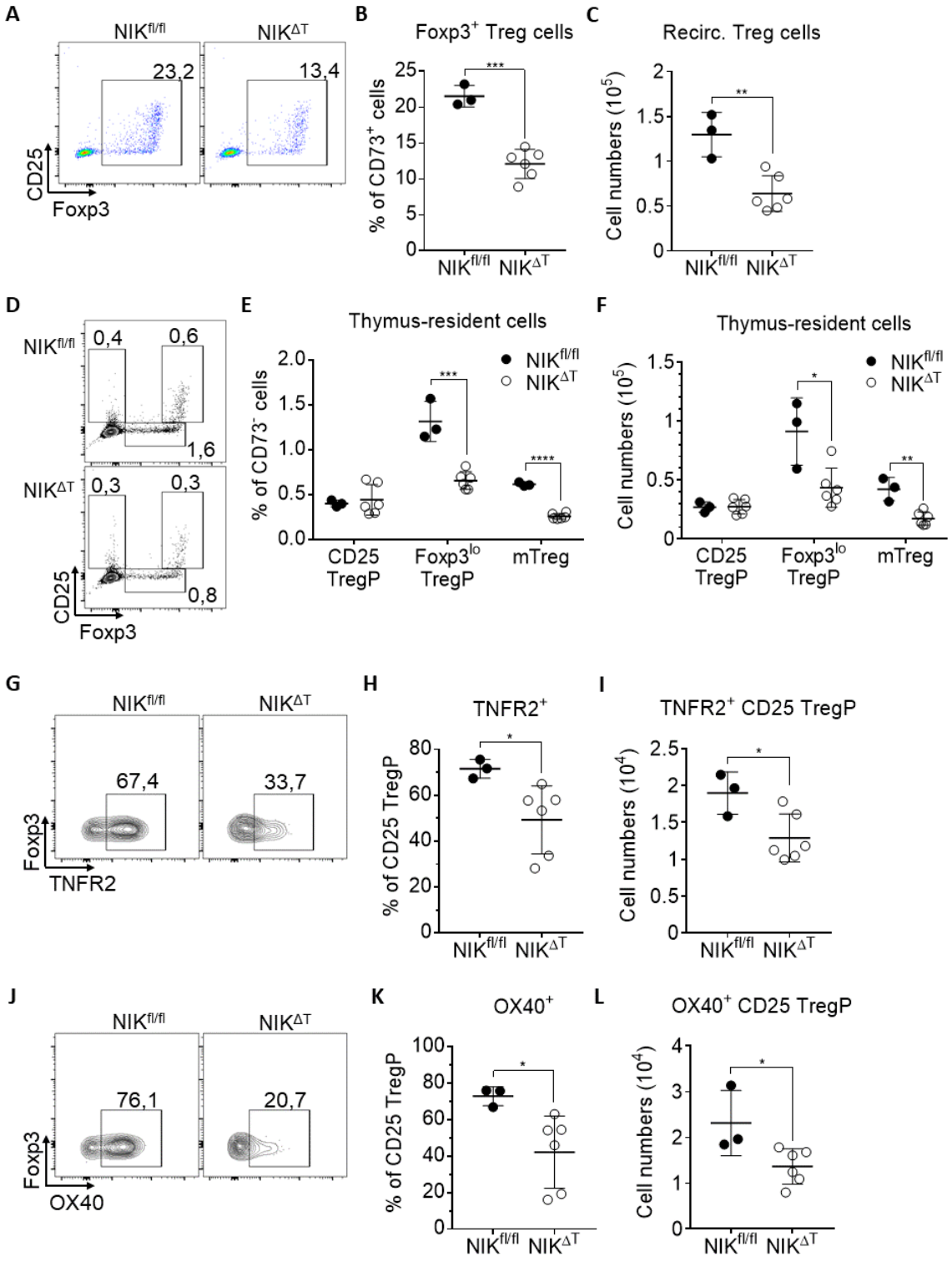

Figure 7: Reduction of thymic Treg cells and Treg cell precursors in mice with conditional deletion of NIK in αβ T cells. Single cell suspensions from the thymus of 6-week-old female $NIK^{\Delta T}$ and $NIK^{fl/fl}$ littermate controls were generated and analysed by flow cytometry. (**A**) Representative *FACS* plot, (**B**) frequencies and (**C**) cell numbers of recirculating ($CD73^+$) $Foxp3^+$ Treg cells. (**D**) Representative *FACS* plot, (**E**) frequencies and (**F**) cell numbers of thymus-resident ($CD73^-$) $CD25^+$ Treg cells precursors (CD25 TregP), $Foxp3^{lo}$ TregP and freshly developed mature Treg cells (mTreg). (**G**) FACS plot, (**H**) percentages and (**I**) cell numbers of $TNFR2^+$ CD25 TregP. (**J**) FACS plot, (**K**) percentages and (**L**) cell numbers of $OX40^+$ CD25 TregP. Data represented as mean ± SD of n ≥ 3 with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 and are representative of at least three independent experiments. Statistical analysis was performed using an (**B**, **C**, **E**, **F**, **I**, **K**, **L**) unpaired two-sided Student's t-test or (**H**) Mann-Whitney test.

It is well established that certain dendritic cell (DC) subsets express CD4 and present antigens to developing thymocytes during thymic T cell development (Saito *et al.*, 2010; Owen, Sjaastad and Farrar, 2019). DCs provide costimulatory signals to T cells through CD80 and CD86 (Shevach, 2018). Next, to assess whether the reduction of TregP as well as mTreg and recirculating Treg cells in NIK$^{\Delta T}$ mice was due to deletion of NIK signalling in CD4^{+} DCs, DC homeostasis and development were analysed by flow cytometry. The percentages of CD11c^{+} and MHC-II^{high} cells were similar between the two groups (**Figure 8A, B**). Moreover, frequencies of CD4^{+} CD86^{+} DCs did not differ between NIK$^{\Delta T}$ and control mice (**Figure 8C, D**). Additionally, same frequencies could be observed for CD4^{+} CD80^{+} DCs (**Figure 8E, F**). The expression levels of CD86 on CD4^{+} CD86^{+} DCs and CD80 on CD4^{+} CD80^{+} DCs were assessed by MFI and were not different between the two groups (**Figure 8G, H**). Collectively, these data suggest that the CD4-Cre did affect neither DC development nor homeostasis.

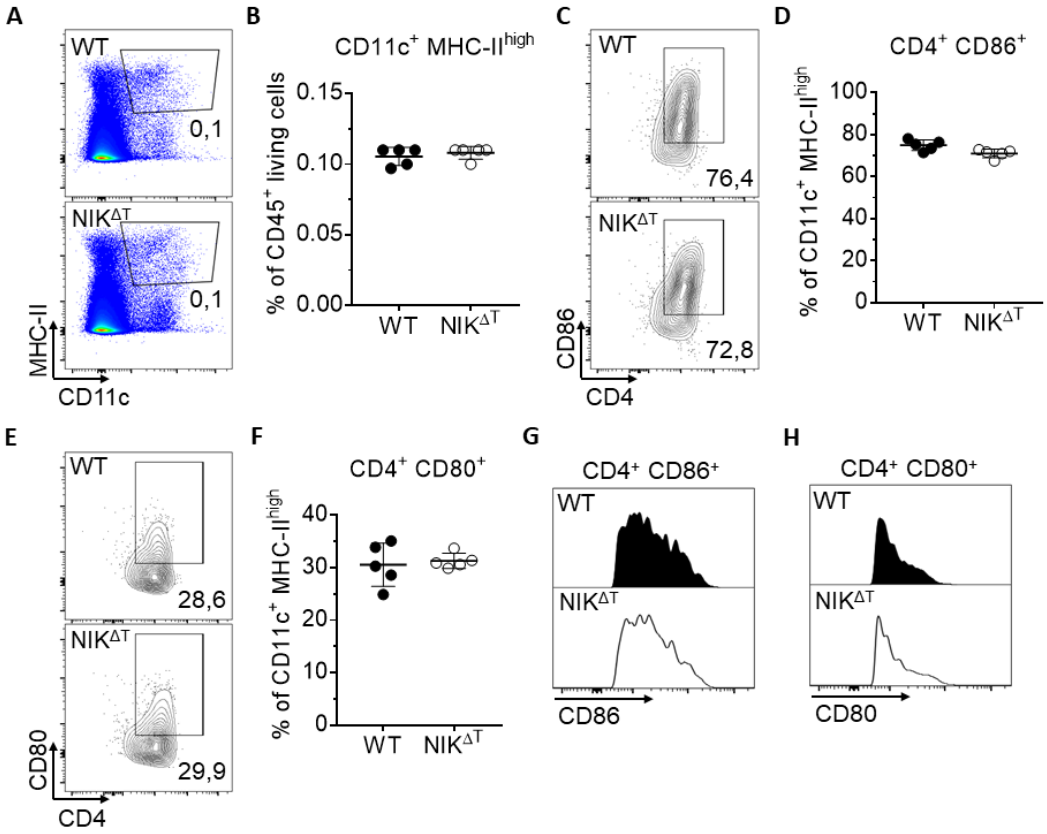

Figure 8: No impaired dendritic cell homeostasis in thymi of mice with conditional deletion of NIK in αβ T cells. Thymi of 7-9-week-old NIK$^{\Delta T}$ and WT mice were digested and single cell suspensions generated. The dendritic cell compartment was analysed by flow cytometry. (A) Representative FACS plot and (B) frequencies of CD11c^{+} MHC-II^{high} cells. (C) Representative FACS plot and (D) frequencies of CD4^{+} CD86^{+} cells. (E) FACS plot and (F) percentages of CD4^{+} CD80^{+} cells. MFI of (G) CD86 on CD4^{+} CD86^{+} cells and of (H) CD80 on CD4^{+} CD80^{+} cells. DCs were pre-gated on cells, single cells and CD45^{+} living cells. Data represent mean ± SD of n = 5 and are representative of two independent experiments. Statistical analysis was performed using (B, D, F) Mann-Whitney test.

Thymic epithelial cells are major antigen-presenting cells during thymic T cell development (Owen, Sjaastad and Farrar, 2019). To exclude an indirect effect of NIK deletion in αβ T cells on the homeostasis

of thymic epithelial cells, this compartment was analysed by flow cytometry. Expectedly, similar frequencies of cortical thymic epithelial cells (cTEC), mature medullary thymic epithelial cells (mTEC) as well as immature mTEC between NIK$^{\Delta T}$ and control mice were found (**Figure 9A, B**). Additionally, the thymic cytokine milieu comprising IL-2 and TGF- β1 as well as CCL20 that could potentially influence thymic T cell expansion or attraction was analysed. Thymic epithelial cells produce CCL20 and thereby recruit recirculating Treg cells back into the thymus (Cowan *et al.*, 2018). IL-2 drives Treg cell maturation and expansion, while TGF-β has been reported to be critical driving negative selection of thymocytes (McCarron *et al.*, 2019; Owen, Sjaastad and Farrar, 2019). We found that thymic mRNA levels of *IL-2*, *CCL20* and *TGF- β1* did not differ between NIK$^{\Delta T}$ and control mice (**Figure 9C**). In summary, these data hint towards a functioning TEC compartment and normal thymic cytokine milieu in NIK$^{\Delta T}$ mice.

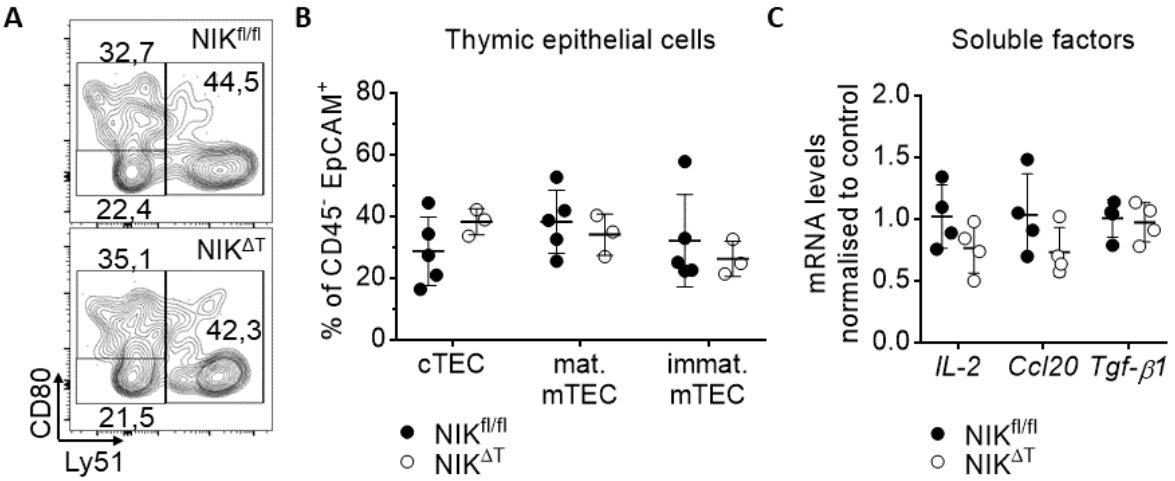

Figure 9: No impaired homeostasis of thymic epithelial cells as well as mRNA levels of *IL-2*, *CCL20* and *TGF-β1* in mice with conditional deletion of NIK in αβ T cells. Thymi of 8-week-old NIK$^{\Delta T}$ mice and littermate controls were digested and single cell suspensions generated. The thymic epithelial cell compartment was analysed by flow cytometry. (A) Representative plot and (B) frequencies of cortical thymic epithelial cells (cTEC) and mature as well as immature medullary thymic epithelial cells (mTEC). TEC were pre-gated on cells, single cells, living cells and CD45$^-$ EpCAM$^+$ cells. (C) mRNA levels of *IL-2*, *CCL20* and *TGF-β1* from thymi of 7-week-old NIK$^{\Delta T}$ mice and littermate controls. Data represent mean ± SD of n ≥ 3 and are representative of two independent experiments. Statistical analysis was performed using (B, C) Mann-Whitney test.

Next, to assess whether the reduction of thymic Treg cells and TregP in NIK$^{\Delta T}$ mice was due to a requirement for NIK in CD4$^+$ cells and, hence, TregP we analysed the thymic T cell compartment of NIK$^{\Delta Treg}$ mice by flow cytometry. Indeed, similar percentages of CD73$^+$ recirculating Foxp3$^+$ Treg cells were found between the two groups (**Figure 10A, B**). Moreover, the frequencies of CD25 TregP, Foxp3^{lo} TregP and mTreg did not differ between NIK$^{\Delta Treg}$ and control mice (**Figure 10C, D**). Taken together, these results provide evidence that the reduction of Treg cells in NIK$^{\Delta T}$ mice stems from a reduction of CD4$^+$ TregP, namely, CD25 TregP and Foxp3^{lo} TregP, whose maintenance depends on NIK signalling.

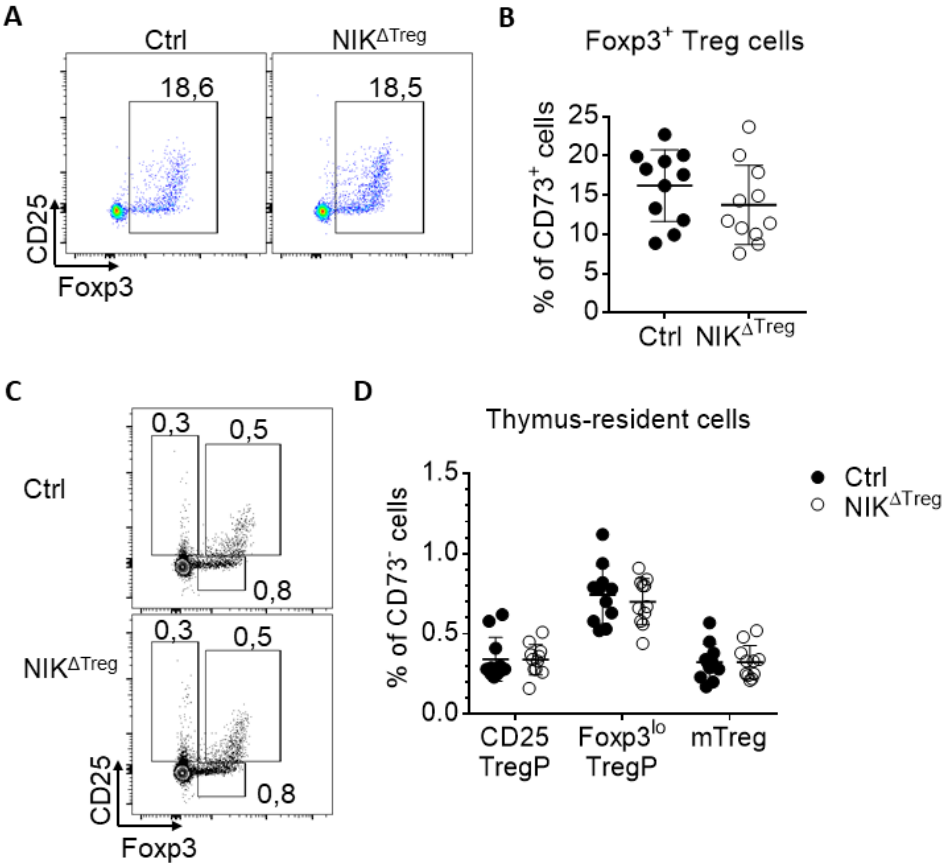

Figure 10: Normal frequencies of thymic Treg cells and Treg cell precursors in mice with conditional deletion of NIK in Foxp3^{+} Treg cells. Single cell suspensions from thymi of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and analysed by flow cytometry. (A) Representative FACS plot and (B) frequencies of recirculating (CD73^{+}) Treg cells. (C) Representative FACS plot and (D) frequencies of CD25 TregP, Foxp3^{lo} TregP and mTreg. Treg cells and TregP were pre-gated on cells, single cells, living cells and CD4 single-positive cells before gating on CD73^{+} cells for recirculating Treg cells and CD73^{-} for thymus-resident cells. Data represent mean ± SD of n = 11 and are pooled from three independent experiments. Statistical analysis was performed using (B, D) Mann-Whitney test.

Owen and colleagues reported that thymic CD25 TregP express TNFR2 in order to expand adequately upon antigen recognition (Owen *et al.*, 2019). Additionally, above data demonstrate a requirement for NIK in the maintenance of CD25 TregP. Hence, the thymic Treg cell and TregP compartment in TNFR2$^{-/-}$ mice was analysed by flow cytometry based on the separation of recirculating (CD73^{+}) and thymus-resident cells (CD73^{-}). Thymic CD4 single-positive cells from TNFR2$^{-/-}$ mice did, indeed, not express TNFR2 (**Figure 11A**). Of note, TNFR2$^{-/-}$ mice did not show a reduction of recirculating Foxp3^{+} Treg cells (**Figure 11B, C, D**). Surprisingly, only CD25 TregP and neither Foxp3^{lo} TregP nor mTreg were found to be reduced in TNFR2$^{-/-}$ mice (**Figure 11E, F**). Consequently, these data provide further evidence that TNFR2 is required for CD25 TregP maintenance.

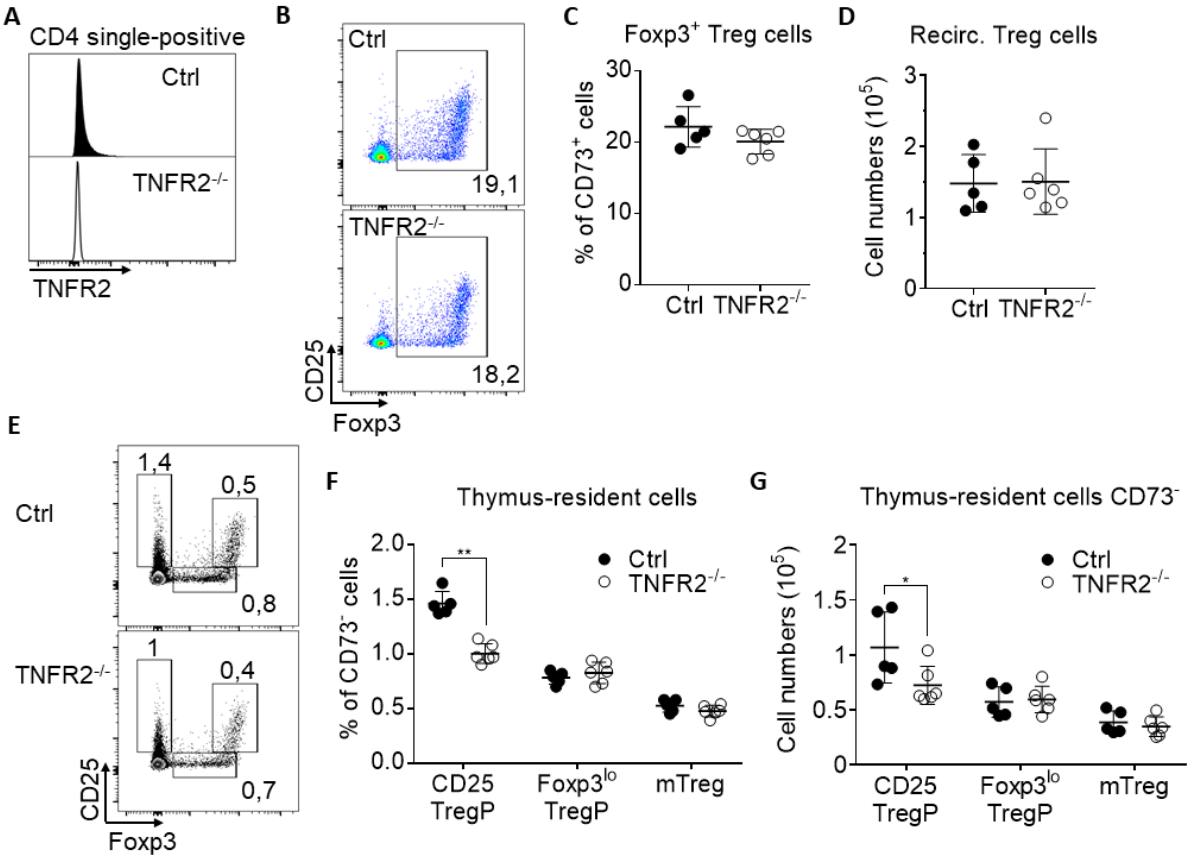

Figure 11: Reduction of CD25 TregP in TNFR2$^{-/-}$ mice. Single cell suspensions from thymi of 9-week-old TNFR2$^{-/-}$ and control mice were generated and analysed by flow cytometry. (A) Representative histogram showing TNFR2 expression on CD4 single-positive cells. (B) Representative FACS plot, (C) frequencies and (D) cell numbers of recirculating (CD73^{+}) Treg cells. (E) Representative FACS plot, (F) frequencies and (G) cell numbers of CD25 TregP, Foxp3^{lo} TregP and mTreg. Treg cells and TregP were pre-gated on cells, single cells, living cells and CD4 single-positive cells before gating on CD73^{+} cells for recirculating Treg cells and CD73^{-} for thymus-resident cells. Data represented as mean ± SD of n ≥ 5 with *p ≤ 0.05, **p ≤ 0.01 and are representative of two independent experiments. Statistical analysis was performed using an (G) unpaired two-sided Student's t-test or (C, D, F) Mann-Whitney test.

In summary, the above results provide evidence that thymic TregP require NIK for their maintenance and suggest that the reduced thymic Treg cell compartment in NIK$^{\Delta T}$ mice stems from a reduction in Foxp3^{lo} TregP and TNFR2^{+} as well as OX40^{+} CD25 TregP.

3.2 Reduction of Foxp3^{+} Treg cells in secondary lymphoid organs upon deletion of NIK in αβ T cells

Next, we investigated whether NIK is cell-intrinsically required for the maintenance of peripheral as well as in particular tissue-resident Treg cells. To begin, we re-analysed peripheral lymphoid organs of the NIK$^{\Delta T}$ strain, in which Lacher *et al.* noted a reduction in Foxp3^{+} Treg cells (Lacher *et al.*, 2018). Single cell suspensions from the spleen were generated and analysed by flow cytometry. First, it was confirmed

that spleens of $NIK^{\Delta T}$ mice show reduced frequencies of $Foxp3^+$ Treg cells in addition to reduced Treg cell numbers (**Figure 12A-C**). Interestingly, the percentages of $CD103^+$ Treg cells (**Figure 12D, E**), $Helios^+$ Treg cells (**Figure 12G, H**) but not $TNFR2^+$ Treg cells (**Figure 12G, J**) were reduced in $NIK^{\Delta T}$ mice as well. Potentially due to overall decreased $Foxp3^+$ Treg cell numbers, cell counts were reduced for all three Treg cell subtypes (**Figure 12F, I and K**). Taken together, these observations show that NIK-deficiency in $CD4^+$ T cells results in a decrease of splenic $Foxp3^+$ Treg cells and Treg cell subtypes.

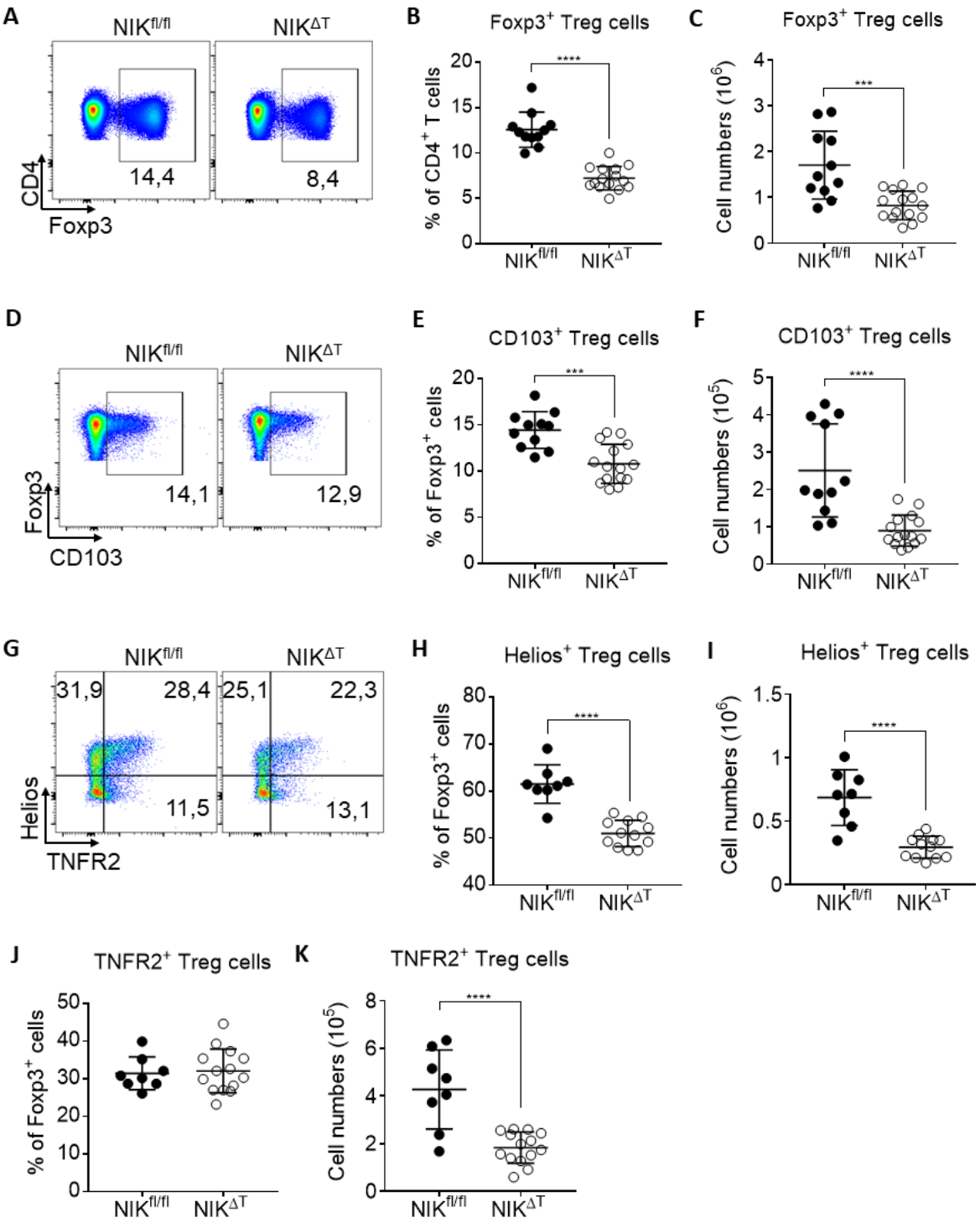

Figure 12: Reduction of Treg cells and their subtypes in the spleen of mice with conditional deletion of NIK in αβ T cells. Single cell suspensions from spleens of 6-10-week-old NIK-deficient mice and control littermates were isolated and analysed by flow cytometry. (**A, B**) percentages and (**C**) cell numbers of Foxp3$^+$ Treg cells. Foxp3$^+$ Treg cells were pre-gated on lymphocytes, single cells, living cells and, eventually, CD4$^+$ T cells. (**D, E**) Percentages and (**F**) cell numbers of CD103$^+$ Treg cells. (**G, H**) Percentages and (**I**) cell numbers of Helios$^+$ Treg cells. (**J**) Percentages and (**K**) cell numbers of TNFR2$^+$ Treg cells. CD103$^+$ Treg cells, Helios$^+$ Treg cells as well as TNFR2$^+$ Treg cells were pre-gated on Foxp3$^+$ Treg cells. Biological replicates with n ≥ 8 are shown as mean ± SD with ***p ≤ 0.001, ****p ≤ 0.0001 and were pooled from (**A-F**) three or (**G-K**) two independent experiments. Statistical analysis was performed using (**C, F, I, K**) unpaired two-sided Student's t-test and (**B, E, H, J**) Mann-Whitney test.

The reduction of effector Treg cell populations, that tend to migrate between secondary lymphoid organs and peripheral tissues, such as CD103$^+$, Helios$^+$ as well as TNFR2$^+$ Treg cells, in the spleen of the NIK$^{\Delta T}$ strain strongly suggested to further analyse the peripheral tissues of these mice (Estrada Brull, Panetti and Joller, 2022). Yet, first the intestine-draining mesenteric lymph nodes (mLN) were analysed by flow cytometry. Indeed, when we investigated the mLN, reduced frequencies (**Figure 13A, B**) as well as cell counts (**Figure 13C**) could be detected for Foxp3$^+$ Treg cells in NIK$^{\Delta T}$ mice in contrast to the mere tendency of reduced Treg cells, that was published by Lacher and colleagues (Lacher *et al.*, 2018). Furthermore, the same could be observed for frequencies (**Figure 13D, E**) and cell numbers (**Figure 13F**) of Helios$^+$ Treg cells. In contrast, NIK deletion in all CD4$^+$ T cells increased the percentages (**Figure 13D, G**) of TNFR2$^+$ Treg cells, whereas the cell numbers of TNFR2$^+$ Treg cells were decreased.

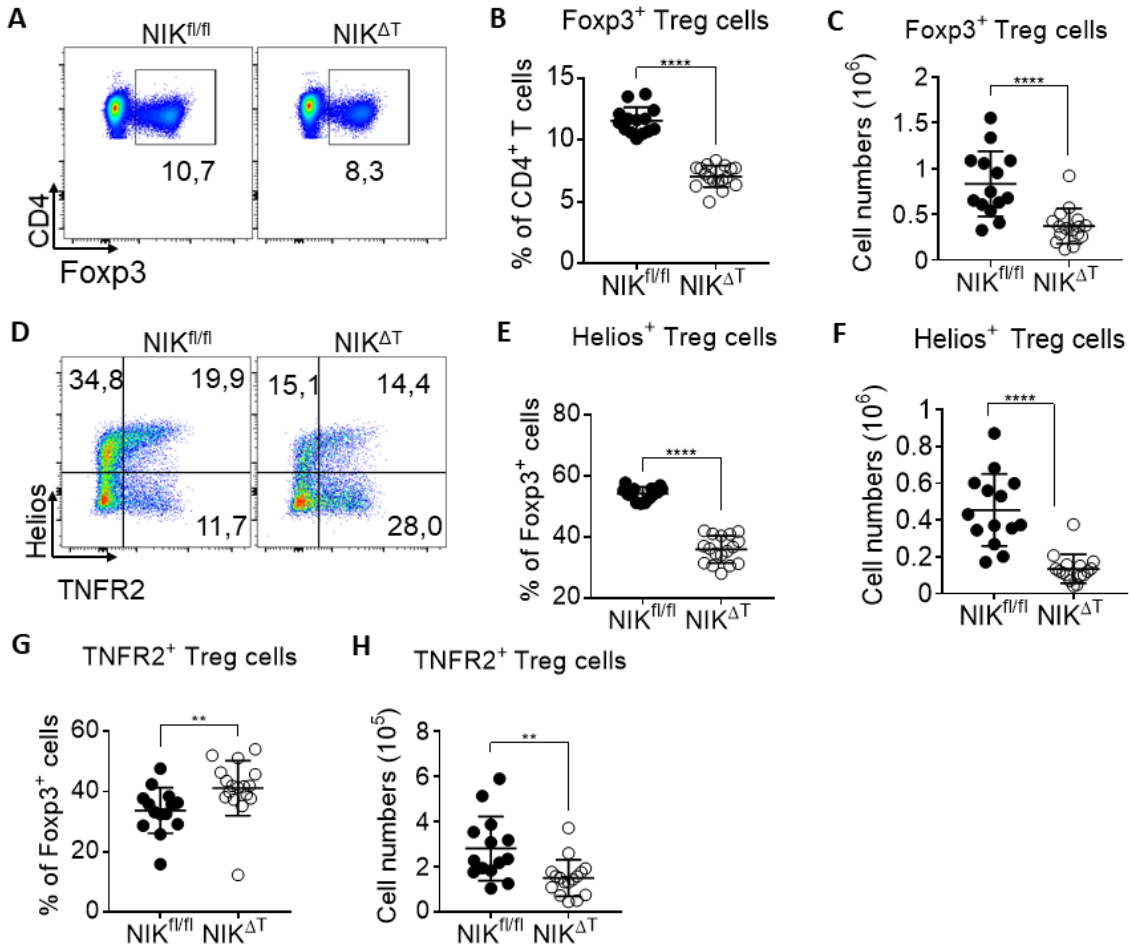

Figure 13: Reduction of Treg cells and their subtypes in mesenteric lymph nodes of mice with conditional deletion of NIK in αβ T cells. mLN of 6-12-week-old steady-state mice of the NIK$^{\Delta T}$ strain and littermate controls were isolated and single cell suspensions generated. Flow cytometric analysis revealed (**A, B**) the percentage and (**C**) cell numbers of Foxp3$^+$ Treg cells. Foxp3$^+$ Treg cells are pre-gated on lymphocytes, single cells, living cells and CD4$^+$ T cells. (**D, E**) Percentage and (**F**) cell numbers of Helios$^+$ Treg cells, in addition to (**G**) percentages and (**H**) cell numbers of TNFR2$^+$ Treg cells. Helios$^+$ Treg cells as well as TNFR2$^+$ Treg cells were both pre-gated on Foxp3$^+$ Treg cells. Biological replicates with n ≥ 14 are represented as mean ± SD with **p ≤ 0.01, ****p ≤ 0.0001 and were pooled from three independent experiments. Statistical analysis was performed using (**H**) unpaired two-sided Student's t-test and (**B, C, E, F, G**) Mann-Whitney test.

In summary, these results suggest that $CD4^+$ T cells require NIK to ensure adequate numbers of $Foxp3^+$ Treg cells and Treg cell subtypes in mLN.

3.3 Reduction of $Foxp3^+$ Treg cells in peripheral tissues upon deletion of NIK in αβ T cells

To date, tissues of $NIK^{\Delta T}$ mice and control littermates, except for the CNS of mice suffering from EAE, have not been analysed. Keeping in mind the emerging role of tissue Treg cells in autoimmune disorders and interesting findings of Treg cell frequencies from the spleen and mLN shown in section 3.2, this paragraph will further analyse the colon of the $NIK^{\Delta T}$ strain. Cells from the colonic lamina propria were isolated and investigated by flow cytometry. Strikingly, the deletion of NIK in $CD4^+$ T cells affected frequencies (**Figure 14A, B**) as well as cell numbers (**Figure 14C**) of colonic $CD4^+$ T cells under steady state homeostasis. While we observed higher percentages (**Figure 14D, E**) of $Foxp3^+$ Treg cells, the overall cell count (**Figure 14F**) was reduced. As previously introduced in section 1.3, intestinal $Foxp3^+$ Treg cells can be distinguished into tTreg cells and microbiota-induced pTreg cells by looking at their transcription factors Helios and RORγt, respectively. Strikingly, the frequencies (**Figure 14G, H**) as well as the cell numbers (**Figure 14I**) of $Helios^+$ Treg cells were strongly reduced in $NIK^{\Delta T}$ mice, while the frequencies and cell count for $ROR\gamma t^+$ as well as double-negative ($Helios^-$, $ROR\gamma t^-$) Treg cells remained unaffected. Furthermore, we found a reduction in percentage (**Figure 14J, K**) and cell numbers (**Figure 14L**) of intestinal $CD103^+$ Treg cells. It would have been interesting to analyse $TNFR2^+$ Treg cells, however, certain epitopes of TNFR2, we believe, had been cleaved off during the harsh enzymatic digestion procedure needed for the isolation of cells from the intestine and were therefore not detectable by FACS analysis. In summary, these results indicate that NIK is highly important for the maintenance of colonic $CD4^+$ T cells, $Foxp3^+$ Treg cells as well as certain Treg cell subtypes, such as $Helios^+$ and $CD103^+$ Treg cells as identified here.

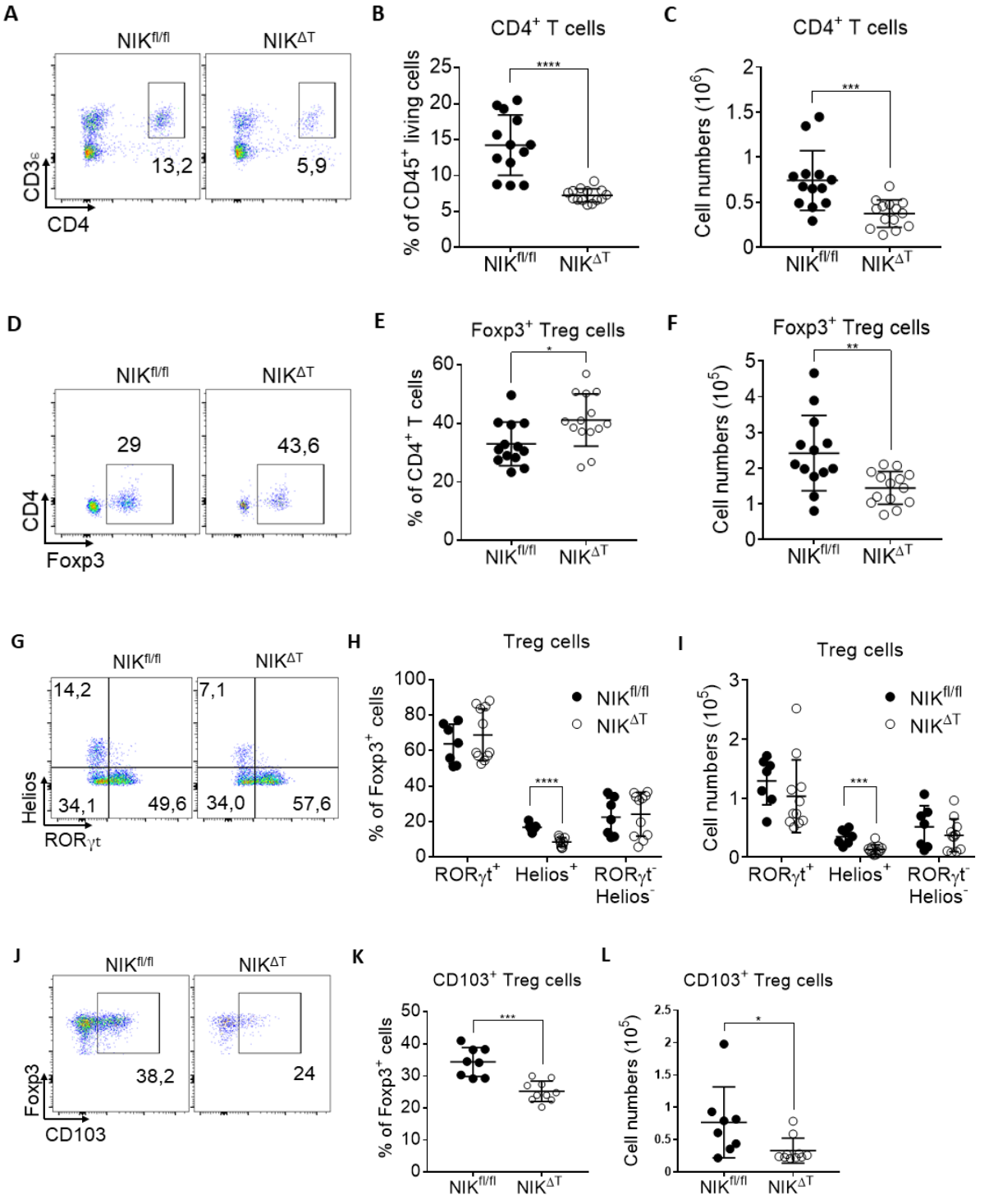

Figure 14: Reduction of Treg cells and their subtypes in the colon of mice with conditional deletion of NIK in αβ T cells. The colon of 6-12-week-old steady-state mice of the NIK$^{\Delta T}$ strain and littermate controls were isolated and single cell suspensions of lamina propria-resident cells generated. Flow cytometric analysis revealed (**A, B**) the percentage and (**C**) cell numbers of CD4$^+$ T cells. CD4$^+$ T cells are pre-gated on lymphocytes, single cells and CD45$^+$ living cells. (**D, E**) Percentage and (**F**) cell numbers of Foxp3$^+$ Treg cells. (**G, H**) Percentages and (**I**) cell numbers of Helios$^+$ Treg cells, RORγt$^+$ Treg cells as well as Treg cells negative for both Helios and RORγt. (**J, K**) Percentages and (**L**) cell numbers of CD103$^+$ Treg cells. CD103$^+$ Treg cells were pre-gated on living cells, CD4$^+$ T cells and Foxp3$^+$ Treg cells. Biological replicates with n ≥ 7 are represented as mean ± SD with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 and were pooled from (**A-F**) three independent experiments and (**G-L**) two independent experiments. Statistical analysis was performed using (**C, F, H, I**) unpaired two-sided Student's t-test and (**B, E, K, L**) Mann-Whitney test.

The liver is connected to the intestine through the portal vein and hence constantly encounters intestinal antigens against which Treg cells confer tolerance and are therefore crucial for maintaining hepatic homeostasis (Crispe *et al.*, 2006; Balmer *et al.*, 2014). Hence, it was assessed whether NIK in $CD4^+$ T cells is important for $Foxp3^+$ Treg cell maintenance in the liver, as was observed for colonic tissue. Therefore, $CD45^+$ cells from the liver were isolated and analysed by flow cytometry. In contrast to the colon, the frequencies (**Figure 15A, B**) as well as cell numbers (**Figure 15C**) of $CD4^+$ T cells did not differ between $NIK^{\Delta T}$ mice and control littermates. However, when looking at $Foxp3^+$ Treg cells, we observed a pronounced reduction in terms of frequencies (**Figure 15D, E**) and cell numbers (**Figure 15F**) in $NIK^{\Delta T}$ mice. Additionally, both, $Helios^+$ as well as $TNFR2^+$ Treg subtypes were reduced both in percentage (**Figure 15G, I**) and cell numbers (**Figure 15H**) when NIK was deleted in all αβ T cells. Taken together, these data show that deletion of NIK in $CD4^+$ T cells results in a reduction of hepatic $Foxp3^+$ Treg cells as well as their $Helios^+$ and $TNFR2^+$ subtypes.

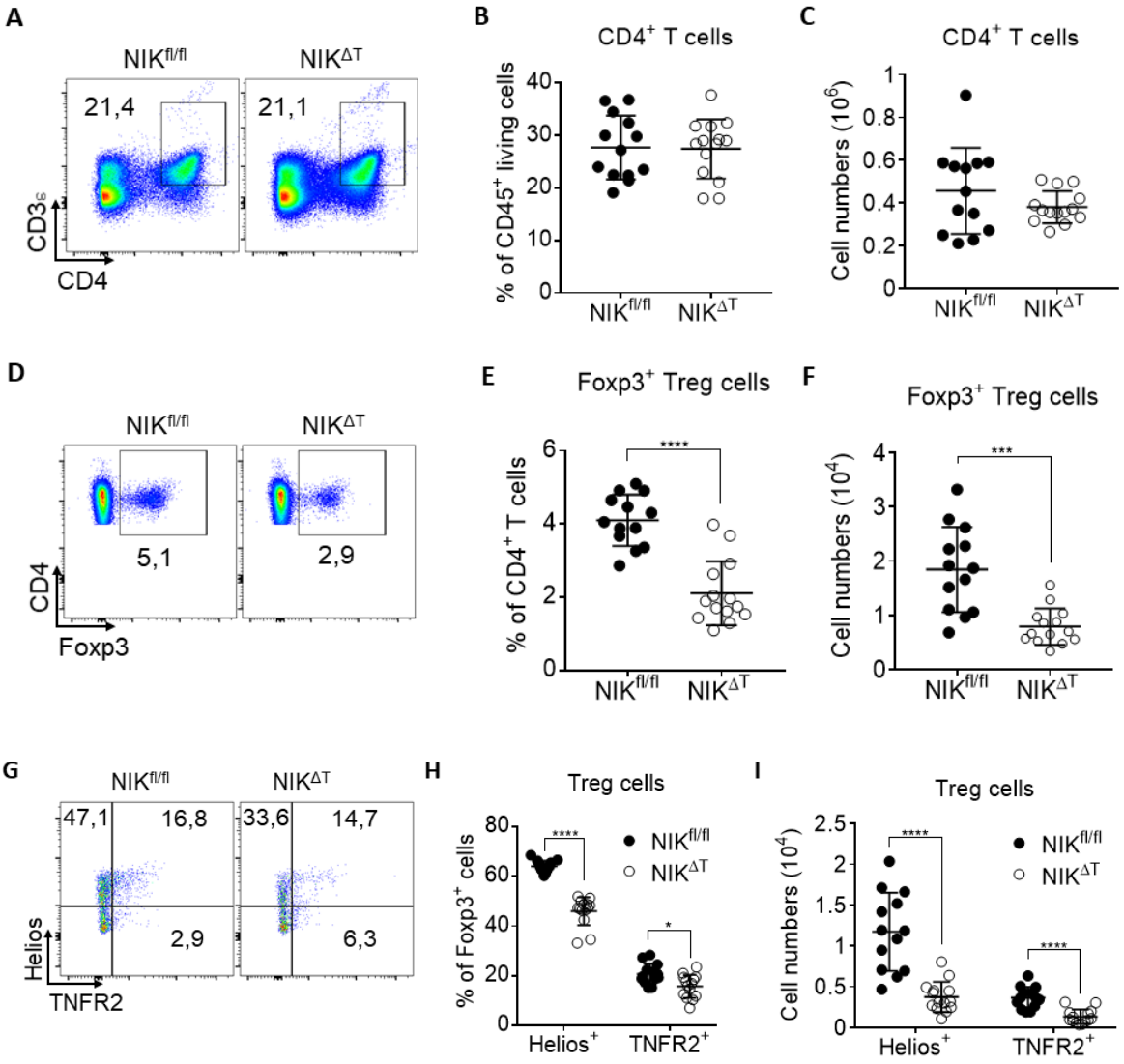

Figure 15: Reduction of Treg cells and their subtypes in the liver of mice with conditional deletion of NIK in αβ T cells. The liver of 6-12-week-old perfused steady-state mice of the NIK$^{\Delta T}$ strain and littermate controls were isolated and single cell suspensions of CD45$^+$ cells generated. Flow cytometric analysis revealed (**A, B**) the percentage and (**C**) cell numbers of CD4$^+$ T cells. CD4$^+$ T cells are pre-gated on lymphocytes, single cells and CD45$^+$ living cells. (**D, E**) show the percentage and (**F**) cell numbers of Foxp3$^+$ Treg cells. (**G, H**) display percentages and (**I**) cell numbers of Helios$^+$ and TNFR2$^+$ Treg cells. Biological replicates with n ≥ 13 are represented as mean ± SD with *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001 and were pooled from three independent experiments. Statistical analysis was performed using (**B, C, F, I**) unpaired two-sided Student's t-test and (**E, H**) Mann-Whitney test.

In summary, the deletion of NIK in all αβ T cells using the CD4-Cre results in a decrease of Foxp3$^+$ Treg cells their subtypes in secondary lymphoid organs and tissues such as the colon and the liver. Keeping in mind the importance of NIK for the maintenance of thymic TregP and reduced thymic Treg cell output in NIK$^{\Delta T}$ mice, the next chapter will elucidate to what extent these findings are cell intrinsic.

3.4 No reduction of Foxp3$^+$ Treg cells and their subtypes in secondary lymphoid organs upon deletion of NIK in Foxp3$^+$ Treg cells

In order to investigate whether the above found reduction of Foxp3$^+$ Treg cells in lymphoid organs and peripheral tissues in NIK$^{\Delta T}$ mice is Treg cell-intrinsic, a mouse strain, in which NIK is conditionally deleted in Foxp3$^+$ Treg cells (NIK$^{\Delta Treg}$), was generated by crossing NIK$^{fl/fl}$ with Foxp3^{Cre} R26^{RFP}. The R26-RFP reporter allows for assessment of recombination efficiency of the Foxp3-Cre and for lineage tracing experiments. Of note, the NIK$^{\Delta Treg}$ strain was very sensitive towards germline deletion that resulted in very few offsprings that could be used for experiments. Hence, mice subjected to following experiments using the NIK$^{\Delta Treg}$ strain could not always be age- and gender-matched in order to ensure large enough sample sizes. Yet, at least one littermate and gender-matched control was present in each experiment. Moreover, 8-18-week-old mice that were subjected to the following experiments looked phenotypically healthy and overall litter sizes were normal. The R26-RFP reporter revealed that more than 80 % of Foxp3$^+$ Treg cells were indeed RFP-positive as assessed in inguinal lymph nodes (**Figure 16A, B**) and the spleen (**Figure 16C**). Floxed alleles of NIK were confirmed in NIK$^{\Delta Treg}$ but not control mice by PCR, so that a deletion of NIK in more than 80 % of Treg cells can be assumed. Around 10% of CD4$^+$ T cells were positive for RFP, hence Treg cells (**Figure 16A**). As defined earlier, the percentages of Foxp3$^+$ Treg cells in the periphery of steady state WT mice usually range from around 7 % to up to 15 % (Wing *et al.*, 2008). Hence, these data confirm that Foxp3-Cre recombination in Foxp3$^+$ Treg cells in the NIK$^{\Delta Treg}$ strain and Foxp3-Cre-positive control mice is comparable to previously published data.

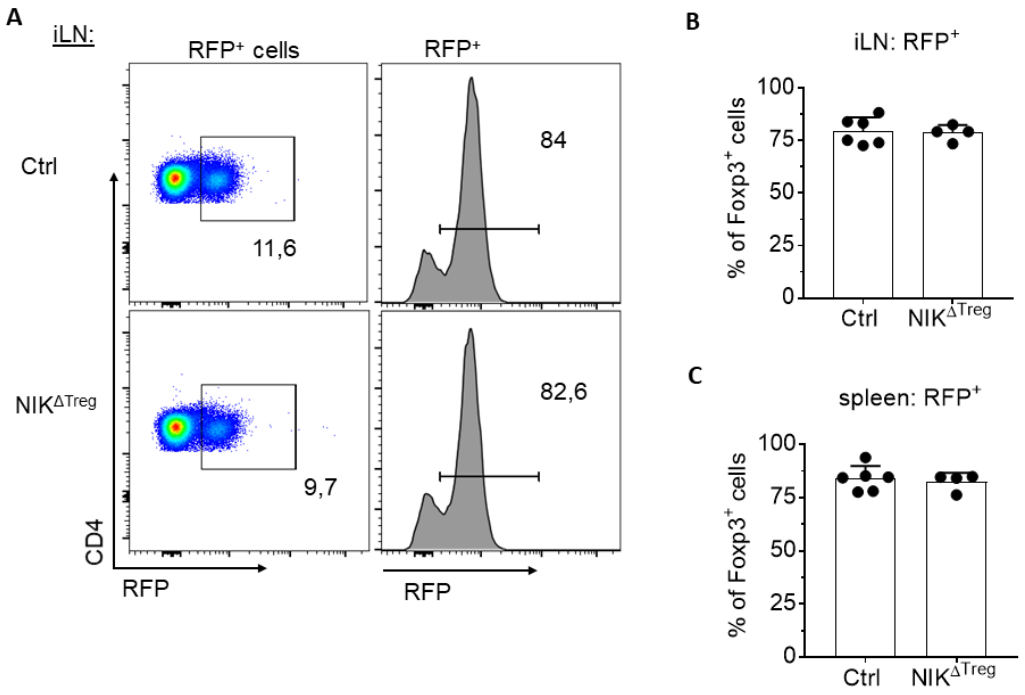

Figure 16: Recombination efficiency of the Foxp3-Cre. Mice carrying a R26-RFP were crossed with mice of the Foxp3-Cre strain, generating the strain Foxp3^{Cre} R26^{RFP}, that were in addition to NIK$^{fl/wt}$ Foxp3^{Cre} R26^{RFP} used as control (ctrl) mice for the NIK$^{\Delta Treg}$ strain. Crossing the Foxp3^{Cre} R26^{RFP} strain with the NIK$^{fl/fl}$ strain generated mice, in which NIK is conditionally deleted in Foxp3^{+} Treg cells (NIK$^{\Delta Treg}$). Single cell suspensions from inguinal lymph nodes (iLN) and the spleen were generated and the expression of a RFP reporter assessed by flow cytometry. (**A**) Example plot of the percentages of RFP^{+} cells among Foxp3^{+} Treg cells for NIK$^{\Delta Treg}$ and control mice from iLN. The percentages of RFP^{+} cells among Foxp3^{+} Treg cells are displayed for (**B**) iLN and (**C**) the spleen. Data in (**B**, **C**) represent the mean ± SD of n ≥ 4, pooled from two independent experiments. Statistical analysis was performed using (**B, C**) Mann-Whitney test.

Next, we assessed the percentages of Foxp3^{+} Treg cells in secondary lymphoid organs of steady state NIK$^{\Delta Treg}$ and control mice. While we detected reduced frequencies of Foxp3^{+} Treg cells in lymphoid organs in the NIK$^{\Delta T}$ strain as outlined in previous chapters, in the NIK$^{\Delta Treg}$ strain, the percentages of Foxp3^{+} Treg cells were similar between the two groups in the spleen, inguinal lymph nodes, Peyer's patches and mLN (**Figure 17A, B**). Moreover, cell numbers of Foxp3^{+} Treg cells from the NIK$^{\Delta Treg}$ strain were not changed for spleen, inguinal lymph nodes (iLN), Peyer's patches (PP) and mLN (**Figure 17C**). These results show that Foxp3^{+} Treg cells do not cell-intrinsically require NIK in order to maintain their homeostasis in peripheral lymphoid organs under steady-state conditions.

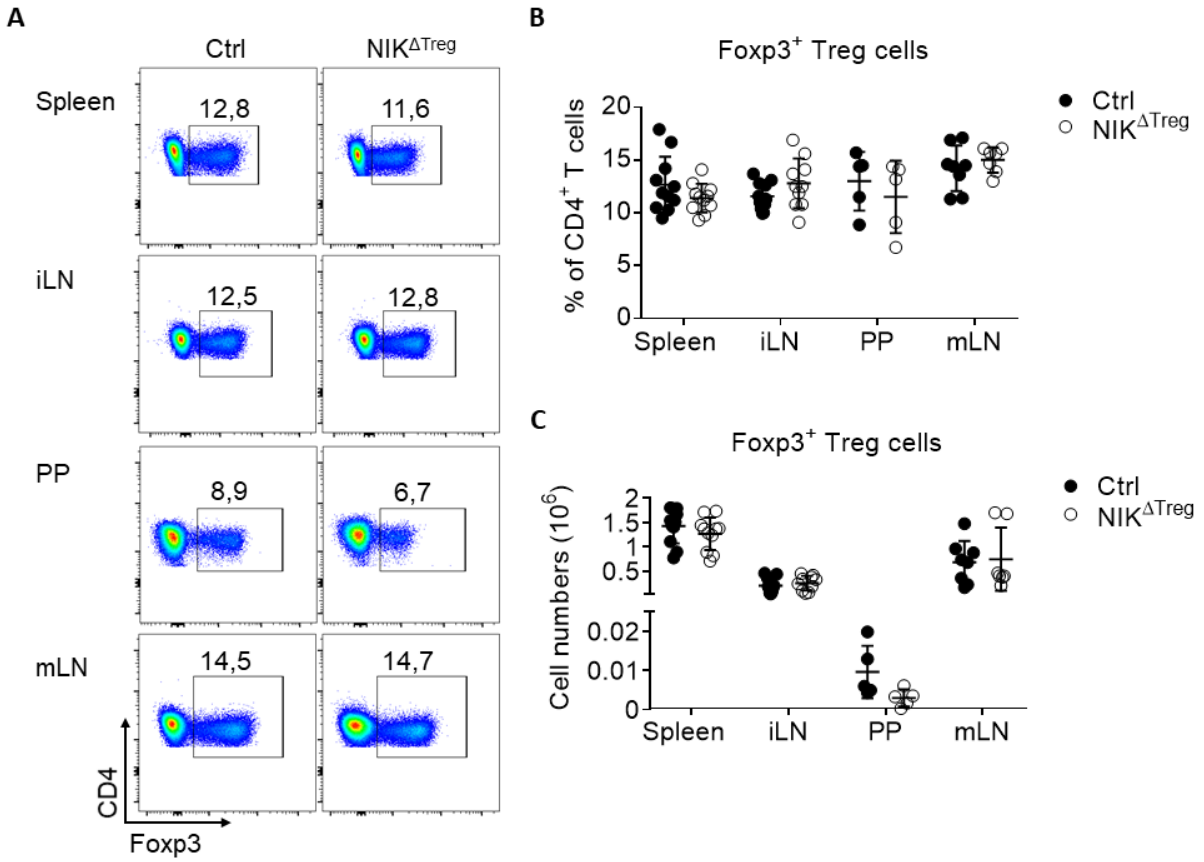

Figure 17: No cell-intrinsic requirement for NIK in maintaining the homeostasis of peripheral Foxp3⁺ Treg cells in secondary lymphoid organs in steady state. Single cell suspensions from the spleen, inguinal lymph nodes, Peyer's patches and mLN of 8-18-week-old NIK$^{\Delta Treg}$ and control (ctrl) mice were generated and analysed by flow cytometry. (**A**) Example plot of Foxp3⁺ Treg cells. (**B**) represents the percentage of Foxp3⁺ Treg cells of CD4⁺ T cells. (**C**) cell numbers of Foxp3⁺ Treg cells are displayed. Foxp3⁺ Treg cells were pre-gated on lymphocytes, single cells, living cells and, eventually, CD4⁺ T cells. Data in (**B, C**) represent the mean ± SD of n ≥ 5, pooled from three independent experiments. Statistical analysis was performed using (**B, C**) unpaired two-sided Student's t-test.

While the deletion of NIK in all αβ T cells (NIK$^{\Delta T}$), see section 3.2, resulted in a reduction of Helios⁺ Treg cells in mLN, it was analysed whether the Helios⁺ Treg cell subtype was reduced in the NIK$^{\Delta Treg}$ strain in comparison to control mice as well. Interestingly, the percentages of Helios⁺ and RORγt⁺ Treg cells were similar in mLN between the NIK$^{\Delta Treg}$ strain and control mice (**Figure 18A, B**). The same could be observed for absolute Treg cell numbers of Helios⁺ and RORγt⁺ Treg cells (**Figure 18C**). Hence, these data provide evidence that NIK is also dispensable for the maintenance of Helios⁺ and RORγt⁺ Treg cells in the periphery under steady state homeostasis.

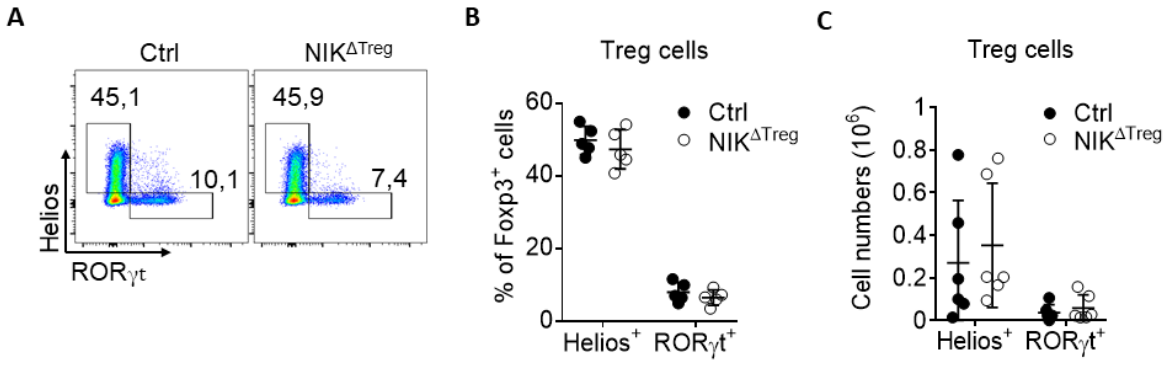

Figure 18: No reduction of Helios$^+$ Treg cells in mesenteric lymph nodes in steady state mice with conditional deletion of NIK in Foxp3$^+$ Treg cells. mLN were obtained from 8-18-week-old NIK$^{\Delta Treg}$ and control mice and single cell suspensions generated. FACS analysis revealed similar (**A, B**) percentages and (**C**) cell numbers of Helios$^+$ as well as RORγt$^+$ Treg cells between NIK$^{\Delta Treg}$ and control mice. Helios$^+$ and RORγt$^+$ Treg cells were pre-gated on lymphocytes, single cells, living cells, CD4$^+$ T cells and Foxp3$^+$ Treg cells. Data in (**B, C**) represent the mean ± SD of n ≥ 5, pooled from three independent experiments. Statistical analysis was performed using (**C**) unpaired two-sided Student's t-test and (**B**) Mann-Whitney test.

Upon activation, Treg cells further differentiate from naïve into effector Treg cells and exert their suppressive functions (Dias *et al.*, 2017). Since mice of the NIK$^{\Delta Treg}$ strain of up to 18 weeks of age did not show any signs of autoimmunity and appeared phenotypically healthy, we examined whether NIK-deficiency had indeed any impact on the capacity of Foxp3$^+$ Treg cells to differentiate into effector Treg cells. Therefore, Foxp3$^+$ Treg cells from single cell suspensions from inguinal lymph nodes and the spleen were analysed by flow cytometry for the expression of surface markers CD62L and CD44. In inguinal lymph nodes under steady state conditions, differences between naïve and effector Treg cells could be observed neither in terms of frequency (**Figure 19A, B**) nor absolute cell numbers between NIK$^{\Delta Treg}$ and control mice (**Figure 19C**). In the spleen, frequencies of naïve and effector Treg cells (**Figure 19D**) as well as cell numbers (**Figure 19E**) were similar between the two groups. Hence, it can be concluded that NIK is not required for the differentiation of Foxp3$^+$ Treg cells into effector Treg cells in the periphery in steady state.

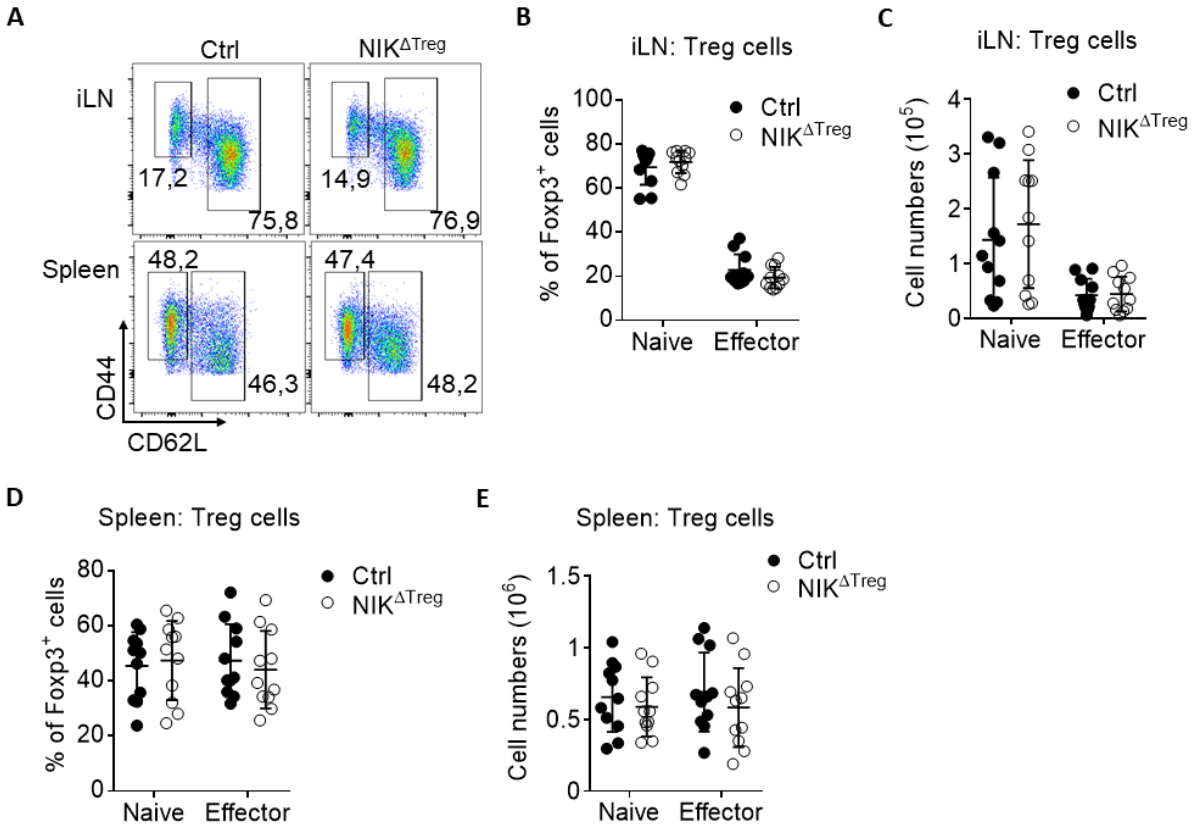

Figure 19: No requirement for NIK in the generation of peripheral effector Treg cells in steady state. Single cell suspensions from the spleen and inguinal lymph nodes of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were obtained and analysed by flow cytometry. (**A**) Example plots of naïve (CD62L^{+}) vs effector (CD44^{high}) Treg cells from inguinal lymph nodes and the spleen of NIK$^{\Delta Treg}$ and control mice. (**B**) Percentages and (**C**) cell numbers of naïve as well as effector Treg cells are displayed for inguinal lymph nodes. (**D**) shows percentages while (**E**) shows cell numbers of naïve and effector Treg cells in the spleen. Naïve vs effector Treg cells were pre-gated on lymphocytes, single cells, living cells, CD4^{+} T cells and, finally, Foxp3^{+} Treg cells. Data represent the mean ± SD of n = 11, pooled from three independent experiments. Statistical analysis was performed using (**B**: Effector**, C, E**) unpaired two-sided Student's t-test and (**B**: Naive**, D**) Mann-Whitney test.

As previously elucidated in chapter 1.1, Treg cells are responsible for the restriction of an immune response by suppressing, amongst others, T cells (Shevach, 2018). Hence, further information on the suppressive capacity of Treg cells can be obtained by looking at the activation of Tconv cells. Thus, we generated splenic single cell suspensions and analysed Tconv cell subpopulations by flow cytometry. Expectedly, the percentage of CD4^{+} and CD8^{+} T cells in the spleen did not differ between NIK$^{\Delta Treg}$ and control mice (**Figure 20A, B**). Furthermore, percentages of naïve and effector CD4^{+} T cells were similar between groups (**Figure 20C, D**) along with comparable frequencies of IFNγ-producing CD4^{+} and CD8^{+} T cells in NIK$^{\Delta Treg}$ and control mice (**Figure 20E, F**). To summarise, these data indicate that NIK is dispensable for Foxp3^{+} Treg cells in maintaining peripheral homeostasis under steady state conditions.

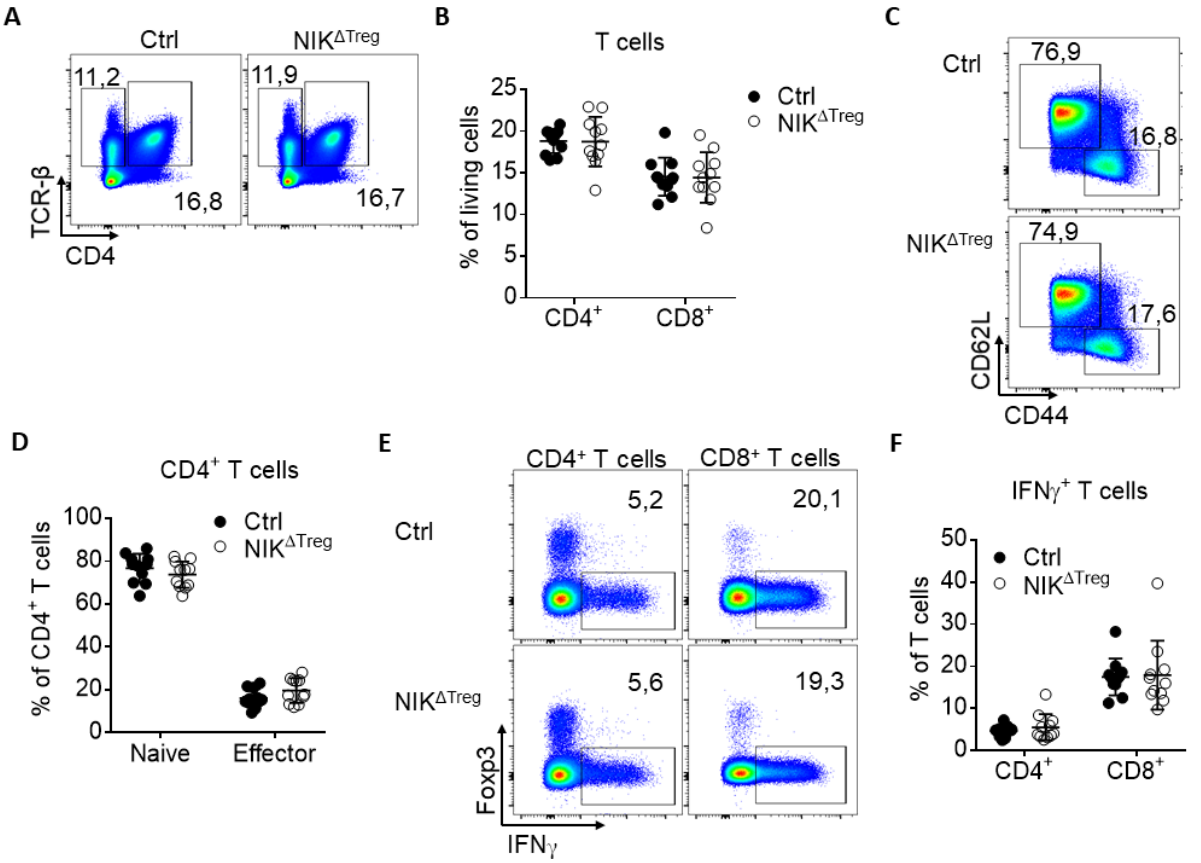

Figure 20: No activation of peripheral conventional T cells in steady state mice with conditional deletion of NIK in Foxp3$^+$ Treg cells. Spleens of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were obtained and single cell suspensions generated for flow cytometric analysis. This figure shows (**A**) example plots and (**B**) percentages of CD4$^+$ and CD8$^+$ T cells (TCRβ$^+$, CD4$^-$). (**C**) gating example and (**D**) percentages of naïve (CD62L$^+$, CD44^{low}) vs Teff cells (CD62L$^-$, CD44^{high}) are displayed. An (**E**) example plot and (**F**) percentages of IFNγ-producing Tconv cells are shown. CD4$^+$ T cells were pre-gated on lymphocytes, single cells and living cells. Data represent the mean ± SD of n = 11, pooled from three independent experiments. Statistical analysis was performed using (**B, D, F**) Mann-Whitney test.

As mentioned earlier in section 1.1, Foxp3$^+$ Treg cells are critical in maintaining immune homeostasis through suppression of immune cell expansion. Thus, further immune cell subtypes that are involved in inflammatory processes were analysed by flow cytometry. Yet, looking at B cells, identified by surface marker CD19, revealed similar frequencies of this cell type in the spleens of NIK$^{\Delta Treg}$ and control mice (**Figure 21A, B**). Since activated B cells have been associated with increased serum IgA levels and autoimmunity (Li *et al.*, 2019), serum IgA levels of NIK$^{\Delta Treg}$ and control mice were assessed by ELISA. Unsurprisingly, NIK$^{\Delta Treg}$ mice did not show elevated IgA levels in comparison to control mice (**Figure 21C**). Furthermore, the percentages of neutrophils and inflammatory monocytes were found to be similar between the two groups (**Figure 21D, E**). Taken together, these data provide further evidence that NIK in Treg cells is not required for the prevention of immune cell activation in steady state mice of up to 18 weeks of age.

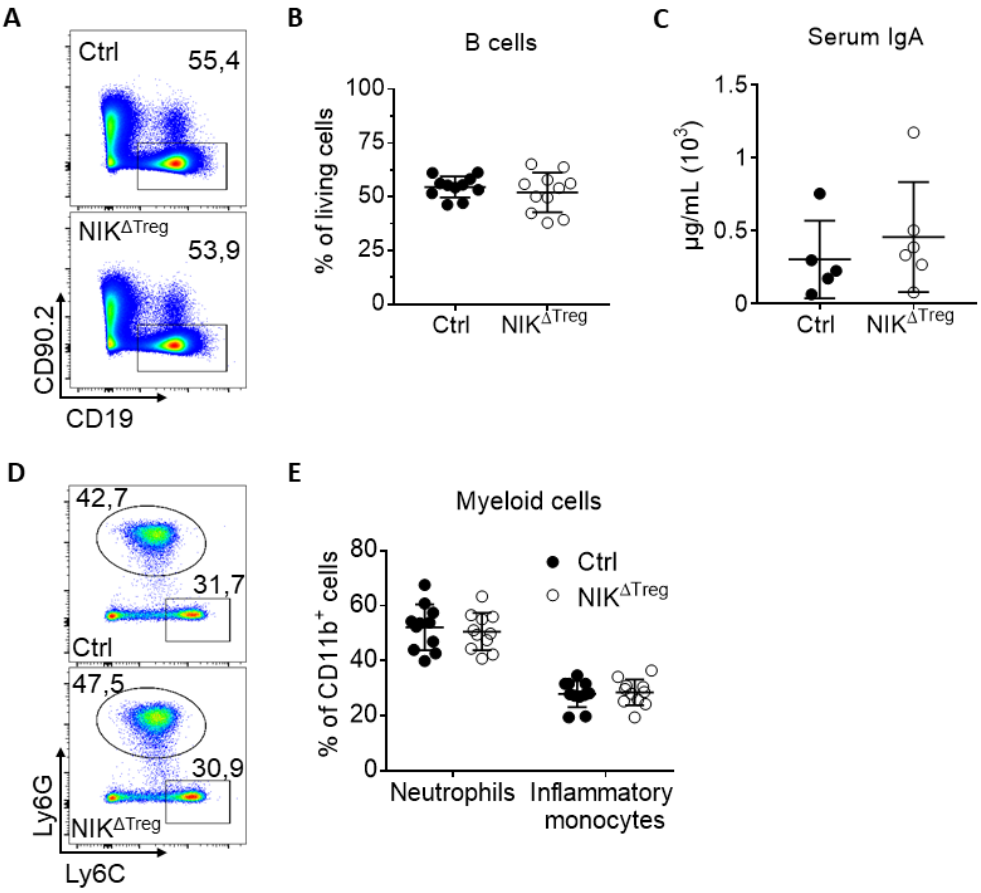

Figure 21: No impairment of the homeostasis of peripheral B cells and myeloid cells in steady state mice with deletion of NIK in Foxp3⁺ Treg cells. Single cell suspensions from spleens of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and B cells, neutrophils as well as inflammatory monocytes analysed by flow cytometry. B cells were pre-gated on lymphocytes, single cells and living cells while myeloid cells were pre-gated on lymphocytes, single cells, living cells, CD90.2$^-$/CD19$^-$ cells and, eventually, CD11b$^+$ cells. (**A, B**) Percentages of B cells as well as (**D, E**) percentages of neutrophils (Ly6G^{high}) and inflammatory monocytes (Ly6C^{high}) are shown. Serum of 8-18-week-old NIK$^{\Delta Treg}$ and control mice was obtained and (**C**) IgA levels determined by ELISA. Data represent the mean ± SD of n = 11 and were pooled from (**A, B, D, E**) three independent experiments or represent the mean ± SD of n ≥ 5 from (**C**) one experiment. Statistical analysis was performed using (**B, C, E**) Mann-Whitney test.

Collectively, the above shown data provide evidence that conditional deletion of NIK in Foxp3$^+$ Treg cells does not affect Treg cell maintenance and suppressive function in the periphery under steady state conditions.

3.5 Reduction of Foxp3$^+$ Treg cells and their subtypes in peripheral tissues upon deletion of NIK in Foxp3$^+$ Treg cells

To examine whether the previously identified reduction of Treg cells in the tissues of NIK$^{\Delta T}$ mice described in chapter 3.2 was Treg cell-intrinsic, the colon as well as the liver of NIK$^{\Delta Treg}$ and control mice were analysed. First, the recombination efficiency of the Foxp3-Cre in the colon was assessed.

Comparable to results from the periphery shown in section 3.4, close to 90 % of Foxp3$^+$ Treg cells were found to express RFP (**Figure 22A, B**). Hence, it can be assumed that NIK was deleted in around 90 % of Foxp3$^+$ Treg cells in the colon of NIK$^{\Delta Treg}$ mice.

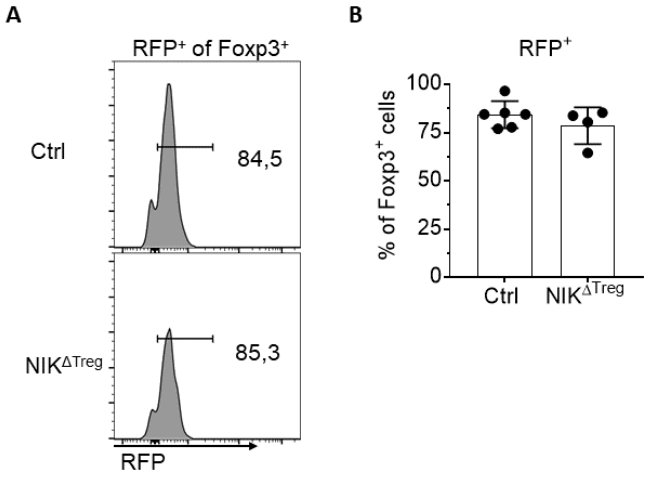

Figure 22: Recombination efficiency of the Foxp3-Cre in Foxp3$^+$ cells in the colon. Single cell suspensions from the colonic lamina propria were generated and the expression of the RFP reporter assessed by flow cytometry. (**A**) Example plot of the percentages of RFP$^+$ cells among Foxp3$^+$ Treg cells for NIK$^{\Delta Treg}$ and control mice from the lamina propria of the colon. (**B**) Percentages of RFP$^+$ cells among Foxp3$^+$ Treg cells. Data in (**B**) represent the mean ± SD of n ≥ 4, pooled from two independent experiments. Statistical analysis was performed using (**B**) Mann-Whitney test.

While no reduction of Foxp3$^+$ Treg cells could be detected in secondary lymphoid organs of NIK$^{\Delta Treg}$ mice, strikingly, we detected a significant decrease in percentage (**Figure 23A, B**) as well as cell numbers (**Figure 23C**) of Foxp3$^+$ Treg cells in the colon of NIK$^{\Delta Treg}$ in comparison to control mice. As described earlier in chapter 1.3, colonic Treg cell subtypes comprise thymus-derived Helios$^+$ Treg cells as well as microbiota-induced RORγt$^+$ Treg cells. In line with previous findings showing that NIK$^{\Delta T}$ mice harbour a reduction in the Helios$^+$ Treg cell compartment, a decrease in frequencies (**Figure 23D, E**) and cell numbers (**Figure 23F**) of Helios$^+$ Treg cells could also be detected in the colon of NIK$^{\Delta Treg}$ mice. Again, the RORγt$^+$ Treg cell fraction as well as the fraction negative for both, Helios and RORγt, remained unaffected (**Figure 23D-F**). In summary, these data confirm that NIK is cell-intrinsically required for the maintenance of Foxp3$^+$ Treg cells and, in particular, for thymus-derived Helios$^+$ Treg cells in the large intestine.

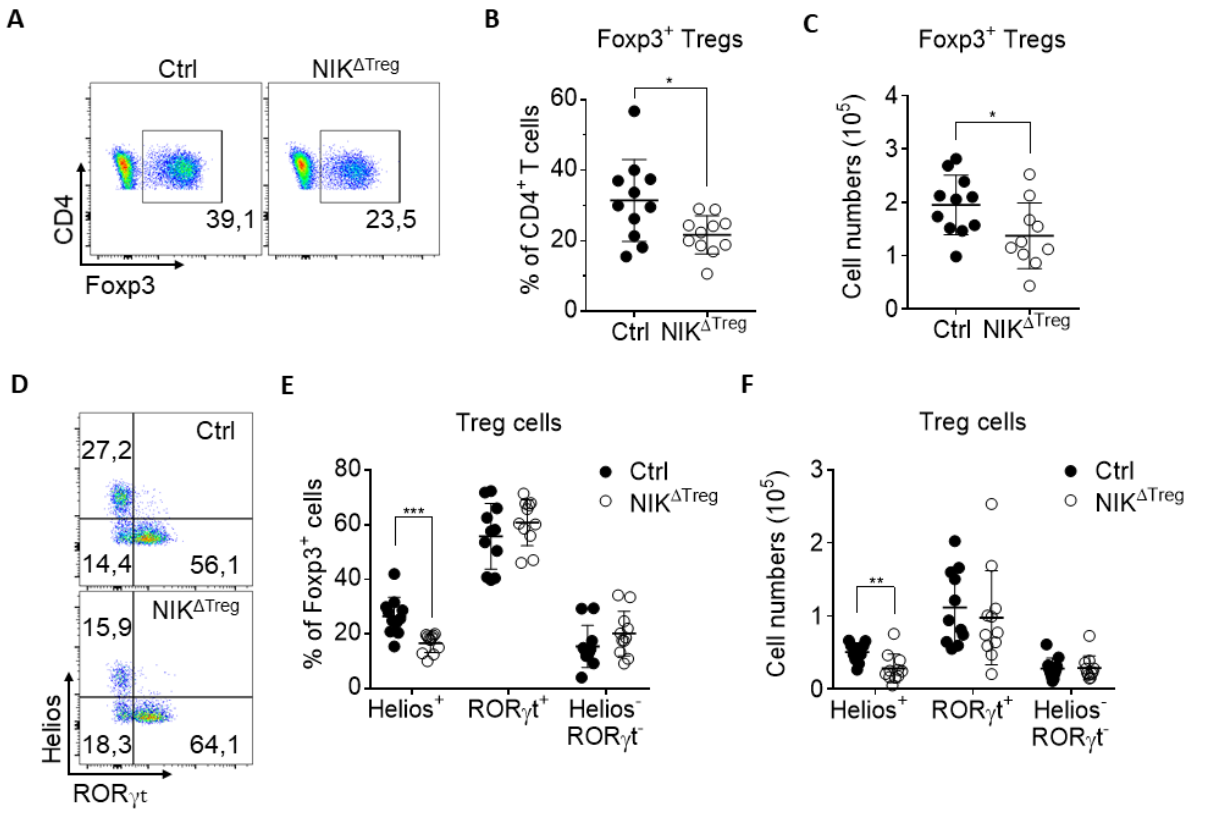

Figure 23: Reduction of Foxp3$^+$ and Helios$^+$ Treg cells in the colon of mice with conditional deletion of NIK in Foxp3$^+$ Treg cells. Single cell suspensions from the lamina propria of the colon of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and Treg cells as well as Treg cell subtypes analysed by flow cytometry. Treg cells were pre-gated on lymphocytes, single cells, CD45$^+$ living cells and, eventually, CD4$^+$ T cells. (**A, B**) Percentages as well as absolute (**C**) cell numbers of Treg cells are shown. (**D, E**) Percentages and (**F**) cell numbers of Helios$^+$, RORγt$^+$ and Helios$^-$ RORγt$^-$ Treg cell subtypes are displayed. Data represent mean ± SD of n = 11 with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and were pooled from three independent experiments. Statistical analysis was performed using (**B, C, E**: Helios$^+$, **F**) unpaired two-sided Student's t-test or (**E**: RORγt$^+$, Helios$^-$ RORγt$^-$) Mann-Whitney test.

Next, based on the reduction of Foxp3$^+$ Treg cells seen in the colon, the expansion of other immune cell populations in the colon was examined. Under steady-state conditions, approximately 20 % of Ly6C^{high} monocytes and around 2 % of Ly6G^{high} neutrophils constitute CD11b$^+$ cells in the colonic lamina propria (Bain *et al.*, 2013; Sullivan *et al.*, 2018). The percentages of Ly6G^{high} neutrophils and Ly6C^{high} monocytes did not differ between NIK$^{\Delta Treg}$ and control mice (**Figure 24A, B**). In line with these findings, we observed that the overall percentages of CD4$^+$ T cells were equal between the two groups (**Figure 24C, D**). However, an increase in frequencies of interferon-γ (IFN-γ)-producing CD4$^+$ T cells could be detected (**Figure 24E, F**). In contrast, merely a tendency towards elevated overall IFNγ-producing CD4$^+$ T cell counts could be observed (**Figure 24G**). Principally, morphological differences of colons between NIK$^{\Delta Treg}$ and control mice assessed by colon length and thickness could not be identified (**Figure 24H**), indicating no spontaneous onset of inflammatory bowel diseases in NIK$^{\Delta Treg}$ mice of 8 to 18 weeks of age.

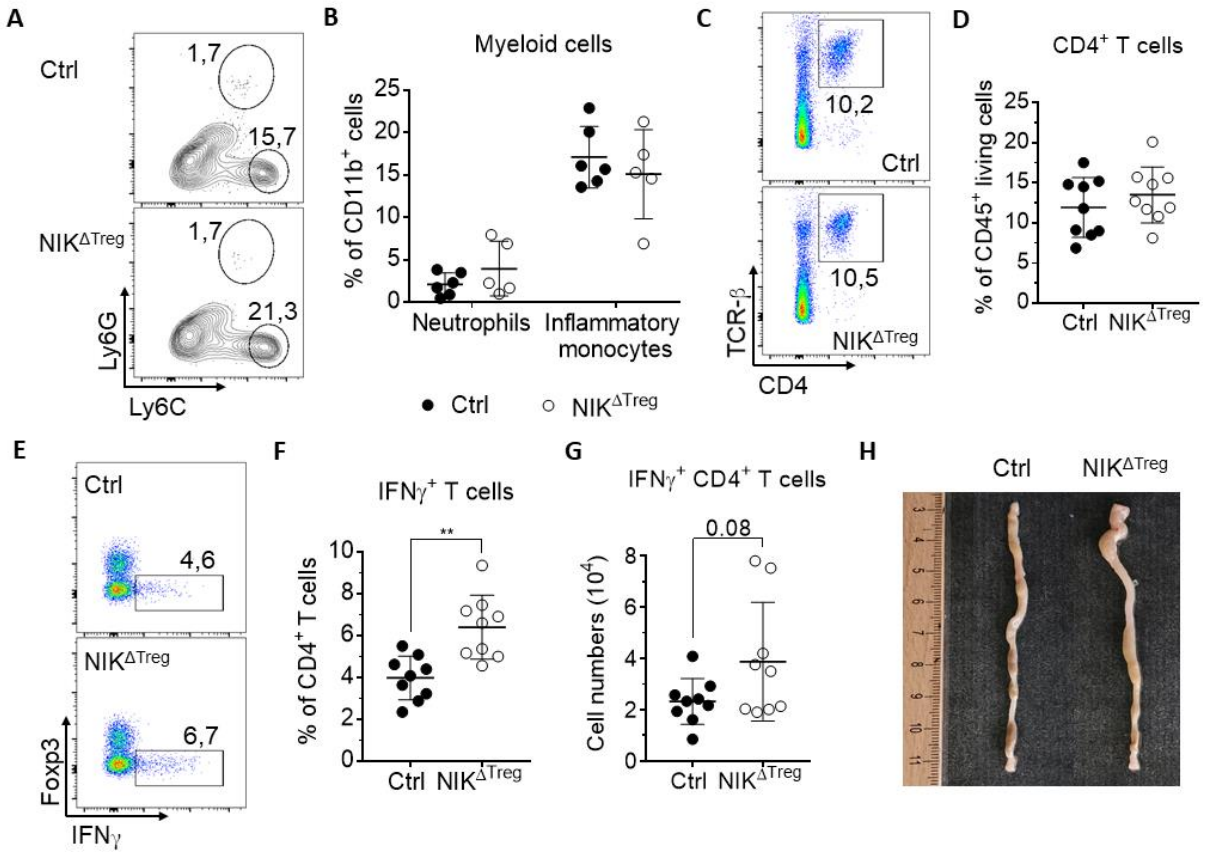

Figure 24: Increase in IFNγ-producing CD4⁺ T cells in the colon of mice with conditional deletion of NIK in Foxp3⁺ Treg cells. Single cell suspensions from the lamina propria of the colon of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and T cells as well as myeloid cells analysed by flow cytometry. T cells were pre-gated on lymphocytes, single cells, CD45$^+$ living cells while myeloid cells were pre-gated on lymphocytes, single cells, CD45$^+$ living cells and, last, CD11b$^+$ cells. (**A, B**) Percentages and (**C**) cell numbers of neutrophils (Ly6G^{high}) and inflammatory monocytes (Ly6C^{high}). (**C, D**) Percentages of CD4$^+$ T cells. (**E, F**) Percentages and (**G**) cell numbers of IFNγ-producing CD4$^+$ T cells. (**H**) Example images of colons of control and NIK$^{\Delta Treg}$ mice. Data represent mean ± SD of n = 11 with **p ≤ 0.01 and were pooled from three independent experiments. Statistical analysis was performed using (**B, C, E**: Helios$^+$**, F**) unpaired two-sided Student's t-test or (**E**: RORγt$^+$, Helios$^-$ RORγt$^-$) Mann-Whitney test.

In particular, within peripheral tissues, the tTreg cell fraction has been associated with non-canonical Treg cell functions such as maintaining tissue homeostasis, regeneration and repair (Panduro, Benoist and Mathis, 2016). Subsequently, colons of NIK$^{\Delta Treg}$ and control mice were examined for histopathological changes by H&E stainings. Proximal colonic sections were characterised by no signs of colitis along with no signs of inflammation, no mucosal destruction comprising no crypt destruction and no goblet cell depletion in both groups (**Figure 25A, B**).

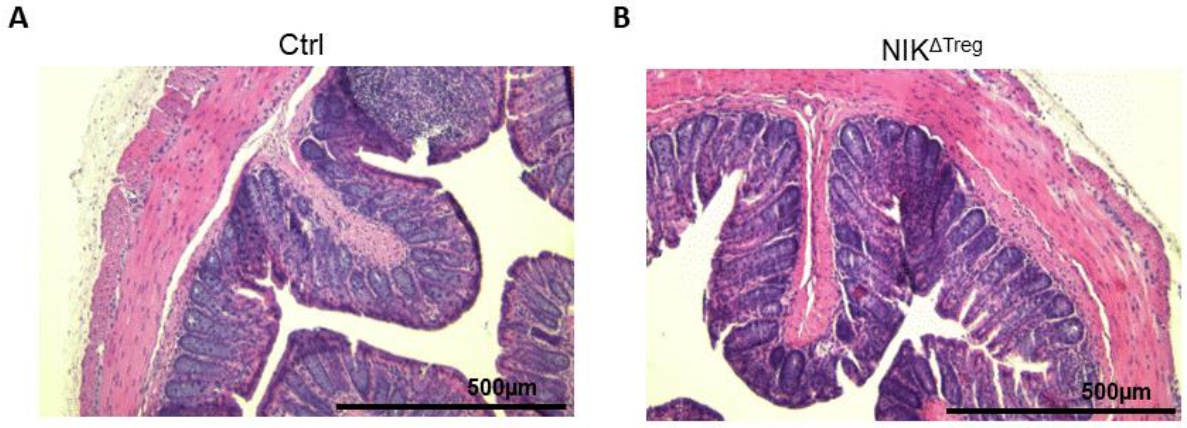

Figure 25: No histopathological differences between colons of NIK$^{\Delta Treg}$ and control mice. Colons of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were obtained. Proximal sections were embedded into paraffin and stained with H&E. Representative H&E stained sections of (**A**) control and (**B**) NIK$^{\Delta Treg}$ mice. Bars indicate 500 µm.

Taken together, our data provide evidence that tTreg cells do not require NIK for the maintenance of tissue homeostasis, regeneration and repair in steady state. Mice of 8 to 18 weeks of age, despite a reduction of Foxp3$^+$ and Helios$^+$ Treg cells in the colonic lamina propria of the NIK$^{\Delta Treg}$ strain, do not develop spontaneous colonic inflammation, which demonstrates that Treg cell-specific deletion of NIK does not affect overall Treg cell function in steady state.

In order to investigate whether Treg cell-specific deletion of NIK affects only colonic tissue-resident Treg cells, also the liver of NIK$^{\Delta Treg}$ and control mice was analysed. First, the recombination efficiency of the Foxp3-Cre in immune cells of the liver was assessed and showed that RFP was expressed in close to 90 % of Foxp3$^+$ cells in both NIK$^{\Delta Treg}$ and control mice (**Figure 26A, B**). Hence, it can be assumed that NIK was deleted in close to 90 % of Treg cells in NIK$^{\Delta Treg}$ mice in the liver. Strikingly, the frequencies of Foxp3$^+$ Treg cells were found to be reduced in the liver as well (**Figure 26C, D**). However, Treg cell-specific deletion of NIK did not affect the percentages of CD62L$^+$ naive and CD44^{high} effector Treg cells (**Figure 26E, F**).

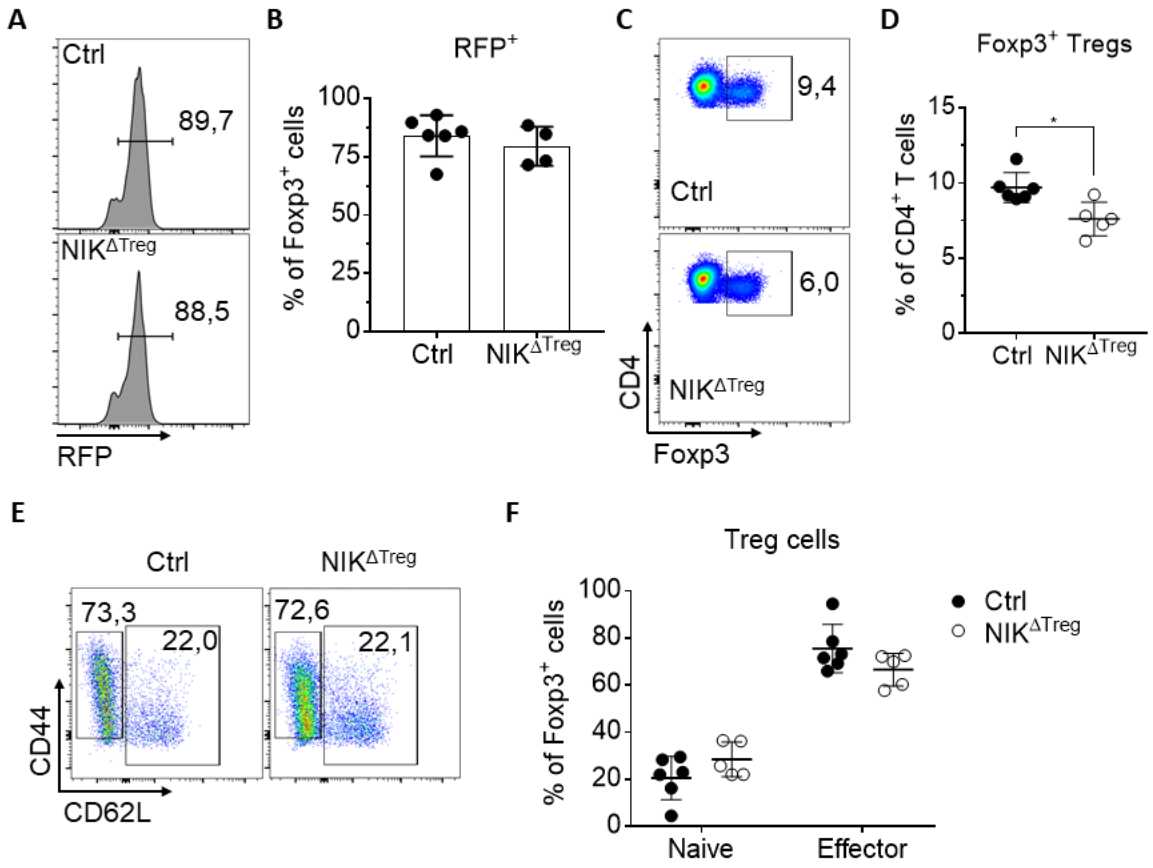

Figure 26: Decreased percentages of Treg cells in the liver of mice with deletion of NIK in Foxp3⁺ Treg cells. Single cell suspensions from the liver of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and Treg cells analysed by flow cytometry. Treg cells were pre-gated on lymphocytes, single cells, CD45⁺ living cells and CD4⁺ T cells. (**A, B**) Percentages of RFP⁺ cells among Foxp3⁺ Treg cells. (**C, D**) Percentages of Foxp3⁺ Treg cells. (**E, F**) Percentages of naive and effector Treg cells. Data represent mean ± SD of n ≥ 4 with *p ≤ 0.05 and were pooled from two independent experiments. Statistical analysis was performed using (**B, D, F**) Mann-Whitney test.

Since a reduction in percentages of Foxp3⁺ Treg cells could be detected, the liver of both NIK$^{\Delta Treg}$ and control mice were examined for histopathological changes by H&E stainings. Yet, H&E stainings revealed no signs of necrosis, no signs of liver fibrosis and, hence, no signs of hepatitis neither in the NIK$^{\Delta Treg}$ strain nor control mice (**Figure 27A, B**).

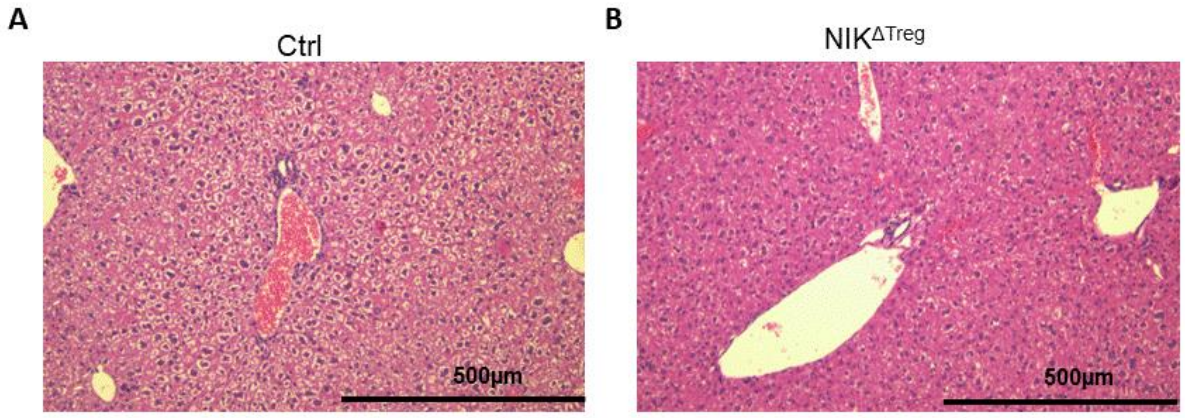

Figure 27: No histopathological differences between livers of NIK$^{\Delta Treg}$ and control mice. The liver of 8-18-week-old NIK$^{\Delta Treg}$ and control mice was retrieved. Lobes were embedded into paraffin and stained with H&E. Representative H&E stained sections of (**A**) control and (**B**) NIK$^{\Delta Treg}$ mice. Bars indicate 500 µm.

Collectively, these data suggest that NIK plays a crucial role for the maintenance of Foxp3$^+$ as well as Helios$^+$ Treg cells in tissues, such as the colon and the liver, that results in a decrease of these cells but is dispensable for the function of Foxp3$^+$ Treg cells and therefore tissue homeostasis as assessed by the effector state of Treg cells and confirmed by morphologically and histopathologically healthy tissues of NIK$^{\Delta Treg}$ mice.

3.6 No requirement for NIK in the maintenance of Foxp3 expression

Since our data provide evidence that NIK expression is required for the maintenance of tissue Treg cells, it was examined whether the reduction of NIK-deficient Treg cells was due to the downregulation of Foxp3. This was investigated in the NIK$^{\Delta Treg}$ strain by making use of a RFP reporter. Upon Foxp3-Cre activation, the RFP reporter is expressed in Foxp3$^+$ Treg cells and the signal is maintained within the Treg cell as long as it is alive. All RFP-positive cells where therefore analysed for Foxp3 expression by flow cytometry to assess whether Treg cells downregulate Foxp3 and lose their Treg identity. We found that colonic RFP-positive cells expressed similar percentages of Foxp3 in control and NIK$^{\Delta Treg}$ mice (**Figure 28A, B**). Additionally, there was no reduction of Foxp3 expression in colonic NIK-deficient Treg cells as assessed by MFI (**Figure 28C**). Equally, NIK-deficient Treg cells from the liver showed neither a downregulation of Foxp3 (**Figure 28D, E**) nor a decrease in Foxp3 MFI (**Figure 28F**). Hence, our data confirm that NIK is not required for Treg cell stability and maintenance of Foxp3 expression.

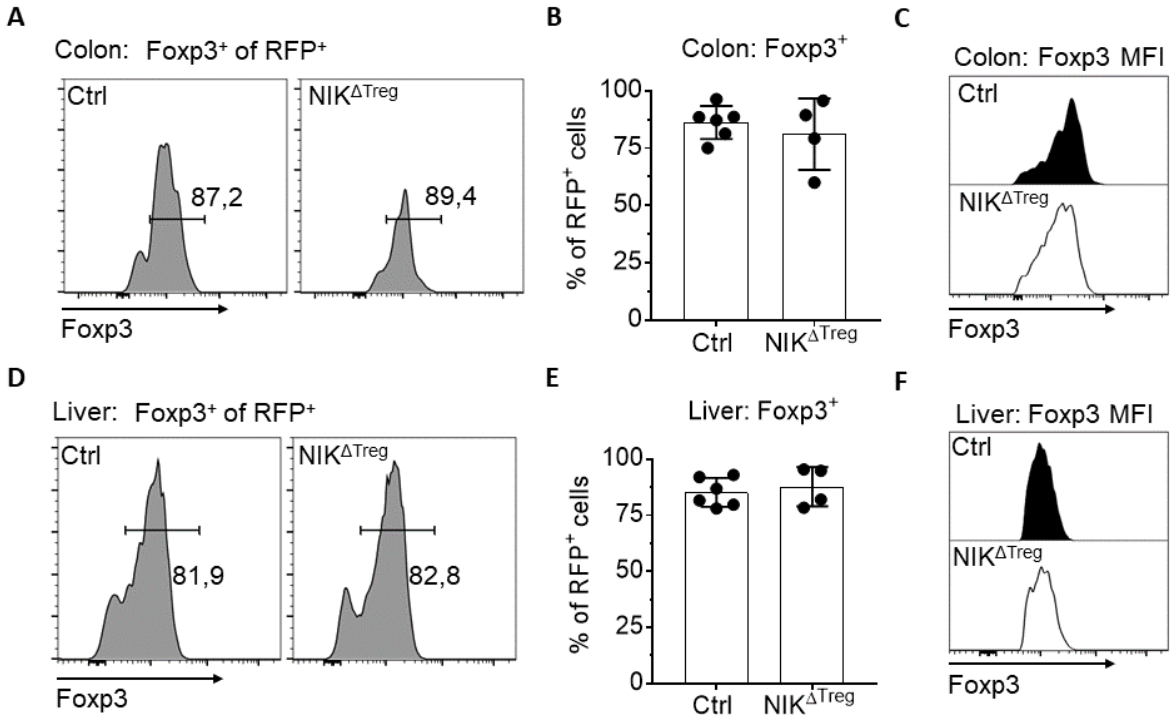

Figure 28: No Foxp3 downregulation upon NIK deficiency. Single cell suspensions from the colon and the liver of 8-18-week-old NIK$^{\Delta Treg}$ and control mice were generated and Treg cells analysed by flow cytometry. Treg cells were pre-gated on lymphocytes, single cells, CD45$^+$ living cells and CD4$^+$ T cells. (**A, B**) Percentages of Foxp3$^+$ Treg cells of RFP$^+$ cells in the colon. (**C**) Foxp3 MFI of colonic Foxp3$^+$ Treg cells. (**D, E**) Percentages of Foxp3$^+$ Treg cells of RFP$^+$ cells from the liver. (**F**) Foxp3 MFI of Foxp3$^+$ Treg cells from the liver. Data represent mean ± SD of n ≥ 4 and were pooled from two independent experiments. Statistical analysis was performed using (**B, E**) Mann-Whitney test.

It has been shown that IL-2 signalling through its receptor CD25 maintains Foxp3 expression and is required for the expansion of Foxp3$^+$ Treg cells in the periphery (Fontenot *et al.*, 2005). Hence, we analysed whether NIK-deficiency disturbs the homeostasis of CD25$^+$ Treg cells or CD25 expression on Treg cells. To do so, Treg cells from spleens were analysed by flow cytometry. Expectedly, no reduction in CD25$^+$ Treg cells in terms of frequencies (**Figure 29A, B**) and cell numbers (**Figure 29C**) could be observed in NIK$^{\Delta Treg}$ mice. In line with stable Foxp3 expression in NIK-deficient Treg cells, CD25 expression on Foxp3$^+$ Treg cells was found to be similar between control and NIK-deficient Treg cells (**Figure 29D**).

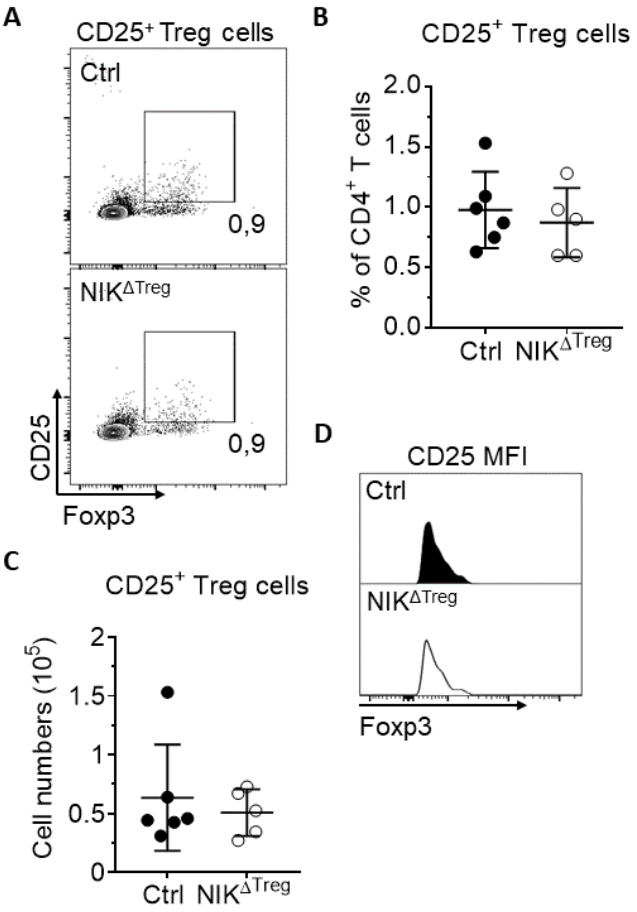

Figure 29: No requirement for NIK in maintenance of peripheral homeostasis of CD25⁺ Treg cells. Single cell suspensions from the spleen of 8-18-week-old $NIK^{\Delta Treg}$ and control mice were generated and Treg cells analysed by flow cytometry. Treg cells were pre-gated on lymphocytes, single cells, living cells and $CD4^+$ T cells. (**A**) Example FACS plot of splenic $CD25^+$ Treg cells. (**B**) Percentages and (**C**) cell numbers of splenic $CD25^+$ Treg cells. (**D**) CD25 MFI of $Foxp3^+$ Treg cells from the spleen. Data represent mean ± SD of n ≥ 5 and were pooled from two independent experiments. Statistical analysis was performed using (**B, C**) Mann-Whitney test.

Collectively, these data show that NIK is dispensable for the maintenance of Foxp3 and CD25 expression and, therefore, Treg cell stability.

3.7 Increase in $Foxp3^+$ Treg cells in peripheral tissues and secondary lymphoid organs upon overexpression of NIK in $Foxp3^+$ Treg cells

We could show that the conditional deletion of NIK in $Foxp3^+$ Treg cells led to a reduction of Treg cells in different tissues, such as the colon and the liver. Since the Treg cell-specific deletion of NIK resulted in a reduction of Treg cells, we examined whether a conditional overexpression of NIK in Treg cells caused an increase in $Foxp3^+$ Treg cells in turn. For that, $Foxp3^{Cre}$ $NIK^{Tg/Tg}$ mice were generated. $Foxp3^{Cre}$ $NIK^{Tg/Tg}$ mice express NIK under the R26 promoter after Foxp3-Cre-mediated excision of a floxed STOP cassette. Expectedly, Treg cell-specific overexpression of NIK resulted in an increase of $Foxp3^+$ Treg cells in the spleen, in the colon as well as in the liver (**Figure 30A, B**). This effect was especially pronounced in the colon as there was a more than 2-fold increase in percentages of $Foxp3^+$ Treg cells in $Foxp3^{Cre}$ $NIK^{Tg/Tg}$ mice compared to controls (**Figure 30A, B**). Of note, the total numbers of cells in spleen, colon and liver did not differ between $Foxp3^{Cre}$ and $Foxp3^{Cre}$ $NIK^{Tg/Tg}$ mice (**Figure 30C**). Morphologically,

spleens and colons of Foxp3^{Cre} NIK$^{Tg/Tg}$ mice at the age of 9 weeks did not display signs of inflammation such as splenomegaly as well as colon shortening and wall thickening, respectively (Figure 30D, E).

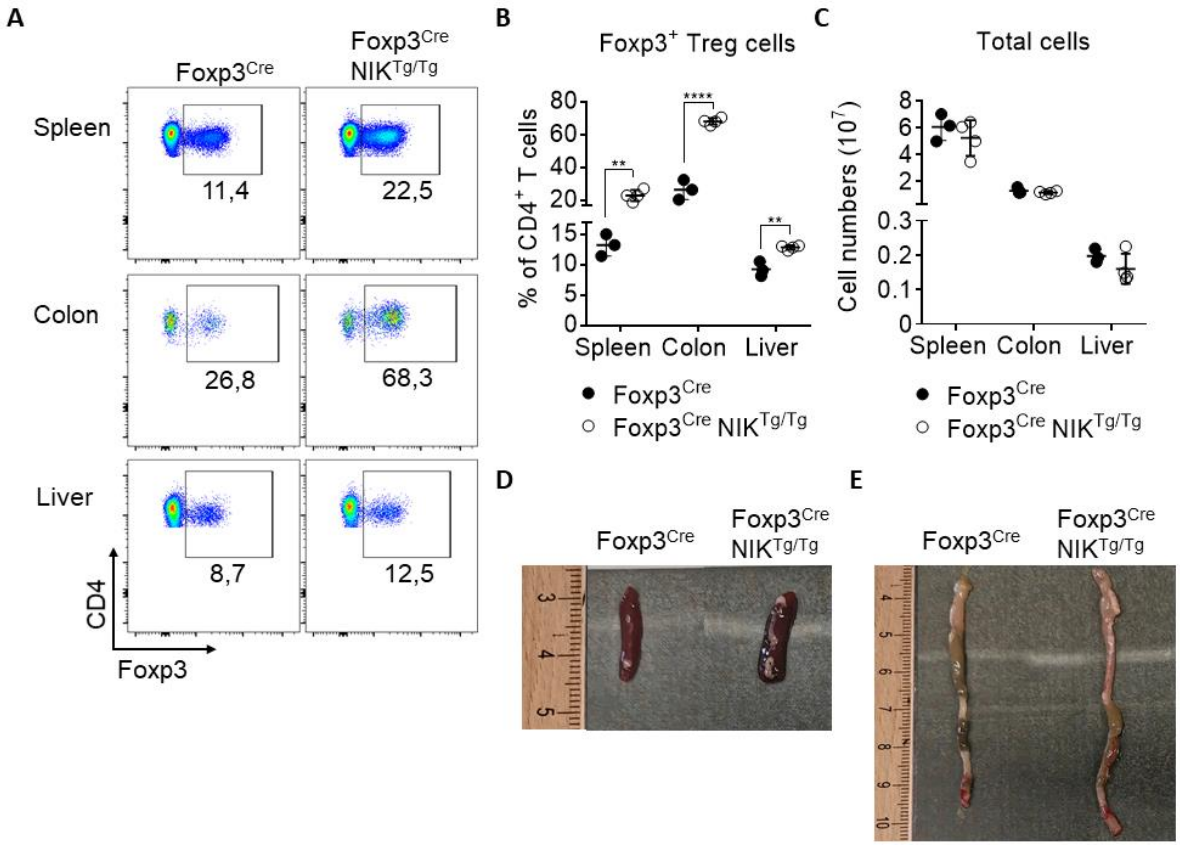

Figure 30: Increase in Treg cells in spleen, colon and liver upon overexpression of NIK in Foxp3$^+$ Treg cells. Single cell suspensions of the spleen, the colonic lamina propria and the liver of 10-week-old Foxp3^{Cre} NIK$^{Tg/Tg}$ and Foxp3^{Cre} control mice were generated and Treg cells analysed by flow cytometry. (**A**) Gating example of Foxp3$^+$ Treg cells from spleen, colon and liver. (**B**) Percentages of Foxp3$^+$ Treg cells from spleen, colon and liver. (**C**) Total living cell numbers of spleen, colon and liver. (**D**) Representative image of spleens of 9-week-old Foxp3^{Cre} controls and 10-week-old Foxp3^{Cre} NIK$^{Tg/Tg}$ mice. (**E**) Representative image of colons of 9-week-old Foxp3^{Cre} controls and 10-week-old Foxp3^{Cre} NIK$^{Tg/Tg}$ mice. Data represent mean ± SD of n ≥ 3 with **p ≤ 0.01, ****p ≤ 0.0001. Data are representative of two independent experiments. Statistical analysis was performed using an (**B, C**) unpaired two-sided Student's t-test.

These data provide evidence that NIK signalling plays a cell-intrinsic role in the maintenance or expansion of Foxp3$^+$ Treg cells.

3.8 Demand for NIK in Foxp3$^+$ Treg cell expansion through the TNF - TNFR2 axis

We detected a cell-intrinsic role for NIK in the maintenance of Foxp3$^+$ Treg cells that resulted in decreased Treg cell numbers in peripheral tissues. Peripheral tissues such as the colon are a rather harsh and inflammatory environment for immune cells (Delgado and Brunner, 2019). We wanted to investigate whether NIK-deficient Treg cells expand to the same extent as WT Treg cells. In order to do so, CD45.2$^+$ CD90.1$^+$ WT and CD45.2$^+$ CD90.2$^+$ NIK-deficient Treg cells were subjected into the same

lymphopenic environment along with CD45.1$^+$ naïve CD4$^+$ T cells that upon conversion into Th1 and Th17 display pro-inflammatory properties (**Figure 31A**). This enabled for a better comparison of expansion between WT and NIK-deficient Treg cells since they were exposed to the same environment. Strikingly, 16 days-post transfer, flow cytometric analysis revealed strongly reduced percentages of NIK-deficient Treg cells in the colon, mLN and spleens (**Figure 31B, C**). As previously introduced in chapter 1.3, intestinal homeostasis has been described to be tightly controlled by TNF. Furthermore, Chen *et al.*, 2013 reported that TNFR2 is required for the expansion of Treg cells, while Rauert *et al.*, 2010 showed that TNFR2 signalling leads to stabilisation of NIK and p100 processing. Consequently, we investigated whether NIK deletion in Foxp3$^+$ Treg cells had any impact on the homeostasis of TNFR2$^+$ Treg cells. Indeed, NIK-deficient TNFR2$^+$ Treg cells were reduced in mLN (**Figure 31D, E**). These data indicate, that TNFR2$^+$ Treg cells require NIK for maintenance.

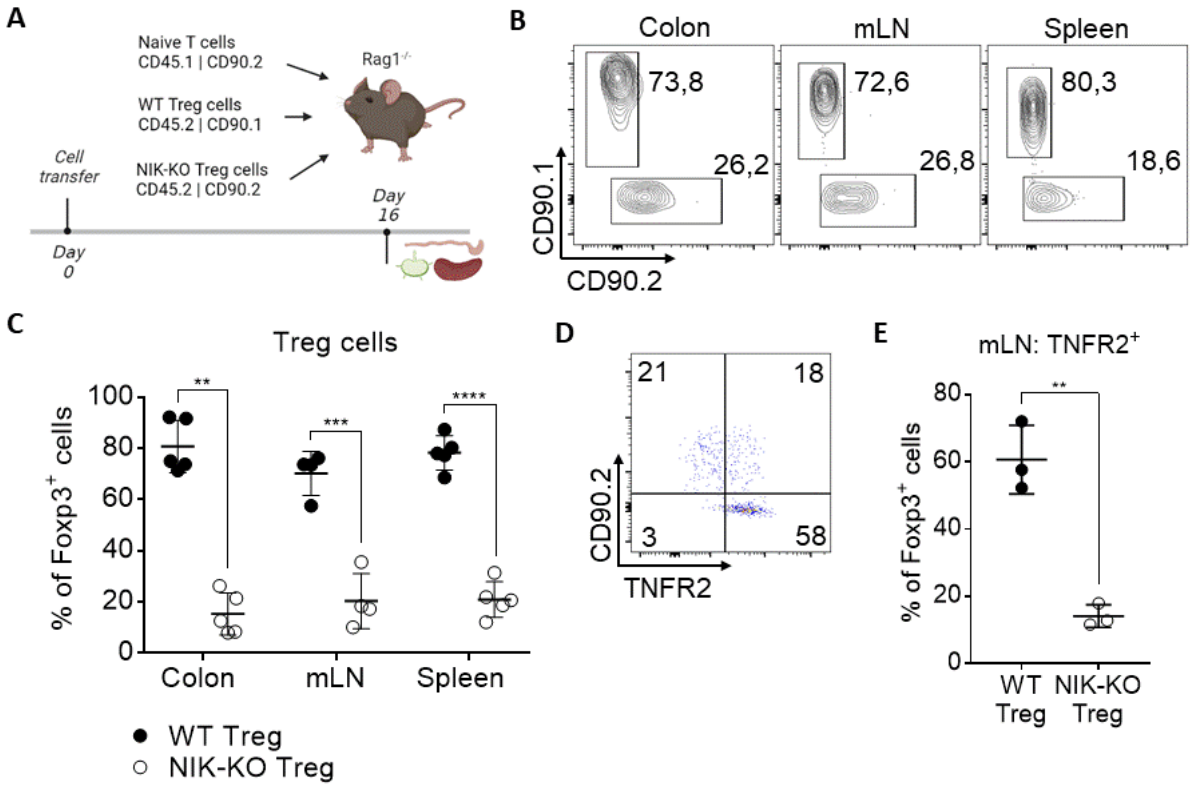

Figure 31: Restricted expansion of NIK-deficient Treg cells *in vivo*. Treg cells were isolated from spleens of NIK$^{\Delta T}$ (NIK-KO Treg cells) mice and Thy1.1 (WT Treg cells) mice by MACS purification. Naive CD4$^+$ T cells congenic for CD45.1 and CD90.2 were isolated from Ly5.1-mice by MACS purification. 5 * 10^5 naive CD4$^+$ T cells were co-transferred along with 2.5 * 10^5 WT Treg cells (CD45.2 and CD90.1) and 2.5 * 10^5 NIK-KO Treg cells (CD45.2 and CD90.2) into Rag1$^{-/-}$ mice. After 2 weeks, single cell suspensions of spleens, mLN and colons from Rag1$^{-/-}$ mice were generated and analysed by flow cytometry. (**A**) Experimental set-up. (**B**) Representative FACS plot showing percentages of transferred Treg cells in the colon, mLN and the spleen. (**C**) Percentages of transferred Treg cells from the colon, mLN and the spleen. Treg cells were pre-gated on lymphocytes, single cells, CD45.2$^+$ living cells and CD4$^+$ Foxp3$^+$ Treg cells. (**D**) Representative plot and (**E**) percentages of CD90.1 WT and CD90.2 NIK-KO Treg cells from mLN expressing TNFR2. Data represent mean ± SD of n ≥ 3 with **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 and are representative of (**B, C**) at least three or (**D, E**) two independent experiments. Statistical analysis was performed using an (**C**: mLN, spleen, **E**) unpaired two-sided Student's t-test or (**C**: Colon) Mann-Whitney test.

To test, whether NIK-deficient Treg cells indeed failed to respond to TNF stimulation, we isolated splenocytes from $NIK^{\Delta T}$ and $NIK^{fl/fl}$ control littermates and stimulated them *in vitro*. Of note, similar numbers of NIK-deficient Treg cells and their WT counterparts were observed upon IL-2 stimulation and without stimulation. However, TNF stimulation induced expansion in WT but not NIK-KO Treg cells (Figure 32 A, B).

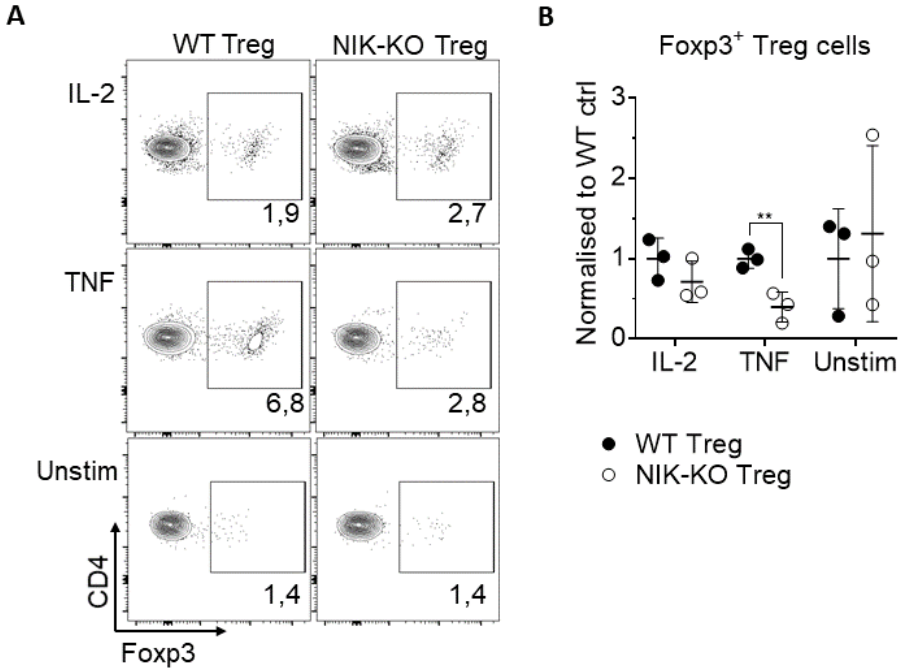

Figure 32: Hampered expansion of NIK-deficient Treg cells upon TNF stimulation. 2 * 10^5 splenocytes from $NIK^{\Delta T}$ mice and control littermates ($NIK^{fl/fl}$) were stimulated with 10 ng/mL IL-2, 10 ng/mL TNF or left unstimulated for 3 days at 37°C. Cultures were analysed by flow cytometry. (**A**) Example plots of $Foxp3^+$ Treg cells upon indicated stimuli. (**B**) $Foxp3^+$ Treg cell counts were normalised to WT controls. $Foxp3^+$ Treg cells are pre-gated on lymphocytes, single cells, living cells and $CD4^+$ T cells. Data represent mean ± SD of n = 3 with **p ≤ 0.01 and are representative of two independent experiments. Statistical analysis was performed using an unpaired two-sided Student's t-test.

Collectively, the above data suggest that NIK is required for the expansion of $Foxp3^+$ Treg cells through the TNF - TNFR2 axis.

3.9 No implication of NIK in the proliferation of $Foxp3^+$ Treg cells

It was reported that conventional $CD4^+$ T cells harbouring a mutation in the *NIK* gene proliferate normally in response to anti-CD3 and anti-CD28 *in vitro* as assessed by thymidine incorporation (Murray *et al.*, 2011). Furthermore, Susan Murray showed that CFSE-labelled Treg cells from NIK-KO mice proliferated to the same extent as their WT counterparts in response to anti-CD3 and co-stimulation provided by APCs *in vitro* (Murray, 2013). However, the observation of decreased NIK-deficient Treg cell populations prompted us to further investigate the proliferative capacity of Treg cells *in vitro* as well as

in vivo. First, Treg cells were isolated, labelled with VCT and stimulated in culture. VCT labels cells intra- and extracellularly. Its signal intensity dilutes out with every cell division. Expectedly, there was no difference between WT and NIK-deficient Treg cells that divided at least once, neither for the anti-CD3, anti-CD28 nor the anti-CD3, anti-CD28, IL-2 condition (**Figure 33A, B**). Thus, *in vitro* results generated here coincide with what is known from the literature.

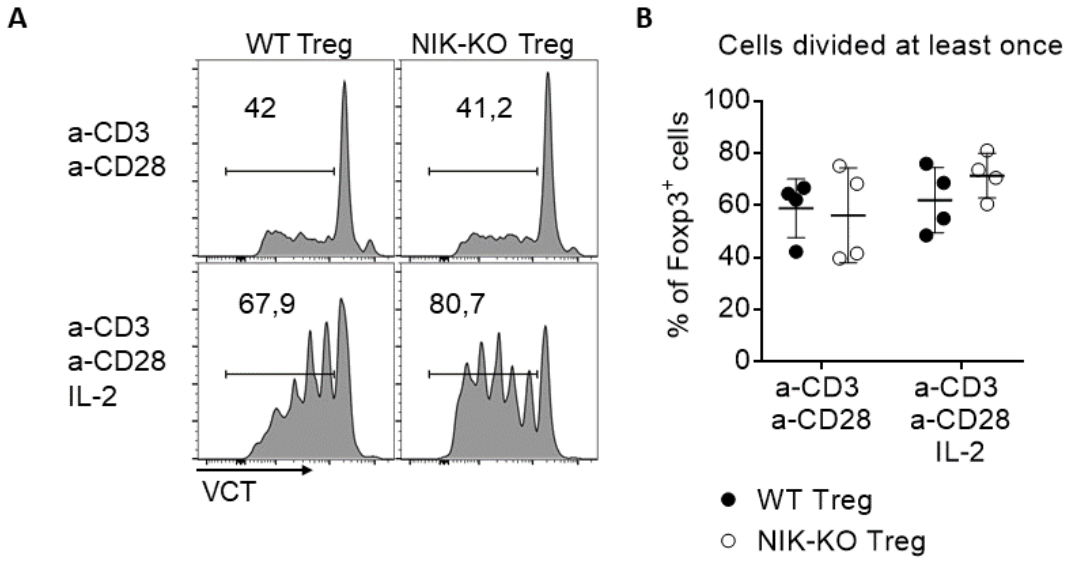

Figure 33: Normal proliferation of NIK-deficient Treg cells *in vitro*. $CD4^+$ T cells from spleens of $NIK^{\Delta T}$ mice (NIK-KO Treg) and control littermates (WT Treg) were isolated by MACS purification (CD4-negative MACS) and stained with VCT. 2 * 10^5 $CD4^+$ T cells were then stimulated with 0,5 µg/mL anti-CD3, 1 µg/mL anti-CD28 and with or without 10 ng/mL IL-2 for 3,5 days at 37°C. (**A**) Example plot and (**B**) percentages of $Foxp3^+$ Treg cells that have proliferated at least once. $Foxp3^+$ Treg cells are pre-gated on lymphocytes, single cells, living cells and $CD4^+$ T cells. Data represent mean ± SD of n = 4 and are representative of three independent experiments. Statistical analysis was performed using an unpaired two-sided Student's t-test.

Next, proliferation of $Foxp3^+$ Treg cells was assessed *in vivo* by Ki67 staining, a nuclear molecule that is expressed in all cycles during mitosis, except for the resting phase G0, and accumulates within the S phase. Under acute inflammatory conditions during T cell transfer colitis in $Rag1^{-/-}$ mice, transferred NIK-deficient Treg cells displayed merely a tendency towards decreased expression levels of Ki67 in comparison to transferred WT Treg cells (**Figure 34A**). Besides, conditional overexpression of NIK in $Foxp3^+$ Treg cells from the colon and the liver did not result in increased Ki67 expression or $Ki67^+$ populations (**Figure 34B, C**), although $Foxp3^+$ Treg cells from tissues of $Foxp3^{Cre}$ $NIK^{Tg/Tg}$ mice were found to be increased in comparison to $Foxp3^{Cre}$ controls, see chapter 3.7. Furthermore, although we found decreased $TNFR2^+$ and $Helios^+$ Treg cell subtypes in the liver and the colon of $NIK^{\Delta T}$ mice, respectively, Ki67 expression of NIK-deficient $Helios^+$ Treg cells from the colon (**Figure 34D**) and NIK-deficient $TNFR2^+$ Treg cells from the liver (**Figure 34E**) was similar to their WT Treg counterpart.

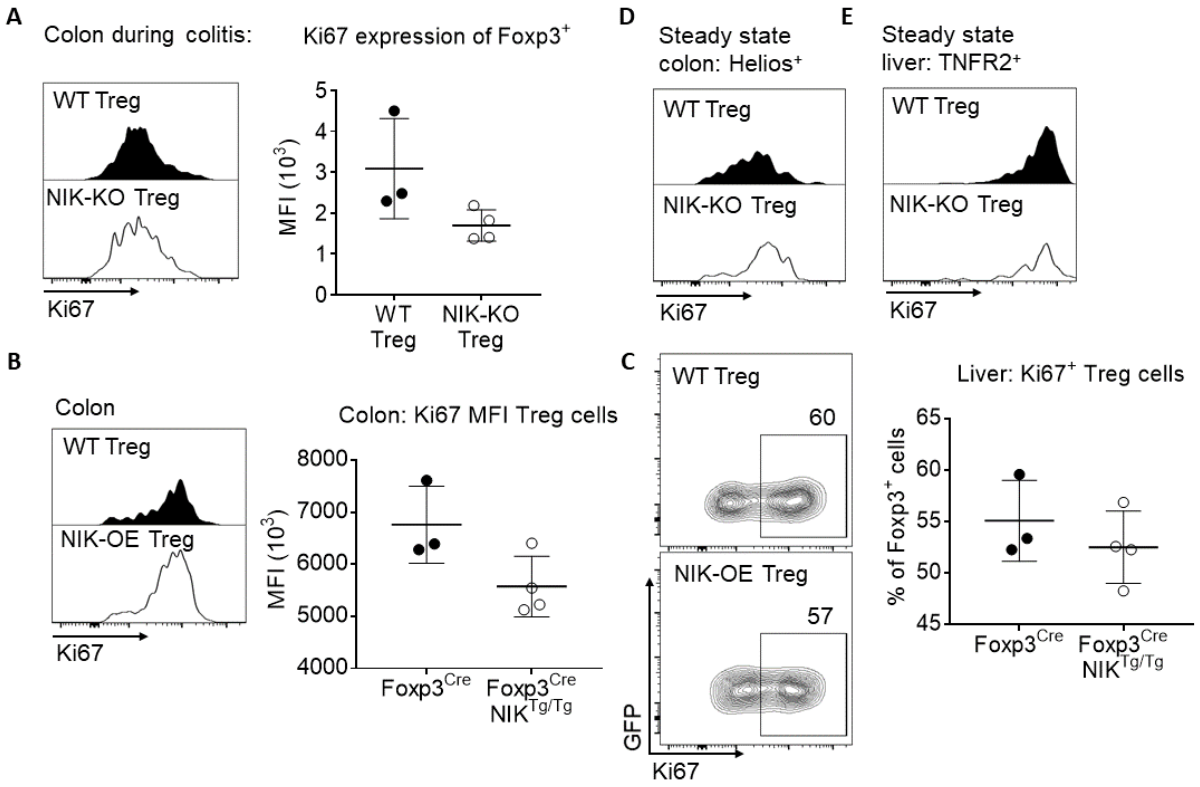

Figure 34: Normal proliferation of NIK-deficient Treg cells *in vivo*. Single cell suspensions from respective organs and mice were generated and analysed by flow cytometry. (**A**) Example plot of Ki67 expression by Foxp3$^+$ Treg cells and respective scatter plot showing MFI of Foxp3$^+$ Treg cells from the colon of Rag1$^{-/-}$ subjected to an adaptive T cell transfer colitis having received either WT or NIK-KO Treg cells (from NIK$^{\Delta T}$ mice). (**B**) Histograms and respective scatter plot of Ki67 expression of Foxp3$^+$ Treg cells isolated from the colon of steady state Foxp3^{Cre} NIK$^{Tg/Tg}$ (NIK-OE) and Foxp3^{Cre} control mice. (**C**) Example plot and respective scatter plot of Ki67$^+$ Treg cells isolated from the liver of steady state Foxp3^{Cre} NIK$^{Tg/Tg}$ and Foxp3^{Cre} control mice. Foxp3$^+$ Treg cells are pre-gated on lymphocytes, single cells, CD45$^+$ living cells and CD4$^+$ T cells. Representative histograms of Treg subtypes from steady state NIK$^{\Delta T}$ mice and NIK$^{fl/fl}$ control littermates isolated from the (**D**) colon and (**E**) liver. Green fluorescent protein (GFP). Data represent mean ± SD of n ≥ 3 and are representative of (**A, D, E**) three independent experiments. Statistical analysis was performed using an (**A, B, C**) unpaired two-sided Student's t-test.

Taken together, these results from *in vitro* and *in vivo* studies suggest that NIK deficiency does not affect the proliferation of Foxp3$^+$ Treg cells.

3.10 No change in mRNA levels of apoptosis-related genes upon NIK stabilisation in Foxp3$^+$ Treg cells

Mice that lack NIK in Foxp3$^+$ Treg cells displayed decreased Treg cell populations, while mice that overexpress NIK in Foxp3$^+$ Treg cells showed an increase in Foxp3$^+$ Treg cells. However, our data suggest that NIK is not implicated in the proliferation of Treg cells. In addition, we showed that NIK is dispensable for the maintenance of Foxp3 expression. As a consequence, we attempted to identify alterations in apoptosis by investigating mRNA levels of *Mcl-1*, *Bak* and *Bax* that are genes known for regulating apoptosis in Treg cells (Shen, Yang and Chen, 2014). Stabilisation of NIK through AT406 in WT Treg cells

did neither induce a significant change in mRNA levels of anti-apoptotic *Mcl-1* nor in pro-apoptotic genes *Bak* and *Bax* (Figure 35A). NIK stabilisation and subsequent p100 processing in AT406-treated WT Treg cells did indeed occur after 4 hours of stimulation as assessed by p100 processing into p52 (Figure 35B).

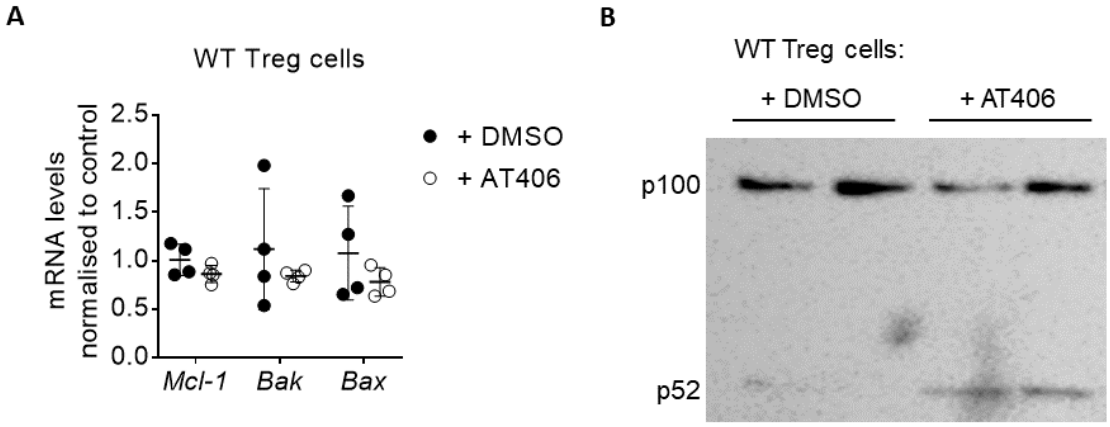

Figure 35: No upregulation of apoptosis-related genes *Mcl-1*, *Bak* and *Bax* upon stabilisation of NIK. Treg cells were isolated from splenic single cell suspensions of C57BL/6 WT mice by MACS purification and treated with either dimethyl sulfoxide (DMSO) or 10 µM AT406 for 4 hours at 37 °C in T cell medium before further analysis. (**A**) Quantification of mRNA levels of apoptosis-related genes by real-time PCR. (**B**) Whole-cell extracts from treated WT Treg cells were subjected to Western blot analysis of the processing of p100 into p52. Shown are technical duplicates. Data represent mean ± SD of n = 4 and are representative for (**A**) three independent experiments and (**B**) one experiment. Statistical analysis was performed using an (**A**) unpaired two-sided Student's t-test.

Taken together, the mechanism behind the defect in the maintenance of NIK-deficient Treg cells is not based on an altered regulation of the genes *Mcl-1*, *Bak* and *Bax* and still remains to be unravelled.

3.11 No requirement for NIK in Treg cell-mediated suppression *in vitro*

In a previous publication, it was shown that NIK is dispensable for the suppressive capacity of Foxp3$^+$ Treg cells *in vitro* (Murray, 2013). Therefore, Treg cells from spleens of NIK$^{\Delta T}$ mice and control littermates were MACS purified and subjected to an *in vitro* Treg suppression assay in which conventional CD4$^+$ T cells were activated and co-cultured with either WT or NIK-deficient Treg cells. The purity of MACS-isolated Treg cells assessed by CD25 expression on CD4$^+$ T cells was similar between WT and NIK-KO Treg cells with around 90 % (Figure 36A). Of note, we found that NIK-deficient Treg cells suppressed the proliferation of VCT-labelled conventional CD4$^+$ T cells to the same extent as WT Treg cells (Figure 36B, C).

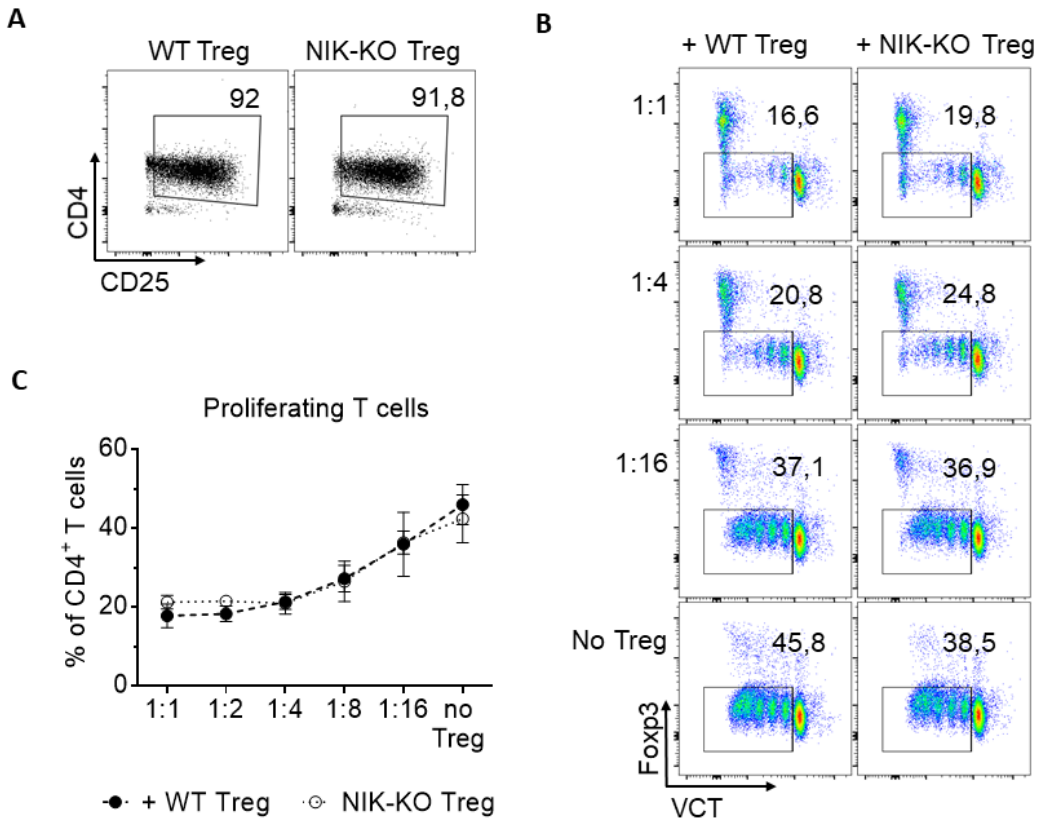

Figure 36: Normal suppressive capacity of NIK-deficient Treg cells *in vitro*. Treg cells were isolated from spleens of $NIK^{\Delta T}$ (NIK-KO Treg cells) mice and $NIK^{fl/fl}$ littermates (WT Treg cells) by MACS purification. WT $CD4^+$ Tconv cells were isolated from $NIK^{fl/fl}$ mice. $CD4^+$ Tconv cells were labelled with VCT and cultured with Treg cells in ratios of 1:1, 1:2, 1:4, 1:8, 1:16 and without Treg cells. Cultures were stimulated with 0.5 µg/mL of anti-CD3 and 6 ng/mL of anti-CD28 at 37 °C for 4 days. (**A**) Example plots of Treg cell purity after MACS separation analysed by flow cytometry. (**B**) Flow cytometric example plots of VCT-labelled proliferating $CD4^+$ Tconv cells plated in different ratios with Treg cells. (**C**) Percentages of VCT-labelled proliferating $CD4^+$ Tconv cells plated in different ratios with Treg cells analysed using flow cytometry. Data represent mean ± SD of n = 4. Data are representative of two independent experiments. Statistical analysis was performed using (**C**) Mann-Whitney test.

Hence, these data provide evidence that NIK is dispensable for the suppressive capacity of Treg cells, in particular for NIK-KO Treg cells from $NIK^{\Delta T}$ mice in *in vitro* settings.

3.12 No suppression of T cell transfer colitis by NIK-deficient $Foxp3^+$ Treg cells

As shown above, NIK-deficient Treg cells displayed normal suppressive capacity in an *in vitro* Treg suppression assay and maintained tissue homeostasis in steady state $NIK^{\Delta Treg}$ mice although they were reduced in the colon and in the liver of these mice. Hence, the performance of NIK-deficient Treg cells was investigated under strong inflammatory conditions within the setting of an adaptive T cell transfer colitis, a mouse model of inflammatory bowel disease (Ostanin *et al.*, 2009), in which lymphopenic $Rag1^{-/-}$ mice are reconstituted with WT naïve $CD4^+$ T cells that after intraperitoneal transfer develop into colitis-inducing Th1 and Th17 cells. The injection of WT Treg cells prevents the onset of colitis. Of note,

Rag1$^{-/-}$ mice that were reconstituted with NIK-deficient Treg cells suffered from weight loss (**Figure 37A**). Additionally, colons of Rag1$^{-/-}$ mice that received NIK-KO Treg cells looked highly inflamed assessed by colon shortening and wall thickening (**Figure 37B**). Furthermore, endoscopic examination revealed that Rag1$^{-/-}$ mice reconstituted with NIK-KO Treg cells suffered from diarrhoea and colonic inflammation characterised by a non-transparent colon wall due to elevated wall thickening as well as an increase in granularity and vascularity at end point, while Rag1$^{-/-}$ mice that received WT Treg cells did not show any signs of colonic inflammation (**Figure 37C**). Additionally, H&E stainings of proximal colon sections were performed and scored using a histological colitis scoring system (adapted from Kihara *et al.*, 2003). Rag1$^{-/-}$ that received WT Treg cells did not show any inflammation and no crypt damage (**Figure 37D, E**). On the contrary, Rag1$^{-/-}$ mice that received NIK-KO Treg cells displayed moderate to severe inflammation into the submucosa and in one case even transmural inflammation, in addition crypt loss that ranged from 1/3 of crypts being basally damaged up to regions characterised by complete crypt loss (**Figure 37D, E**). Collectively, these data reveal that NIK-deficient Treg cells fail to prevent T cell-mediated colitis.

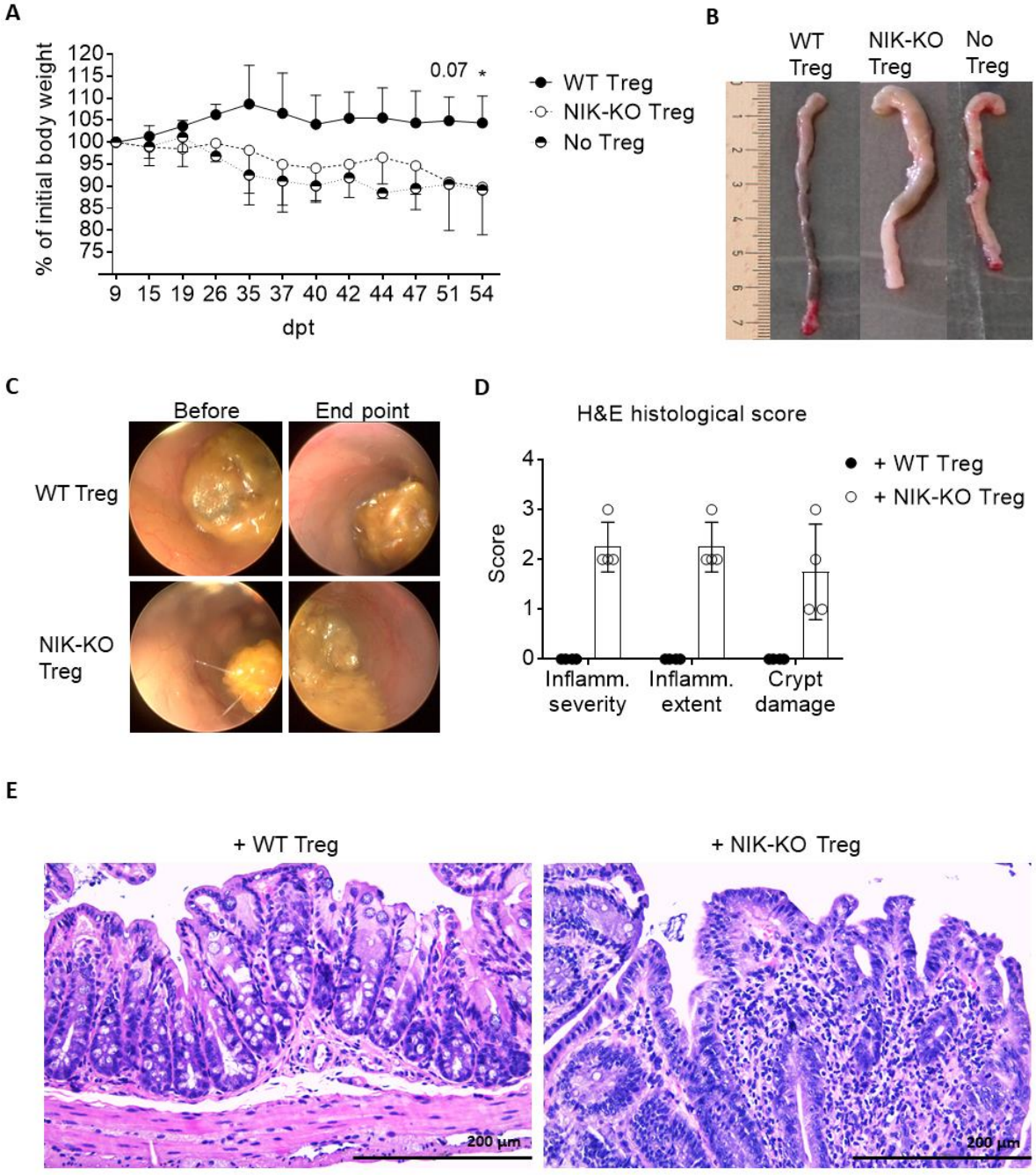

Figure 37: No prevention of adaptive T cell transfer colitis by NIK-deficient Treg cells. Treg cells were isolated from spleens of $NIK^{\Delta T}$ (NIK-KO Treg cells) mice and $NIK^{fl/fl}$ littermates (WT Treg cells) by MACS purification. Naive $CD4^+$ T cells were isolated from C57BL/6 mice congenic for CD45.1 by MACS purification. 5 * 10^5 naive $CD4^+$ T cells were either transferred alone or co-transferred along with 5 * 10^5 WT Treg cells or 5 * 10^5 NIK-KO Treg cells into $Rag1^{-/-}$ mice. (**A**) Weight of $Rag1^{-/-}$ mice measured over the period of 54 days. (**B**) Representative example photographs of colons from $Rag1^{-/-}$ having received WT Treg cells, NIK-KO Treg cells or no Treg cells at end point of the experiment. (**C**) Representative endoscopic images of the colons of $Rag1^{-/-}$ having received either WT or NIK-KO Treg cells before the start and at the end point (dpt 54) of the experiment. (**D**) Histological scores of H&E sections from the proximal colon, at experimental end point, of $Rag1^{-/-}$ mice reconstituted with either WT or NIK-KO Treg cells. (**E**) Representative images of H&E stainings of the proximal colon of $Rag1^{-/-}$ mice at end point of the experiment. Bars indicate 200 µm. Data represent mean ± SD of $n \geq 3$ with $*p \leq 0.05$ for $Rag1^{-/-}$ mice reconstituted with WT and NIK-KO Treg cells. $Rag1^{-/-}$ mice reconstituted with naive $CD4^+$ T cells only n = 5 at start point and n = 2 at end point. Data are representative of (**A, B**) at least three or (**C, D, E**) two independent experiments. Statistical analysis was performed using (**A**) Mann-Whitney test.

Next, FACS analysis revealed increased cell counts of total living cells in the spleen and colon of $Rag1^{-/-}$ mice that received NIK-KO Treg cells provide further evidence that these cells failed in preventing colitis-mediated inflammation (**Figure 38A**). In line with this, increased cell numbers of transferred $CD45.1^{+}$ cells that produce IFNγ, IL-17A as well as both IFNγ and IL-17A could be detected in $Rag1^{-/-}$ reconstituted with NK-KO Treg cells (**Figure 38B**). Of note, similar numbers of $Foxp3^{+}$ Treg cells could be observed in the colon as well as in the spleen of $Rag1^{-/-}$ mice (**Figure 38C**). Yet, despite a highly inflammatory environment, NIK-deficient Treg cells were stable did not downregulate Foxp3 (**Figure 38D, E**). Interestingly, NIK-deficient Treg cells showed comparable expression levels of CTLA-4 and Foxp3 (**Figure 38F, G**) suggesting normal suppressive capacity per se looking at those two markers. However, the ratio of Treg cells divided by effector T cells (Teff) is significantly lower in $Rag1^{-/-}$ mice that received NIK-KO Treg cells (**Figure 38H**). Yet, the question remains whether the underlying inflammation in $Rag1^{-/-}$ mice that received NIK-KO Treg cells is at least partially due the lack of expansion of NIK-deficient Treg cells.

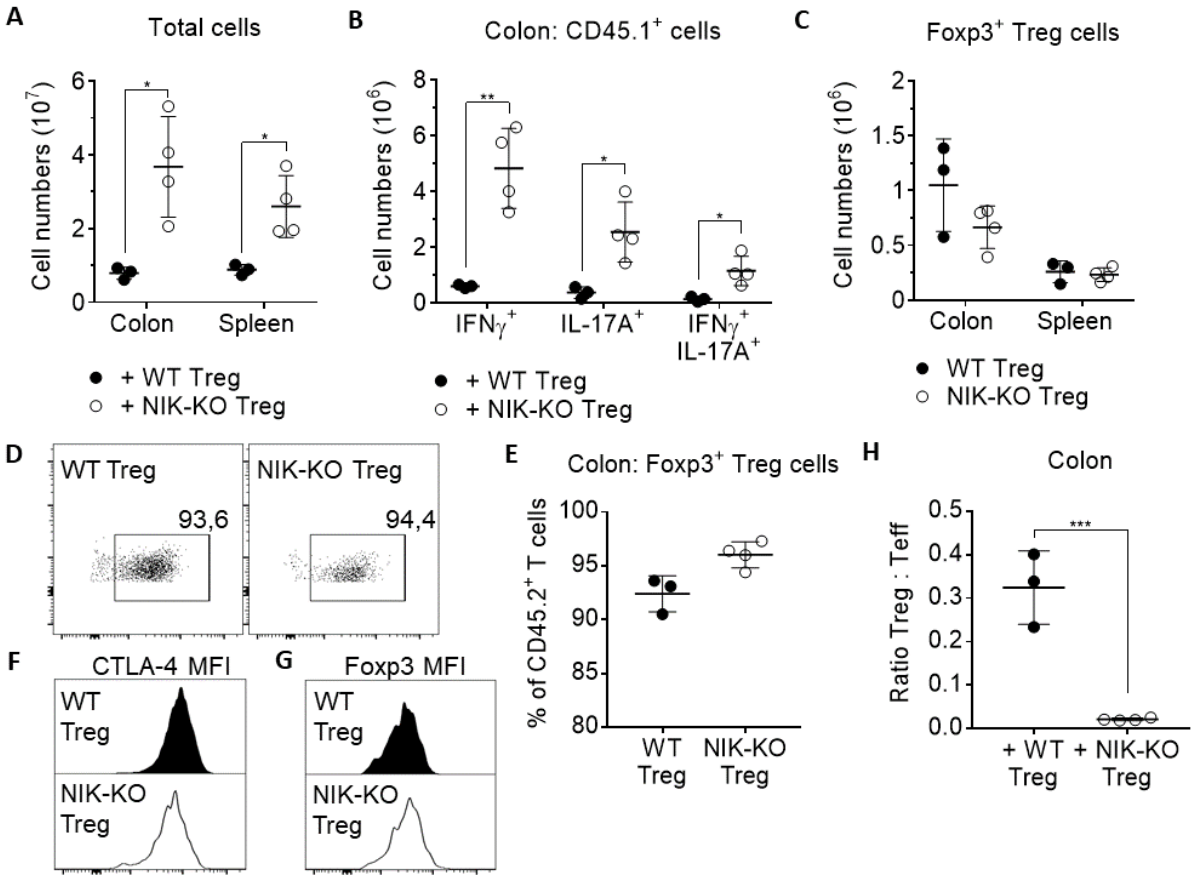

Figure 38: No suppression of expansion of pro-inflammatory cells in a colitis setting by NIK-deficient Treg cells. Treg cells congenic for CD45.2 were isolated from spleens of $NIK^{\Delta T}$ (NIK-KO Treg cells) mice and $NIK^{fl/fl}$ littermates (WT Treg cells) by MACS purification. Naive $CD4^+$ T cells congenic for CD45.1 were isolated from C57BL/6 mice by MACS purification. 5 * 10^5 naive $CD4^+$ T cells were either transferred alone or co-transferred along with 5 * 10^5 WT Treg cells or 5 * 10^5 NIK-KO Treg cells into $Rag1^{-/-}$ mice to induce an adaptive T cell transfer colitis. At end point, single cell suspensions of spleens and colons from $Rag1^{-/-}$ mice were generated and analysed by flow cytometry. (**A**) Cell count for total living cells from colon and spleen. (**B**) Cell numbers of $CD45.1^+$ pro-inflammatory cells from the colon. (**C**) Cell counts of $Foxp3^+$ Treg cells from spleen and colon. (**D**) Example plot of the remaining percentage of $Foxp3^+$ Treg cells of transferred $CD45.2^+$ cells in the colon. (**E**) Percentage of $CD45.2^+$ cells that are positive for Foxp3. $Foxp3^+$ Treg cells were pre-gated on lymphocytes, single cells, living cells, $CD4^+$ T cells and CD45.2. (**F**) Representative histogram of CTLA-4 median fluorescence intensity (MFI) of $Foxp3^+$ Treg cells from the colon. (**G**) Representative histogram of Foxp3 MFI of $Foxp3^+$ Treg cells from the colon. (**H**) Ratio of $CD45.2^+$ $Foxp3^+$ Treg cells divided by $CD45.1^+$ Teff cells in the colon. Data represent mean ± SD of n ≥ 3 with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and are representative of three independent experiments. Statistical analysis was performed using an (**A, B, C, H**) unpaired two-sided Student's t-test or (**D**) Mann-Whitney test.

In summary, we show that NIK-deficient Treg cells fail to prevent a T cell-mediated colitis and suggest that NIK is required for adequate maintenance of Treg cells under inflammatory conditions.

4 Discussion

NIK is the key player of non-canonical NF-κB signalling that upon stabilisation induces IKKα phosphorylation that in turn leads to processing of p100 into p52 and eventual translocation of p52:RelB heterodimers into the nucleus (Senftleben *et al.*, 2001; Xiao, Fong and Sun, 2004; Sun and Ley, 2008). While NIK has been established to be critical for Teff cell maintenance and function downstream of TNFR superfamily receptors (Sun, 2017), relatively little is known about its role in Treg cells. Findings in regards to peripheral maintenance and Treg cell development even contradict each other. One report showed that there is a cell-intrinsic role for NIK in Treg cell maintenance in peripheral lymphoid organs (Murray, 2013). Lacher *et al.* found that conditional deletion of NIK in Foxp3$^+$ Treg cells did not result in a reduction of Foxp3$^+$ Treg cells in peripheral lymphoid organs, suggesting that NIK does not play a cell-intrinsic role in peripheral Treg cell maintenance (Lacher *et al.*, 2018). Yet, a reduction in frequency of Foxp3$^+$ Treg cells in primary and secondary lymphoid organs became evident when NIK was specifically deleted in CD4$^+$ T cells (Lacher *et al.*, 2018). This suggests that NIK is implicated in thymic Treg cell development. However, the other report came to the conclusion that NIK is not critical for thymic Treg cell development (Murray, 2013). Moreover, there are no publications about the role of NIK in tissue Treg cell homeostasis. *In vitro*, it was reported that NIK is dispensable for the suppression of Tconv cells (Murray, 2013). To date, there are no findings in regards to whether NIK is required for Treg cell suppressive function the *in vivo*. We propose a role for NIK in the maintenance of thymic TregP, tissue Treg cells and Treg cells under inflammatory conditions during colitis. These novel observations are discussed below.

4.1 NIK ascertains Treg cell precursor homeostasis during thymic Treg cell development

Our results suggest a novel role for NIK in thymic Treg cell development. TNFR2$^+$ CD25 TregP, OX40$^+$ CD25 TregP as well as Foxp3^{lo} TregP populations were decreased in thymi of mice that lack NIK in all αβ T cells. These findings provide evidence that NIK is required for the maintenance of TregP populations. Owen *et al.* found that thymic TregP arise from two distinct progenitor populations, the CD25 TregP and the Foxp3^{lo} TregP (Owen *et al.*, 2019). Indeed, Mahmud and colleagues showed that TNFR superfamily members expressed on Treg cell progenitors are crucial for thymic Treg cell development which was abrogated by triple knockout of OX40, TNFR2 and GITR *in vivo* (Mahmud *et al.*, 2014). Here, we show that this effect was at least in part mediated by NIK. NIK is the key player of non-canonical NF-κB signalling that is induced by upstream degradation of TRAF3 upon receptor engagement and eventually leads to degradation of p100 into p52 (Sun, 2012). TNFR2 as well as OX40 engage TRAF3 that results in eventual p100 processing (Wajant, Henkler and Scheurich, 2001; Rauert *et al.*, 2010; Murray *et al.*,

2011). Hence, this suggests that thymic TregP maintenance requires NIK-mediated non-canonical NF-κB signalling. TNFR2 and OX40 along with other TNFR superfamily members were reported to be crucial for Teff cell maintenance (Sun, 2017). Indeed, signalling through TNFR superfamily member CD27 in thymic Treg cells induced by CD70-expressing mTEC and thymic DCs prevented apoptosis of thymic Treg cells (Coquet *et al.*, 2013). However, Mahmud *et al.* further showed that CD27 expression on CD25 TregP did not correlate with OX40, TNFR2 and GITR expression upon TCR signalling and that stimulation of CD25 TregP with CD70 did not result in increased mature Treg cell numbers while stimulation with OX40L, GITRL and TNF did (Mahmud *et al.*, 2014). This suggests that CD27 is rather implicated in preventing apoptosis in mature Treg cells than in the conversion of TregP into mature Treg cells. Of note, other TNFR superfamily members CD137 and CD30 are not expressed on TregP (Mahmud *et al.*, 2014).

In addition to $OX40^+$ and $TNFR2^+$ TregP, $Foxp3^{lo}$ TregP were decreased in $NIK^{\Delta T}$ mice. The $Foxp3^{lo}$ TregP subset was reported to be dependent on canonical NF-κB signalling since it was reduced in mice deficient for NF-κB1, while the CD25 TregP population remained unaffected (Mahmud *et al.*, 2014). Signalling interactions of non-canonical NF-κB with canonical NF-κB were previously demonstrated to occur for example upon constitutive expression of NIK that resulted in the activation of canonical NF-κB signalling through the IKK complex (Zarnegar *et al.*, 2008). Thus, we cannot rule out that the effect of NIK deficiency on $Foxp3^{lo}$ TregP might at least partially stem from reduced activation of the canonical NF-κB pathway. The same holds true for the CD25 TregP subset. Moreover, NIK has been established to be crucial for the maintenance of activated Tconv cells, called Teff cells (Sun, 2017). Due to their thymic developmental programme, Treg cells are at baseline more activated than naïve $CD4^+$ T cells since they show intermediately strong interactions with presented autoantigens (Owen, Sjaastad and Farrar, 2019). Thus, it is well imaginable that NIK signalling is of equal importance for Teff cells as well as TregP.

$TNFR2^{-/-}$ mice were shown to harbour a reduction in thymic as well as splenic $Foxp3^+$ Treg cells (Chen *et al.*, 2013). This prompted us to investigate whether these mice show a reduction in CD25 TregP, $Foxp3^{lo}$ TregP or mature thymic Treg cells, in particular by excluding recirculating Treg cells using the marker CD73. Indeed, a reduction in CD25 TregP but neither $Foxp3^{lo}$ TregP nor mature Treg cells could be observed. Yet, these findings are surprising for two reasons. First, as demonstrated by Mahmud *et al.*, GITR, OX40 and TNFR2 are co-expressed on CD25 TregP and redundantly drive the conversion of CD25 TregP into mature Treg cells (Mahmud *et al.*, 2014). Secondly, we could observe neither a decrease of mature nor recirculating Treg cells as Chen *et al.* reported for thymic and splenic Treg cells (Chen *et al.*, 2013). Differences between animal facilities might lead to different activation statuses of immune cells and levels of pro-inflammatory cytokines such as IL-2 and TNF present within mice. Due to increased TNF levels, WT controls might have higher frequencies of CD25 TregP in our animal facility in comparison

with elsewhere. Upon conversion into mature Treg cells, Foxp3^{+} Treg cells depend on IL-2 for proliferation (Fontenot *et al.*, 2005). That might be a reason for similar levels of mature and recirculating Treg cells in our TNFR2$^{-/-}$ mice in comparison to other laboratories.

Thymic CD25 TregP and Foxp3^{lo} TregP subsets as well as mature Treg cells were normal in mice with conditional deletion of NIK in Treg cells. Interestingly, recirculating Foxp3^{+} Treg cells, that are positive for CD73, were reduced in thymi of NIK$^{\Delta T}$ but not NIK$^{\Delta Treg}$ mice, which suggests that this reduction stems from a decreased Treg cell progenitor population that results in a decreased output of mature Treg cells in NIK$^{\Delta T}$ mice. In line with this, we detected a decrease in Foxp3^{+} Treg cells in the spleen and lymph nodes of NIK$^{\Delta T}$ but not NIK$^{\Delta Treg}$ mice, which might be again resulting from a reduced Treg cell output in NIK$^{\Delta T}$ but not NIK$^{\Delta Treg}$ mice.

In the NIK$^{\Delta T}$ strain, NIK is conditionally deleted using the CD4-Cre, hence, all CD4^{+} cells are targeted by the Cre. The thymus harbours a signal regulatory protein α (SIRPα)-positive DC subset that expresses CD4 and that is implicated in antigen-presentation to T cells during thymic selection processes (Saito *et al.*, 2010; Owen, Sjaastad and Farrar, 2019). One could argue that NIK deletion using the CD4-Cre might occur in SIRPα-positive DCs and affect their homeostasis and antigen presentation capabilities. Of note, Hofmann *et al.* claimed that NIK signalling in DCs but not T cells is required for the generation of Teff cells capable of inducing EAE (Hofmann *et al.*, 2011). Indeed, DCs were reported to be decreased in *aly/aly* mice along with their expression of surface molecules CD80, CD86 and MHC-II (Tamura *et al.*, 2006). However, several other groups have reported a cell-intrinsic requirement of NIK in T cells for the induction of EAE (Li *et al.*, 2016; Lacher *et al.*, 2018). Murray *et al.* demonstrated that CD4^{+} T cells with mutations in NIK failed to cause GvHD (Murray *et al.*, 2011). Going back to thymic DCs, our data indicate that DC homeostasis and development in NIK$^{\Delta T}$ mice is unaffected showing equal percentages of both thymic CD4^{+} CD80^{+} DC and CD4^{+} CD86^{+} DC subsets characterised by normal expression levels of CD80, CD86 and MHC-II. Besides, it was shown that NIK is dispensable for the development and homeostasis of intestinal DCs (Jie *et al.*, 2018). Yet, functional antigen-presenting capacities of DCs from NIK$^{\Delta T}$ mice were not tested in the scope of this thesis. However, indirect effects of the CD4-Cre deletion on thymic cytokine levels that are implicated in T cell development can be excluded as we report that mRNA levels of whole thymic tissues for *IL-2*, *CCL20* and *TGF-β1* in NIK$^{\Delta T}$ mice were comparable to those of WT controls. Moreover, as demonstrated by Haftmann *et al.*, mTEC crucially require NIK for proper education of thymic Treg cells and prevention of autoimmunity (Haftmann *et al.*, 2021). Yet, we showed that NIK deficiency in CD4^{+} cells did not indirectly affect the homeostasis of cTEC and mTEC. Taken together, these results provide evidence that the reduction of CD25 TregP, Foxp3^{lo} TregP as well as mature Treg cells in NIK$^{\Delta T}$ mice stem from a cell-intrinsic requirement of NIK and not from a defect in APC homeostasis or development.

4.2 Requirement for NIK in tissue Treg cell maintenance

As previously mentioned, non-canonical NF-κB was shown to be required for the maintenance of Teff cells (Sun, 2017). NIK-deficient T cells failed to induce EAE as well as GvHD highlighting a role for NIK in Teff cell maintenance and function (Murray *et al.*, 2011; Li *et al.*, 2016; Lacher *et al.*, 2018). Indeed, EAE pathogenicity was further shown to be mediated by non-canonical transcription factor p52, which suggests that NIK as part of non-canonical NF-κB is required for EAE-inducing effector functions of $CD4^+$ T cells (Yu *et al.*, 2014). Yet, the role of NIK in Treg cell maintenance remains disputed. Previous publications have investigated Treg cell maintenance in peripheral lymphoid organs such as the thymus, spleen and lymph nodes. While Susan Murray suggested a cell-intrinsic role for NIK in peripheral Treg cell maintenance, in contrast, Lacher *et al.* found normal percentages of $Foxp3^+$ Treg cells in secondary lymphoid organs when specifically deleting NIK using the Foxp3-Cre (Murray, 2013; Lacher *et al.*, 2018). To date, nothing is known about the role of NIK for Treg cells in tissues.

We confirmed that the deletion of NIK in $Foxp3^+$ Treg cells did not result in decreased peripheral Treg cell populations, showing normal numbers in the spleen, Peyer's patches, mesenteric as well as inguinal lymph nodes. When conditionally deleting NIK in all αβ T cells using the CD4-Cre we did, however, observe a reduction of $Foxp3^+$ Treg cells in the periphery. Yet, as discussed in the previous section 4.1, this reduction probably resulted from a decreased output of newly developed thymic Treg cells. In the periphery, Treg cells depend on IL-2 for expansion (Fontenot *et al.*, 2005; Estrada Brull, Panetti and Joller, 2022). We found that NIK-deficient Treg cells expressed normal levels of CD25 in the periphery and proliferated normally in response to IL-2 *in vitro* and therefore match very well the requirements for being maintained in the periphery. Hence, our data suggest that NIK is dispensable for the maintenance of $Foxp3^+$ Treg cells in secondary lymphoid organs.

Importantly, a reduction of $Foxp3^+$ Treg cells was detected in the colon as well as in the liver in both $NIK^{\Delta Treg}$ and $NIK^{\Delta T}$ mice nevertheless. These data provide evidence that NIK is cell-intrinsically required for the maintenance of tissue-resident Treg cells instead. Indeed, when we overexpressed NIK using the Foxp3-Cre, an increase of $Foxp3^+$ Treg cells was found in the colon, the liver and in the spleen. Since there was no obvious sign for disturbed homeostasis in these young mice, we assume that increased Treg cell numbers do not stem from increased inflammation. Yet, enforced overexpression of genes in mice is very artificial but it nevertheless shows proof of principle. Whether the increase in the Treg cell population upon NIK overexpression can be exclusively attributed to non-canonical NF-κB signalling remains questionable, since enforced expression of NIK was shown to activate the canonical NF-κB signalling as well (Zarnegar *et al.*, 2008). Despite an increased $Foxp3^+$ Treg cell population, we further showed that NIK overexpression did not render $Foxp3^+$ Treg cells more proliferative. Besides, despite a reduction of $Foxp3^+$ tissue Treg cells in $NIK^{\Delta Treg}$ mice, a defect in proliferation assessed by FACS Ki67

stainings could not be observed. Additionally, we found that NIK-deficient Treg cells proliferated normally *in vitro* as assessed by VCT staining. Given that NIK-deficient Treg cells do not show a defect in proliferation, we investigated whether diminished maintenance resulted from increased apoptosis. Anti-apoptotic gene *Mcl-1* is reported to be elevated during homeostatic proliferation in Treg cells, while pro-apoptotic genes *Bak* and *Bax* restrain the Treg cell pool (Shen, Yang and Chen, 2014). Of note, we neither detected differences in mRNA levels of anti-apoptotic gene *Mcl-1* nor pro-apoptotic genes *Bak* and *Bax* upon stabilisation of NIK. Since neither a defect in proliferation nor apoptosis could be detected, it might be of interest to further investigate Treg cell exhaustion and/ or anergy. Additionally, decreased percentages of NIK-deficient tissue Treg cells was not due to downregulation of Foxp3. This is not surprising since non-canonical NF-κB transcription factors, until now, were not shown to bind to the CNS locus of Foxp3^{+} Treg cells (Lee and Lee, 2018).

Generally, it is suggested that naïve Treg cells migrate between secondary lymphoid organs and the blood circulation, while effector Treg cells tend to migrate into peripheral tissues upon reactivation by cognate antigens in secondary lymphoid organs (Estrada Brull, Panetti and Joller, 2022). Yet the question arises of whether NIK is required for adequate migration behaviour of Treg cells into tissues. Of note, we observed that frequencies of CD44^{high} effector Treg cells did not differ in the peripheral lymphoid organs and tissues, as exemplified for the liver, between NIK$^{\Delta Treg}$ and control mice. Moreover, Lacher and colleagues found that NIK-deficiency did not affect antigen-specific activation and subsequent migration of antigen-specific 2D2 T cells into the CNS (Lacher *et al.*, 2018). Additionally, comparing bone marrow chimeras generated from bone marrow of *aly/aly* and WT-like mice revealed that *aly/aly* lymphocytes home properly to peripheral lymph nodes (Miyawaki *et al.*, 1994). Interestingly, while we did not observe a reduction of CD44^{high} effector Treg cells in the liver, we did nonetheless observe a decrease of CD103^{+} Treg cells in the colon as well as in the spleen of NIK$^{\Delta T}$ mice. CD103 is an integrin expressed on activated effector Treg cells that home to peripheral tissues and sites of inflammation (Huehn *et al.*, 2004; Guo *et al.*, 2008; Smigiel *et al.*, 2014). This supports very well previous observations that NIK is required for Teff cell and in this case effector Treg cell maintenance.

In the colon, Foxp3^{+} Treg cell populations comprise of thymus-derived Helios^{+} and microbiota-induced RORγt^{+} Treg cells (Ohnmacht *et al.*, 2015; van der Veeken *et al.*, 2022). In tissues, Treg cells exert additional tasks to their canonical functions utilised in secondary lymphoid organs. While the Helios^{+} tTreg cell population is suggested to participate in tissue homeostasis and repair, the RORγt^{+} pTreg cell subset mediates tolerance against the commensal microbiome (Panduro, Benoist and Mathis, 2016). Here, we show that particularly the Helios^{+} tTreg cell subset is decreased in the colon in both NIK$^{\Delta Treg}$ and NIK$^{\Delta T}$ strains. The liver is connected to the colon through the portal vein but in contrast to the colon harbours effector Treg cells that are mainly thymus-derived Helios^{+} Treg cells (Chen *et al.*, 2016;

Delacher *et al.*, 2017). In addition to the colon, we saw that the Helios$^+$ Treg cell population is also reduced in the liver. Peripheral tissues are a rather harsh environment where immune cells constantly encounter foreign antigens and in which pro-inflammatory cytokines such as TNF are present already during homeostasis (Panduro, Benoist and Mathis, 2016; Delgado and Brunner, 2019). Colonic Helios$^+$ Treg cells co-express GATA-3 that Treg cells require to be maintained in an inflammatory environment and to be functionally competent (Wohlfert *et al.*, 2011). Helios has been reported to increase Foxp3 stability and is mainly co-expressed in effector Treg cell subsets amongst others CD103$^+$ and TNFR2$^+$ Treg cells (Thornton *et al.*, 2019). Moreover, upon co-transfer of WT and NIK-deficient Treg cells into Rag1$^{-/-}$ mice we noted a reduction of NIK-deficient TNFR2$^+$ Treg cells. *In vitro*, we showed that NIK-deficient Treg cell numbers did not increase upon TNF treatment. This provides evidence that NIK is required for the expansion of TNFR2$^+$ Treg cells through TNF - TNFR2 axis. Indeed, as mentioned above, TNF induces NIK stabilisation and subsequent p100 processing in TNFR2$^+$ human Treg cells (Rauert *et al.*, 2010). TNFR2 equips Treg cells for expansion under inflammatory conditions, since synergistic stimulation with TNF and IL-6 resulted in increased expansion in comparison to treatment with either TNF or IL-6 alone (Chen *et al.*, 2013). Moreover, TNFR2 agonists expanded in particular thymic-derived Treg cells *in vivo* and rendered them more suppressive (Chopra *et al.*, 2016; Fischer *et al.*, 2018; Lamontain *et al.*, 2019). Hence, these data provide further evidence that NIK is required for the maintenance of effector Treg cells, such as TNFR2$^+$ Treg cells, under harsh conditions such as in disease state or in peripheral tissues.

Thymic-derived tissue Treg cells are thought to mediate tissue homeostasis, regeneration and repair (Estrada Brull, Panetti and Joller, 2022). Although we saw a reduction of thymus-derived Helios$^+$ Treg cells in the colon as well as in the liver of NIK$^{\Delta Treg}$ mice, histological analyses displayed signs of inflammation neither for the colon nor the liver under homeostatic conditions. These observations propose that NIK is dispensable for the maintenance of tissue homeostasis in steady state mediated by Treg cells. Of note, we found that the colonic RORγt$^+$ subpopulation remained unaffected by NIK deficiency and potentially compensated for the reduced Helios$^+$ Treg cell population in the prevention of inflammation. Indeed, RORγt$^+$ Treg cells were reported to be the major cell type restricting colonic inflammation (Yang *et al.*, 2016). In line with this, we could not detect increased neutrophil, inflammatory monocyte and total CD4$^+$ T cell populations within the colonic lamina propria of NIK$^{\Delta Treg}$ mice. In addition to Foxp3$^+$ Treg cells, other cell populations such as IL-10-producing Tr1 and TGF-β-producing Th3 cells exert regulatory functions (Yu *et al.*, 2021). These populations were not analysed but might contribute to further intestinal homeostasis and prevention of inflammation. Surprisingly, we did note an increase in IFN-γ-producing Th1 cells in the colon of NIK$^{\Delta Treg}$ mice although no other signs of inflammation could be detected neither by histology nor by FACS. IFN-γ is mainly associated with driving intestinal pathologies as a pro-inflammatory cytokine (Langer *et al.*, 2019). However, it was shown that

IFN-γ also has protective functions for example by ameliorating cartilage damage and joint inflammation in a murine arthritis model (Lee *et al.*, 2017). Hence, a shifted balance towards increased IFN-γ levels might not necessarily result in tissue damage. Overall, we provide evidence that NIK is dispensable for tissue homeostasis mediated by Treg cells under steady state conditions.

4.3 NIK is essential for Treg cells to suppress T cell transfer colitis

NIK has been reported to be dispensable for *in vitro* Treg cell suppressive function (Murray, 2013). In line with this, we confirmed that NIK is not required for the suppression of proliferation of VCT-labelled conventional $CD4^+$ T cells *in vitro*. Of note, conditional overexpression of NIK in $Foxp3^+$ Treg cells resulted in defective suppressive capacity of Treg cells *in vitro* along with the production of pro-inflammatory cytokines by Treg cells. The authors further reported that a small population of mice with enforced expression of NIK in $Foxp3^+$ Treg cells died between the age of 6 and 8 months due to severe lung inflammation (Polesso *et al.*, 2017). Yet, overexpression models are very artificial. To date though, no publications have performed functional assays assessing the *in vivo* suppressive capacity of NIK-deficient Treg cells.

We are the first to report that NIK-deficient Treg cells failed to prevent T cell-mediated colitis in $Rag1^{-/-}$ mice. IKKα is a downstream signalling partner of NIK (Sun and Ley, 2008). Expectedly, it was demonstrated that IKKα-deficient Treg cells showed hampered ability in suppressing a T cell-mediated colitis in $Rag1^{-/-}$ mice (Chen *et al.*, 2015). As mentioned above, signalling through TNFR superfamily members such as TNFR2 induces NIK stabilisation (Rauert *et al.*, 2010). TNFR2 was shown to be essential for Treg cells to prevent T cell transfer colitis (Chen *et al.*, 2013). Interestingly, the TNF - TNFR2 axis was reported to be crucial for tTreg but not iTreg cells in the prevention of transfer colitis (Housley *et al.*, 2011). Furthermore, the importance of TNFR2 for Treg cell suppressive capacity was shown in prevention of EAE and GvHD, just to name a few (Chopra *et al.*, 2016; Atretkhany *et al.*, 2018).

When we performed T cell transfer colitis, we saw that $Rag1^{-/-}$ mice that received NIK-deficient Treg cells succumbed to weight loss characterised by mucosal inflammation and crypt damage on a histological scale. On cellular level analysed by FACS, NIK-deficient Treg cells could not prevent the expansion of colonic Th1 and Th17 cells. These findings support that NIK-deficient Treg cell did not suppress T cell transfer colitis. Yet, while Treg cell proliferation did not seem to be affected assessed by Ki67 staining, colonic Treg cell numbers were equal between $Rag1^{-/-}$ mice that received either WT or NIK-deficient Treg cells. A pro-inflammatory environment, however, leads to an increase in $Foxp3^+$ Treg cells since their expansion depends on for example IL-2 consumption (Fontenot *et al.*, 2005). Hence, if NIK-deficient Treg cells were expanded normally, an increase of NIK-deficient Treg cells over WT Treg cells should have been observed, since WT Treg cells were not exposed to inflammation as their suppressive

capacity was not affected. Thus, NIK seems to be important for Treg cell maintenance under inflammatory conditions which, when affected, results in abrogated suppressive capacity of the Treg cell population as an adequate expansion cannot be maintained. Because proliferation does not seem to be altered in NIK-deficient Treg cells, a possible explanation might be that NIK-deficient Treg cells are more prone to apoptosis. Indeed, T cells, in this case thymocytes, lacking NIK downstream molecule IKKα were shown to proliferate normally but were more susceptible to apoptosis (Ren *et al.*, 2002). In line with this, already in steady state, we saw impaired Treg cell maintenance in tissues of $NIK^{\Delta Treg}$ mice characterised by decreased frequencies and numbers of $Foxp3^+$ Treg cells as well as $Helios^+$ Treg cells. When we co-transferred WT and NIK-deficient Treg cells into the same $Rag1^{-/-}$ host, we noted a massive defect in expansion of NIK-deficient Treg cells in comparison to WT Treg cells. Moreover, we reported above that tissue-resident $Foxp3^+$ Treg cells showed normal proliferative capacity as assessed by Ki67 staining. Anew, defective expansion of NIK-deficient Treg cells along with Teff cells under inflammatory conditions results in colitis development in $Rag1^{-/-}$. Yet, the question remains whether NIK-deficient Treg cells were suppressive if injected in large numbers comparable to those of WT Treg cells under inflammatory conditions. In steady state, we showed that deletion of NIK in $Foxp3^+$ Treg cells did not result in the development of spontaneous colitis of the $NIK^{\Delta Treg}$ strain. H&E stainings did not reveal any signs of colonic inflammation. In addition, $NIK^{\Delta Treg}$ mice did not succumb to a scurfy phenotype characterised by dysfunctional Treg cells resulting in multi-organ infiltration as described in previous reports (Brunkow *et al.*, 2001). Moreover, NIK-deficient Treg cells subjected to the T cell transfer colitis experiment showed normal expression levels of the suppressive marker CTLA-4 and stable Foxp3 expression. This suggests that under steady state conditions NIK is dispensable for Treg cells to maintain homeostasis. In summary, however, we provide evidence that NIK is critically required for the maintenance of a suppressive Treg cell population under inflammatory conditions during colitis.

5 Summary

While NIK has been reported to be dispensable for the activation of naïve CD4$^+$ T cells, it was shown to play a crucial part in the maintenance and function of Teff cells. However, its role in thymic development of Treg cells and in the maintenance of Treg cells in lymphoid organs remains controversial. Moreover, the requirement of NIK for Treg cells resident in peripheral tissues such as the colon or the liver as well as *in vivo* suppression have not been addressed yet.

By conditionally deleting NIK in αβ T cells (NIK$^{\Delta T}$) in C57BL/6 mice, we detected the requirement for NIK in thymic CD25 TregP and Foxp3^{lo} TregP along with a reduction in mature Foxp3$^+$ Treg cells in the thymus, secondary lymphoid organs and peripheral tissues. Yet, flow cytometric analysis revealed normal numbers of Foxp3$^+$ Treg cells in the thymus and secondary lymphoid organs but a reduction of Foxp3$^+$ Treg cells peripheral tissues such as the colon and the liver in mice with conditional deletion of NIK in Foxp3-expressing cells (NIK$^{\Delta Treg}$). In particular, a reduction of the thymus-derived Helios$^+$ Treg cell population was detected in the colon of these mice. Nevertheless, histological analysis of colonic and hepatic tissue sections from NIK$^{\Delta Treg}$ mice did not show any signs of inflammation. *In vitro* stimulation with IL-2 did not reveal a defect in proliferation of NIK-deficient Treg cells. Besides, Ki67 staining showed normal proliferation of both *ex vivo*-isolated NIK-deficient and NIK overexpressing Treg cells. Although *in vitro* NIK stabilisation in WT Treg cells resulted in normal mRNA levels of apoptosis-related genes *Mcl-1*, *Bak* and *Bax*, co-transfer of WT and NIK-deficient Treg cells into the same lymphopenic host revealed a striking defect in the maintenance of NIK-deficient Treg cells. In particular, the maintenance of TNFR2$^+$ Treg cell population was observed to depend on NIK signalling. Indeed, *in vitro* stimulation of splenocytes with TNF expanded WT but not NIK-deficient Treg cells. Importantly, while NIK-deficient Treg cells were able to suppress Tconv cells *in vitro*, they failed to prevent T cell-mediated colitis in Rag1$^{-/-}$ mice *in vivo*.

In summary, being a critical factor for TregP maintenance, our data suggest a novel role for NIK in thymic Treg cell development. While NIK is dispensable for the maintenance of Treg cells in lymphoid organs, it is crucial, however, for the maintenance of certain effector Treg cell subsets residing in peripheral tissues and lymphopenic hosts. Although there is no requirement for NIK in Treg cell-mediated tissue homeostasis of the colon and the liver in steady state, it is essential for Treg cells to suppress T cell-mediated colitis *in vivo*.

6 Zusammenfassung

Für konventionelle T-Zellen ist die Funktion der NF-κB-induzierenden Kinase (NIK) gut erforscht; für die Aktivierung naiver $CD4^+$-T-Zellen ist sie entbehrlich, spielt aber eine entscheidende Rolle bei der Erhaltung und Funktion von konventionellen Effektor-T-Zellen. In Bezug auf regulatorische T-Zellen hingegen existiert kein Konsensus darüber, ob NIK eine zellintrinsische Funktion in der Erhaltung von regulatorischen T-Zellen in lymphoiden Organen spielt. Darüber hinaus ist umstritten, ob NIK für die Entwicklung von regulatorischen T-Zellen im Thymus erforderlich ist. Desweiteren wurde bis dato nicht erforscht, ob regulatorische T-Zellen NIK für das Überleben in periphären Geweben wie zum Beispiel Kolon und Leber sowie für die Prävention einer T-Zell-vermittelten Kolitis benötigen.

Deletion von NIK in αβ-T-Zellen ($NIK^{\Delta T}$) in C57BL/6-Mäusen resultierte in einer Reduktion der zwei thymischen Vorläuferpopulationen von regulatorischen T-Zellen; den CD25 TregP und den $Foxp3^{lo}$ TregP. Diese Observation ging einher mit einer weiteren Reduktion von gereiften $Foxp3^+$ regulatorischen T-Zellen im Thymus, in den sekundären lymphoiden Organen sowie in den periphären Geweben. In Mäusen mit einer Deletion von NIK in Foxp3-exprimierenden Zellen ($NIK^{\Delta Treg}$) konnte hingegen durch durchflusszytometrische Analysen keine Verringerung an regulatorischen T-Zellen in primären und sekundären lymphoiden Organen, jedoch in periphären Geweben wie Kolon und Leber festgestellt werden. Inbesondere die aus dem Thymus stammende Subpopulation der $Helios^+$ regulatorischen T-Zellen war im Kolon nur vermindert zu detektieren. Histologische Analysen konnten eine zellintrinsische Funktion von NIK in regulatorischen T-Zellen bei der Erhaltung der Gewebshomöostase des Kolons und der Leber in $NIK^{\Delta Treg}$-Mäusen unter Normalbedingungen ausschließen. Trotz einer Verminderung der Anzahl der NIK-defizienten regulatorischen T-Zellen in den Geweben, konnte anhand einer *in vitro*-Stimulation mit IL-2 kein Defekt in Bezug auf Proliferation festgestellt werden. Auch eine Ki67-basierte Färbung identifizierte normal proliferierende *ex vivo*-isolierte NIK-defiziente und NIK-überexprimierende regulatorische T-Zellen. NIK-Defizienz in regulatorischen T-Zellen führte daher nicht zu einer beeinträchtigten Proliferation und auch die *in vitro*-Stabilisierung von NIK in regulatorischen T-Zellen aus Wildtyp-Mäusen resultierte in unveränderten mRNA-Spiegeln von Apoptosegenen *Mcl-1*, *Bak* und *Bax*. Der Ko-Transfer von regulatorischen T-Zellen aus Wildtyp-Mäusen und NIK-defizienten regulatorischen T-Zellen in einen lymphopenischen Wirt zeigte jedoch, dass NIK-defiziente regulatorische T-Zellen, insbesondere TNFR2-positive regulatorische T-Zellen, einen akuten Defekt in der Erhaltung aufweisen. In der Tat expandierte die *in vitro*-Stimulation mit TNF regulatorische T-Zellen aus Wildtyp-Mäusen aber nicht NIK-defiziente regulatorische T-Zellen. Letztlich konnten NIK-defiziente regulatorische T-Zellen, trotz gegebener supprimierender Funktion *in vitro*, die Entwicklung einer T-Zell-mediierten Kolitis in $Rag1^{-/-}$-Mäusen nicht verhindern.

Zusammenfassend suggerieren die hier erarbeiteten Ergebnisse eine bis dato unerforschte Rolle von NIK in der Aufrechterhaltung von thymusresidenten regulatorischen T-Zell-Vorläuferpopulationen und somit in der Entwicklung von regulatorischen T-Zellen im Thymus. Obwohl NIK für die Aufrechterhaltung von regulatorischen T-Zellen in lymphoiden Organen keine notwendige Rolle spielt, ist die Aufrechterhaltung bestimmter regulatorischer Effektor-T-Zell-Populationen in periphären Organen wie Kolon und Leber sowie in lymphophenischen Wirten hingegen stark abhängig von NIK. Wenngleich regulatorische T-Zellen NIK für den Erhalt der Gewebshomeöstase von Leber und Kolon im Gleichgewichtszustand nicht benötigen, so hat diese Kinase in regulatorischen T-Zellen jedoch eine essentielle Funktion in der Prävention von T-Zell-mediierter Kolitis in Rag1$^{-/-}$-Mäusen *in vivo*.

7 References

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9 Versicherung

Hiermit versichere ich gemäß § 12, (2) der Promotionsordnung vom 01.04.2018: Ich habe die heute als Dissertation vorgelegte Arbeit selbst angefertigt und ausschließlich die angegebenen Quellen und Hilfsmittel verwendet. Ich habe oder hatte die jetzt als Dissertation vorgelegte Arbeit noch an keiner anderen deutschen oder ausländischen Hochschule oder vergleichbaren Einrichtung zur Erlangung eines akademischen Grades eingereicht. Ich habe noch kein Promotions- PhD,- oder ein vergleichbares Graduierungsverfahren im Promotionsfach erfolglos beendet. Ich habe noch kein Promotions- PhD,- oder ein vergleichbares Graduierungsverfahren im Promotionsfach erfolgreich beendet. Für die Anfertigung der vorgelegten Arbeit wurde keine entgeltliche Hilfe Dritter, insbesondere eine Promotionsberatung oder –vermittlung in Anspruch genommen. Die vorliegende Doktorarbeit wurde von Prof. Dr. [redacted] (Erstbetreuer) und Prof. Dr. [redacted] (Zweitbetreuerin) betreut.

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11 Publications

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