The Role of IKK-induced NF-κB1 p105 Proteolysis in T Lymphocytes

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A thesis presented for the degree of Doctor of Philosophy of the University of London, 2008

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Statement of Declaration

I, Srividya Sriskantharajah, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Proteolysis of NF-κB1 p105 is vital for its function as a precursor to p50 and as an IκB. This occurs in two ways, both mediated by the proteasome. A constitutive proteolytic removal of the p105 C-terminus, termed processing, generates the mature transcription factor p50. In contrast, a signal-induced p105 proteolysis is triggered by phosphorylation of serines 927 and 932 in the p105 PEST region by the IKK complex. This promotes p105 poly-ubiquitination and subsequent complete degradation.

IKK-induced p105 proteolysis has been demonstrated to regulate the kinase activity of the MAP3K TPL-2, since all detectable TPL-2 is found in a complex with p105. Furthermore, NF-κB1 p105 retains Rel subunits in the cytoplasm *via* interaction with the p105 C-terminal ankyrin repeat region. However, it is unclear whether IKK-induced p105 proteolysis contributes to NF-κB activation, though this process would be expected to release Rel subunits to translocate into the nucleus.

A large body of evidence exists to suggest a major role for NF-κB in T cell development and function. To investigate the significance of IKK-induced p105 degradation in T cells, a knock-in mouse strain, *Nfkb1*^{S927A,S932A}, in which serines 927 and 932 of NF-κB1 p105 were mutated to alanine residues was analysed. Previous work has shown that constitutive processing of p105^{S927A,S932A} to p50 occurs normally, but this mutated p105 is refractory to IKK-induced proteolysis.

Work presented here demonstrates that whilst p105 mutation did not affect thymic differentiation of $CD4^+$ and $CD8^+$ T cells, numbers of $CD4^+CD25^+$ regulatory T cells, memory-phenotype $CD4^+$ T cells and thymic NKT cells were significantly reduced. Analysis of BM chimeras revealed cell autonomous and non-haematopoietic defects required for generation of these sub-populations. *In vitro* experiments indicated that TCR-induced proliferation was significantly impaired in *Nfkb1*^{S927A,S932A} $CD4^+$ T cells, due partly to reduced interleukin-2 production. In contrast, p105 mutation had no effect on $CD4^+$ T cell survival. These defects were not due to a lack of TPL-2 activity, based on analysis of TPL-2-deficient mice.

This study presents evidence to suggest a critical role for IKK-induced p105 proteolysis in regulating NF-κB activation in T lymphocytes.

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Abbreviations

α	Antibody
d	Day
°C	Degrees Celsius
h	Hour
kDa	Kilo Dalton
K_d	Dissociation constant
min	Minute
ml	Millilitre
µl	Microlitre
ng	Nanogram
%	Percentage

αGal–Cer	α–galactosylceramide
ABIN-2	A20-binding inhibitor of NF-kB-2
AICD	Activation induced cell death
AP-1	Activator protein 1
APC	Antigen presenting cell
BAFF	B cell activating factor
BCL2	B cell lymphoma-2
BCL10	B cell lymphoma-10
β-TRCP	β-transducin repeat containing protein
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophages
BRDU	5-Bromo-2-deoxyuridine
CARD	Caspase recruitment domain
CARMA1	CARD/MAGUK 1
CFSE	Carboxyfluorescein succinimidyl ester
cSMAC	Central supramolecular activation cluster
CBM	CARMA1/BCL10/MALT1

CTLA-4	Cytotoxic T–lymphocyte antigen–4
CYLD	Cylindromatosis
DAG	Diacyl glyceroll
DC	Dendritic cell
DD	Death domain
DN	Double negative (CD4-CD8-)
DP	Double positive (CD4+CD8+)
DUB	Deubiquitinase
EAE	Experimental autoimmune encephalomyelitis,
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal related kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FOXP3	Forkhead box protein P3
GEF	Guanine exchange factor
GITR	Glucocorticoid–induced T–lymphocyte receptor
GMCSF	Granulocyte-macrophage colony stimulating factor
GRR	Glycine-rich region
HSC	Haematopoietic stem cells
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X–linked
IRAK	Interleukin-1 receptor-associated kinase 4
JAK	Janus-associated kinase
JNK	c-Jun amino-terminal kinase
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-associated motifs

LAT	Linker for activation of T cells
LN	Lymph nodes
LPS	Lipopolysaccharide
LTβ	Lymphotoxin β
MAGUK	Membrane-associated guanylate kinase 1
MALT1	Mucosal-associated lymphoid tissue 1
MAP3K	Mitogen activated protein kinase kinase kinase
MEK	MAP kinase/ERK kinase
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NFAT	Nucleating factor of activated T cells
NF-κB	Nuclear factor of κB
NIK	NF-κB-inducing kinase
NK	Natural killer cells
NKT	Natural killer T cells
NLS	Nuclear localisation signal
PAMP	Pathogen-associated molecular patterbn
PBS	Phosphate buffered solution
PDK1	Phosphoinositide-dependent kinase1
PDBU	Phorbol dibutyrate
PEST	Proline-Glutamic Acid-Serine-Threonine
PI-3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PRR	Pattern recognition receptor
pSMAC	Peripheral supramolecular activation clusters
RAG	Recombination activating genes

RANTES	Regulated upon activation, normal T cell expressed and secreted
RHD	Rel homology domain
RIP	Receptor interacting protein
ROR	Retinoic acid receptor (RAR)–related orphan receptor
SCF	Skp1/Cil-1/F-box
SEM	Standard error of the mean
SLP-76	SH2-domain-containing leukocyte protein of 76kDa
SOCS	Suppressors of cytokine signalling
spMHC	Self-peptide:MHC complex
SP	Single positive ($CD4^+$ or $CD8^+$)
SRC	SH2 region containing
STAT	Signal transducers and activators of transcription
SYK	Spleen tyrosine kinase
T- BET	T-box expressed in T cells
TAD	Transcriptional activation domain
TAK1	TNFR-associated kinase-1
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cells
Th-POK	Thelper-inducing PO/Kruppel factor
TLR	Toll-like receptor
TMEM	Memory T cells
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TPL-2	Tumour progression locus-2
T_{REG}	Natural regulatory T cells
Vα14i NKT	CD1d–restricted NKT cells
VCAM	Vascular cell adhesion molecule
WT	Wild-type
ZAP-70	ζ-chain associated protein of 70kDa

1. Introduction

1. Introduction

1.1 NF–κB transcription factors

Inducible regulation of gene expression is critical in responding to environmental changes and in maintaining homeostasis. Important in this role are the nuclear factor–κB (NF–κB)/Rel proteins. NF–κB was first described as a nuclear protein required for the B cell-specific transcription of the immunoglobulin kappa light gene (Sen and Baltimore, 1986). Subsequent research has identified the NF–κB proteins as a family of evolutionarily conserved inducible transcription factors, which regulate a wide range of genes involved in the immune response and inflammation. Therefore, dysregulated NF–κB activation is associated with a variety of chronic inflammatory disorders, including asthma, rheumatoid arthritis and inflammatory bowel disease (reviewed in (Barnes and Karin, 1997)). In addition, a role for NF–κB in human cancers linked to chronic inflammation is now emerging (Karin and Greten, 2005).

NF–κB activation In unstimulated cells, NF–κB are found as dimers in the cytoplasm, retained in an inactive state through association with inhibitors of NF–κB (IκBs) (Baeuerle and Baltimore, 1988, Baeuerle et al., 1988). IκBs bind and mask the nuclear localisation signals (NLS) of NF–κB transcription factors, inhibiting their translocation to the nucleus and thus their transcriptional activity. A range of agonists including cytokines, antigens and pathogen–derived molecules triggers NF–κB induction (Beinke and Ley, 2004). Activation of NF–κB proceeds

through a cascade of phosphorylation, ubiquitination and degradation events, resulting in the destruction of IκB (reviewed in (Ghosh et al., 1998) (see Figure 1.1). Released NF–κB dimers can then enter the nucleus and bind their cognate response elements in the promoters and enhancers of target genes (Kunsch et al., 1992). From these κB sites, NF–κB transcription factors can recruit corepressors or coactivators, thereby modulating gene expression.

1.1.1 NF–κB/Rel proteins

1.1.1.1 Structure

The term NF–κB refers to a family of transcription factors, composed of dimeric complexes of NF–κB/Rel proteins (see Figure 1.2). Five such proteins, transcribed from separate genes, have been described in mammals; NF–κB1 p50, NF–κB2 p52, RelA (p65), RelB and c–Rel (Sen and Baltimore, 1986, Ruben et al., 1991). NF–κB proteins can form homo– or heterodimers with each other, with the exception of RelB, which can only form heterodimers with p50 and p52 (Baeuerle and Henkel, 1994). NF–κB proteins are characterised by the presence of an N–terminal 300–amino acid region, known as the Rel–homology domain (RHD), which is vital for their function, mediating subunit–dimerisation, nuclear localisation and binding to DNA (Ghosh et al., 1998).

RelA, RelB and c–Rel are transcribed as mature transcription factors, and contain a C–terminal transcriptional activation domain (TAD), which recruits the coactivators necessary to promote gene transcription (Sheppard et al., 1999). In

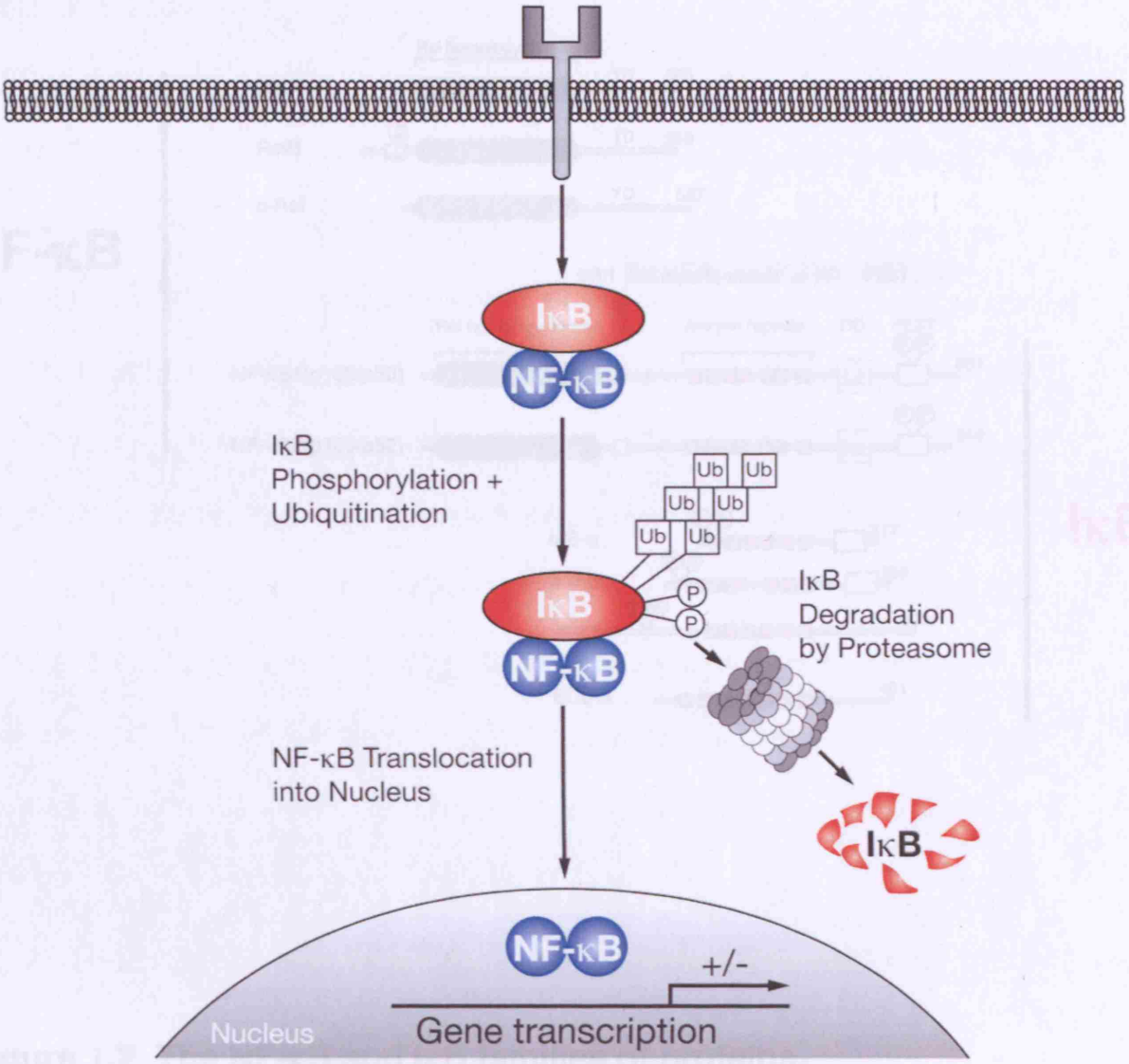


Figure 1.1 General mechanism of NF-κB activation

A number of agonists induce the phosphorylation (P) of IκB proteins, and trigger the subsequent polyubuiquitination (Ub) of IκBs by an E3 ligase complex. This modification targets IκBs for degradation by the 26S proteasome. Associated NF-κB dimers are released to enter the nucleus and bind to κB elements in the promoter of target genes, regulating transcription. Abbreviations: IκB, inhibitor of NF-κB; NF-κB, nuclear factor κB.

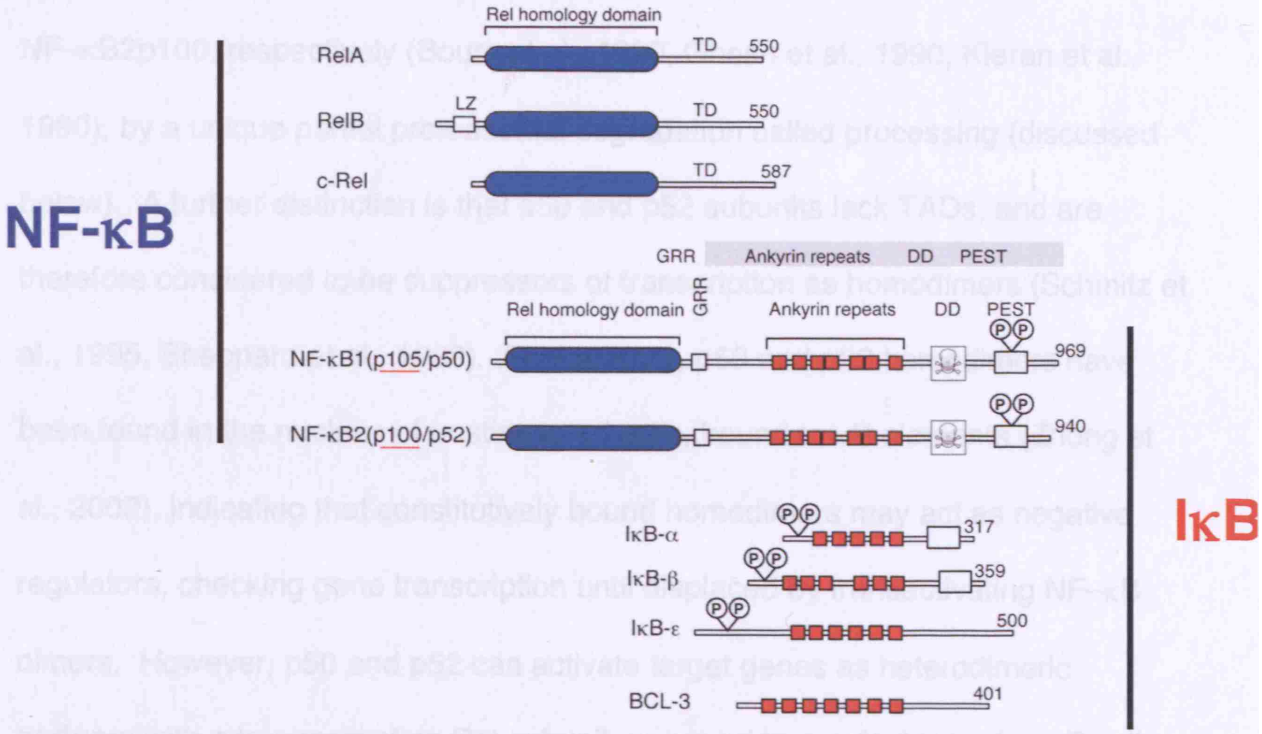


Figure 1.2 The NF-κB and IκB families of proteins.

Rel/nuclear factor-κB (NF-κB) proteins in mammals consists of RelA (also known as p65), RelB, c-Rel, p50 and p52 and are characterized by their N-terminal Rel homology domain (RHD;blue). The products of the *Nfkb1* and *Nfkb2* genes are the long p105 and p100 proteins, which are precursors of p50 and p52, respectively. These proteins dimerize in varous combinations to form NF-κB ttranscription factor complexes. The IκB proteins contain multiple ankyrin repeats (red), which bind the RHD of NF-κB subunits, masking the nuclear localisation signal to prevent their nuclear translocation. This family includes p105 and p100. The number of amino acids in each protein is indicated. DD, death domain; GRR, glycone rich-region; LZ, leucine zipper; TD, transcativation domain; PEST, proline-glutamic acid-serine-threonine enriched region.

contrast, p50 and p52 are generated from the longer precursors NF–κB1p105 and NF–κB2p100, respectively (Bours et al., 1990, Ghosh et al., 1990, Kieran et al., 1990), by a unique partial proteasomal degradation called processing (discussed below). A further distinction is that p50 and p52 subunits lack TADs, and are therefore considered to be suppressors of transcription as homodimers (Schmitz et al., 1995, Sheppard et al., 1999). Furthermore, p50 and p52 homodimers have been found in the nucleus of unstimulated cells, bound to κB elements (Zhong et al., 2002), indicating that constitutively bound homodimers may act as negative regulators, checking gene transcription until displaced by transactivating NF–κB dimers. However, p50 and p52 can activate target genes as heterodimeric partners with a transactivating Rel subunit, or bound to a cofactor such as B cell lymphoma 3 (Bcl–3) or IκBζ (Li and Verma, 2002, Dechend et al., 1999, Kerr et al., 1992).

1.1.1.2 Specificity of NF–κB dimers

The role of Rel proteins appears to be both cell–type specific and distinct, as demonstrated by the different phenotypes of Rel–knockout mice (Gerondakis et al., 2006) (summarised in Table 1.1). This can partly be explained by the different expression patterns of Rel subunits. For example, RelA and p50 are widely expressed, whilst RelB appears to be restricted to B cells, regions of the thymus, lymph nodes (LN) and Peyer's patches (Gerondakis et al., 1999). Expression of c–Rel is also limited, with levels highest in haematopoietic cells and lymphocytes (Li

and Verma, 2002). However, NF–κB dimers also show specificity for the genes they regulate, achieved by the degenerate nature and variable sequences of the κB site, such that different NF–κB dimers display different binding affinities to κB consensus regions (Leung et al., 2004, Fujita et al., 1992, Kunsch et al., 1992). An additional level of complexity has been shown by work carried out in the Baltimore lab, which reveal that mutation of just one base–pair in the κB site can alter the coactivators recruited by NF–κB dimers, and subsequently change the expression of the gene (Leung et al., 2004). These data suggest that the κB site not only determines NF–κB specificity, but also conformation of the bound NF–κB dimers.

1.1.1.3 Post–translational modifications

Although degradation of IκB proteins is the key step for NF–κB activation, a number of groups have identified post–translational modifications, including phosphorylation and acetylation, that can modify the transcriptional activity of NF–κB (reviewed in (Perkins, 2006)). The regulated phosphorylation of RelA has been studied most extensively, and been demonstrated to enhance interactions with transcriptional coactivators such as CREB–binding protein (CBP) (Zhong et al., 1998).

1.1.2 IκB proteins

1.1.2.1 Structure

The subcellular localisation of NF–κB is controlled by the IκB family of proteins, which are characterised by the presence of multiple ankyrin repeats (see Figure

1.2). Crystal structures for IκB bound to RelA/p50 dimers have revealed that it is through these ankyrin motifs that IκBs interact with the RHD of NF–κB proteins, sequestering the NLS (Huxford et al., 1998, Jacobs and Harrison, 1998).

Nine IκB proteins have been identified, and can be generally divided into three groups (Ghosh et al., 1998). Firstly, the classical IκBs, which contain N–terminal regulatory regions required for stimulus–induced degradation; IκBα, IκBβ, and IκBε (Haskill et al., 1991, Davis et al., 1991). The second group are the NF–κB1 p105 and NF–κB2 p100 precursors, which contain a C–terminal region that regulates their signal-induced proteolysis (Liou et al., 1992). Finally, are the nuclear IκB proteins; B cell lymphoma (Bcl)–3, IκBζ and $IκB_{NS}$ (Kerr et al., 1992).

1.1.2.2 NF–κB binding

Interaction studies have demonstrated that IκBs display different affinities to the NF–κB dimers to which they bind (Whiteside et al., 1997). IκBα predominantly binds RelA/p50 or RelA/c–Rel heterodimers, and has low affinity for p50 homodimers. In contrast, p105 readily binds p50 homodimers, and seems to be their main inhibitor (Liou et al., 1992, Ishikawa et al., 1998). Furthermore, RelB can only bind p100, which is critical in the alternative pathway of NF–κB activation *(Section 1.1.4)*. However, p100 has broad affinity for Rel subunits, and a previously uncharacterised pathway has recently been described, where p100 can act as a classical IκB, with LTβ able to activate RelA/p50 dimer release (Basak et al., 2007).

1.1.2.3 Kinetics of IκB degradation and resynthesis

Genetic studies have revealed both distinct and redundant functions for IκBα, IκBβ, and IκBε in mouse embryonic fibroblasts (MEFs) (Gerondakis et al., 2006), despite similar NF–κB dimer binding specificities. These differences have been suggested to be due to IκB isoform–specific kinetics of degradation and resynthesis, which then determines the nature of NF–κB activity (Hoffmann et al., 2002). For example, signal–induced IκBα proteolysis occurs rapidly, in a matter of minutes, and newly synthesised IκBα is detectable an hour after stimulation (Ghosh and Karin, 2002). The result is a transient expression of NF–κB after IκBα proteolysis. Conversely, degradation and resynthesis of IκBβ has been observed to be slower than for IκBα, leading to sustained NF–κB activity (Thompson et al., 1995). This suggests differences in regulation of these proteins, which is underlined by the finding that IκBβ cannot compensate for a lack of IκBα, except when placed under the control of the IκBα promoter (Cheng et al., 1998). Consistent with this observation, NF–κB regulates the transcription of IκBα, but not IκBβ. Furthermore, different signals may be required to trigger activation of NF–κB bound to different IκBs. Indeed, IκBβ requires a stronger stimulus than IκBα for degradation, which in T cells can be provided by CD28 costimulation (Harhaj et al., 1996b). Therefore, the nature of NF–κB activity is regulated both by the activating signal and the IκB protein to which dimers are bound.

1.1.2.4 IκBα termination of NF–κB activation

Newly synthesised IκBα can attenuate NF–κB signalling by entering the nucleus, due to the presence of a NLS on IκBα, to competitively remove DNA–bound NF–κB subunits (Arenzana-Seisdedos et al., 1997). These IκB:NF–κB dimer complexes are then returned to the cytoplasm *via* nuclear export signals (NES) on IκBα proteins, eventually returning the cell to a quiescent state (Arenzana-Seisdedos et al., 1997). Consequently, in the absence of IκBα, a marked delay in termination of NF–κB activation is observed in haematopoietic cells (Klement et al., 1996, Beg et al., 1995a). Furthermore, IκBα is a transcriptional target for NF–κB, creating a negative feedback loop whereby the transcription of IκBα by NF–κB also terminates NF–κB activity (Sun et al., 1993). The result is an oscillation in levels of active nuclear NF–κB dimers, a characteristic of NF–κB signalling which is important in maintaining expression of NF–κB–dependent genes (Nelson et al., 2004).

1.1.3 Canonical pathway of NF–κB activation

Degradation of IκB is a necessary step for NF–κB activation. This event is triggered by phosphorylation of IκB, which targets the protein for K48–linked polyubiquitination and subsequent destruction by a proteolytic enzyme complex, the proteasome (Alkalay et al., 1995b, Alkalay et al., 1995a, DiDonato et al., 1996). The most studied pathway leading to NF–κB activation, known as the canonical pathway, is induced by a wide range of stimuli and leads to degradation of the

prototypical inhibitor, IκBα (see Figure 1.3). The NF–κB dimers bound by IκBα, predominantly RelA/p50 and p50/c–Rel heterodimers, are then released to translocate to the nucleus (Beg et al., 1995a). The degradation of IκBβ and IκBε appear to be regulated in a similar manner (Li and Verma, 2002).

1.1.3.1 Phosphorylation

Phosphorylation of IκBs is mediated by the IκB kinase (IKK) complex, which is rapidly activated after agonist stimulation. This complex consists of the NF–κB essential modulator (NEMO or IKKγ) an adaptor protein required for the activation of the canonical pathway (Li and Verma, 2002), and the kinase subunits IKK1 (IKKα) and IKK2 (IKKβ) (Mercurio et al., 1993, Regnier et al., 1997, DiDonato et al., 1997, Woronicz et al., 1997, Zandi et al., 1997). Both IKK kinases can phosphorylate IκBα on specific residues in the N–terminus; serine 32 and serine 36, although IKK1 phosphorylation of IκBα has been suggested to be less efficient than that of IKK2 (Pasparakis et al., 2002, Karin and Ben-Neriah, 2000, Hayden and Ghosh, 2004, Hacker and Karin, 2006, Verma et al., 1995).

1.1.3.2 Ubiquitination

The phosphorylated serines on IκBα act as docking sites for the β–transducin repeat containing protein (βTrCP) receptor subunit of the ubiquitin E3 ligase Skp1/Cul 1/F–box (SCF) (Kroll et al., 1999, Spencer et al., 1999, Winston et al., 1999, Yaron et al., 1997). This complex, together with the E2 enzyme UbcH5 (Perkins, 2006), are necessary to catalyse the K48–linked ubiquitination of

adjacent lysine residues on IκBα, targeting the IκB for degradation by the 26S proteasome (Alkalay et al., 1995b, DiDonato et al., 1996, Lin et al., 1995, Karin and Ben-Neriah, 2000). The released RelA/p50 and p50/c–Rel hetero–dimers can then translocate to the nucleus, bind κB elements of target genes and regulate their transcription.

1.1.3.3 Nuclear shuttling

Analysis of crystal structures has revealed that IκBα binding masks the NLS of RelA but not of p50 when bound to RelA/p50 heterodimers. Consequently, the presence of a NES on IκBα and the exposed NLS of p50 leads to a constant shuttling of IκBα:RelA/p50 complexes between the nucleus and cytoplasm (Ghosh and Karin, 2002). Degradation of IκBα after activation of the canonical pathway therefore shifts the equilibrium towards a nuclear localisation of RelA/p50 dimers.

1.1.4 Alternative pathway to NF–κB activation

In unstimulated cells, p100 molecules are mainly found bound to RelB in the cytoplasm (Solan et al., 2002). The significance of this interaction was unclear, until the recent identification of a limited number of agonists, including B cell activating factor (BAFF), CD40–L and lymphotoxin β (LTβ), which could stimulate a partial proteolysis, termed processing, of p100 to generate p52/RelB dimers (Coope et al., 2002, Dejardin et al., 2002, Claudio et al., 2002). The resulting p52/RelB dimers translocate to the nucleus and activate gene transcription, in a pathway known as the alternative pathway of NF–κB activation (see Figure 1.3).

This pathway specifically requires IKK1 and the NF–κB-inducing kinase (NIK) *(discussed in Section 1.1.7)* (Senftleben et al., 2001a).

1.1.4.1 Function

Processing of p100 regulates a different subset of NF–κB dimers compared to the canonical pathway. Consequently, gene array analysis has revealed that the target genes modulated by each pathway are also distinct (Gardam et al., 2008). Furthermore, particular strains, including *Ltbr1*$^{-/-}$, *Nik*$^{-/-}$, *Relb*$^{-/-}$ and *Nfkb2*$^{-/-}$ mice, whose cells are deficient in p100 processing, display similar phenotypes where: splenic architecture is disrupted, Peyer's patches and LNs are absent, and B cell survival and maturation are impaired (Yin et al., 2001, Koike et al., 1996, Caamano et al., 1998, Futterer et al., 1998). Therefore, these data suggest a specific function for the alternative pathway, distinct to the canonical pathway. However, a recent study suggests that the alternative pathway may play a more diverse role than in secondary lymphoid organogenesis and B cell function, where the authors propose a role for the alternative pathway in the survival and differentiation of thymocytes triggered by Notch3 (Vacca et al., 2006).

1.1.4.2 Constitutive processing of p100

A low level of constitutive p100 processing has been described to occur *in vivo*, resulting in active nuclear RelB/p52 heterodimers in unstimulated cells (Xiao et al., 2004, Qing and Xiao, 2005). This appears to occur in a cell–type specific manner. Consistent with this idea, *Relb*$^{-/-}$ mice have reduced basal NF–κB activity in thymic and splenic cells (Weih et al., 1995). However, constitutive p100

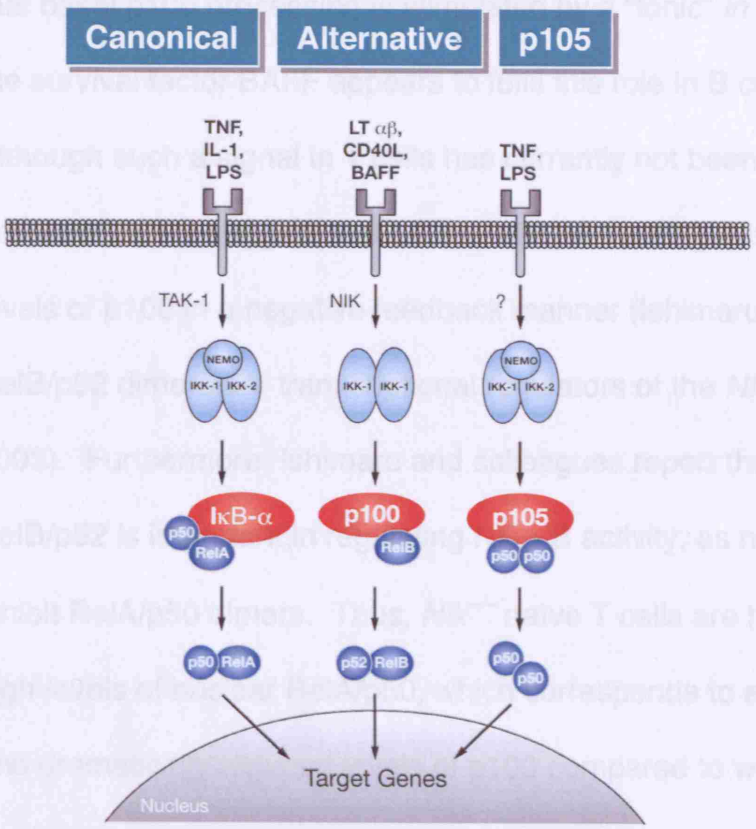


Figure 1.3 Pathways to NF-κB activation.

The canonical pathway (left) is the most-studied pathway leading to NF-κB activation, and is activated by a number of agonists, including those shown. Following receptor ligation, the IKK complex (IKK1-IKK2-NEMO) is activated to catalyse the rapid phosphorylation of the prototypical IκB, IκBα. Subsequent release and nuclear translocation of NF-κB dimers is important for regulating genes involved in immune and inflammatory responses and cell survival. IκBβ and IκBε are similarly regulated, although with different degradation kinetics. In contrast, the alternative pathway (centre) is activated by a subset of stimuli, and is vital for secondary lymphoid organogenesis, B cell survival and maturation. In the alternative signalling pathway, NIK-mediated phosphorylation of IKK1 induces the slow processing of p100 to p52. This leads to the release and nuclear translocation of p52/RelB dimers. Activation of the p105 pathway (right) requires the canonical IKK complex, which catalyses the phosphorylation of the p105 PEST region and triggers its degradation. Associated NF-κB dimers, primarily thought to be p50 homodimers, are released to translocate to the nucleus to regulate gene transcription. Abbreviations: BAFF, B cell activating factor; IL-1, interleukin-1; IκB, inhibitor of NF-κB; IKK, inhibitor of NF-κB kinase; LPS, lipopolysaccharide; LT, lymphotoxin; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase;TAK1, TGFβ-activated kinase 1; TNF, tumour necrosis factor; PEST, proline-glutamic acid-serine-threonine enriched region.

processing was found to require IKK1 and NIK in B cells, similar to inducible processing of p100 (Kayagaki et al., 2002). Consequently, it has been proposed that basal p100 processing is stimulated by a "tonic" *in vivo* signal. Furthermore, the survival factor BAFF appears to fulfil this role in B cells (Kayagaki et al., 2002). Although such a signal in T cells has currently not been identified, analysis of NIK–deficient T cells suggest that basal p100 processing determines steady–state levels of p100 in a negative feedback manner (Ishimaru et al., 2006), since RelB/p52 dimers are transcriptional regulators of the *Nfkb2* gene (Novack et al., 2003). Furthermore, Ishimaru and colleagues report that upregulation of p100 by RelB/p52 is important in regulating NF–κB activity, as newly synthesised p100 can inhibit RelA/p50 dimers. Thus, $Nik^{-/-}$ naïve T cells are hyperproliferative and have high levels of nuclear RelA/p50, which corresponds to a block in p100 processing and dramatically reduced levels of p100 compared to wild-type (WT) cells (Ishimaru et al., 2006).

1.1.5 Inhibitor IκB kinase (IKK) complex

1.1.5.1 Kinases

NF-κB-activating signals converge at the multi–protein IKK complex, and therefore activation of IKK is the requisite step for the induction of NF-κB activity. Protein purification, micro sequencing and molecular cloning have identified three components of the IKK complex: two kinases, IKK1 and IKK2, and a non–catalytic regulatory subunit, NF–κB essential modulator (NEMO) (Mercurio et al., 1993, Regnier et al., 1997, DiDonato et al., 1997, Woronicz et al., 1997, Zandi et al.,

1997). IKK1 and IKK2 are highly homologous proteins, sharing 52% sequence homology and each containing an N–terminal kinase domain (Mercurio et al., 1993, Regnier et al., 1997, DiDonato et al., 1997, Woronicz et al., 1997, Zandi et al., 1997). IKK1 and IKK2 dimerisation occurs through leucine zipper interactions, and is a necessary step for their kinase activity (Mercurio et al., 1993, Woronicz et al., 1997, Zandi et al., 1997). The prevalent form of active IKK in mammalian cells contains both IKK1and IKK2 (DiDonato et al., 1997, Mercurio et al., 1997). However, IKK complexes displaying heterogeneity in terms of their subunit composition have been described. For example, studies with recombinant IKK1 and IKK2 in insect cells indicate that IKK1 and IKK2 can both form homo- and heterodimers (Mercurio et al., 1999, Zandi et al., 1998), although heterodimers display higher kinase activity than either homodimer *in vitro* (Huynh et al., 2000). Furthermore, in IKK1 or IKK2-deficient cell lines, homodimeric forms of the remaining kinase are complexed with NEMO (Hu et al., 1999, Li et al., 1999c, Li et al., 1999b).

Targeted disruption of the IKK genes has revealed distinct phenotypes, which initially suggested that the role of IKK1 and IKK2 in NF–κB activation were non-redundant (summarised in Table 1.1). *Ikk2*$^{-/-}$ mice die at embryonic day 12.5–14.5 from TNF–mediated hepatic apoptosis due to reduced NF–κB activity in hepatocytes (Li et al., 1999c). In contrast, *Ikk1*$^{-/-}$ mice die within a few hours of birth with severe developmental defects and impaired keratinocyte differentiation (Hu et al., 2001, Takeda et al., 1999, Li et al., 1999a). Furthermore, RelA–

deficiency is also lethal, and *Rela*$^{-/-}$ mice have a similar phenotype to *Ikk2*$^{-/-}$ mice (Beg et al., 1995b). Therefore, only IKK2 was believed to be necessary for the activation of RelA in the canonical pathway induced by pro–inflammatory cytokines. However, recent work has suggested that IKK1 can activate RelA downstream of some canonical signalling pathways, including after IL–1 stimulation (Solt et al., 2007). The kinase activity of IKK1–NEMO complexes is also speculated to be responsible for the low levels of NF–κB activity present in TCR and TNFR1–stimulated *Ikk2*$^{-/-}$T cells (Schmidt-Supprian et al., 2003). Consistent with these data, complete ablation of NF–κB activity in epithelial cells requires deletion of both IKK1 and IKK2 (Nenci et al., 2007). Moreover, some redundant functions of IKK1 and IKK2 is suggested by the appearance of severe neural defects in *Ikk1*$^{-/-}$/*Ikk2*$^{-/-}$ mice not seen in either single knockouts (Li et al., 2000).

1.1.5.2 NEMO

NEMO appears to provide a link between the IKK complex and upstream signals (Rothwarf et al., 1998). This role has recently been suggested to be mediated through the ubiquitin-binding domain of NEMO, which is able to specifically bind to proteins expressing K63–ubiquitin chains (Wu et al., 2006a, Ea et al., 2006). These interactions lead to the formation of ubiquitin signalling networks, which are vital for NF–κB activation. Consistent with these data, NEMO-deficient cells show a complete lack of NF–κB activity through the canonical pathway (Nenci et al., 2007, Rudolph et al., 2000). Furthermore, NEMO-deficient mice die early *in utero* due to massive liver apoptosis and have a broader range of defects compared to

Ikk2$^{-/-}$ mice (Schmidt-Supprian et al., 2000). Therefore, in contrast to IKK1 or IKK2, NEMO is essential for the canonical pathway of NF–κB activation (Poyet et al., 2000).

1.1.6 IKK activation

1.1.6.1 Adaptor proteins

The various activating stimuli of NF–κB utilise common signalling intermediates. Of these, TNF receptor–associated factor (TRAF) proteins are essential for both the canonical and alternative pathways leading to NF–κB activation, whilst receptor interacting proteins (RIP) are required for the canonical pathway downstream of several agonists (Hayden and Ghosh, 2008). Activation of the IKK complex requires phosphorylation of serine residues within the activation loop of IKK1 or IKK2 (Johnson et al., 1996). However, the kinases involved are distinct between the canonical and alternative pathways of NF–κB activation.

1.1.6.2 IKK in the canonical pathway

Several kinases have been proposed to activate IKK upstream in the canonical pathway, including protein kinase C (PKC) isozymes or mitogen activated protein kinase kinase kinases (MAP3K). However, these studies were mainly carried out *in vitro*, and therefore, the IKK kinases involved *in vivo* are unclear (Hayden and Ghosh, 2008). Increasing evidence suggests that the MAP3K TGFβ (transforming growth factorβ)–activated kinase–1 (TAK1), is responsible for phosphorylation and activation of the canonical pathway downstream of RIP (Sato et al., 2005).

Furthermore, TAK1–dependent phosphorylation of IKK2 in the activation loop has been observed (Wang et al., 2001). Another potential IKK–kinase may be mitogen–associated extracellular kinase kinase kinase (MEKK3), a MAP3K identified to be critical in TNF–mediated activation of NF–κB (Blonska et al., 2005, Yang et al., 2001). In contrast, there is some evidence that a kinase may not be required to activate IKK at all, since trans–autophosphorylation has been observed after overexpression of IKK1 or IKK2 as a consequence of oligomerization (Zandi et al., 1998).

1.1.6.3 IKK in the alternative pathway

The mechanisms leading to activation of the alternative pathway are distinct to the canonical pathway of NF–κB activation. Firstly, induction of p100 processing requires IKK1, but not IKK2 and NEMO. RIP is also not necessary, which has been presumed to be due to the lack of NEMO in the alternative IKK complex (Hayden and Ghosh, 2008). Instead, interaction studies and kinase assays have suggested that NIK directly phosphorylates and activates IKK1 (Regnier et al., 1997, Xiao et al., 2001, Xiao et al., 2004). Indeed, cells from *aly/aly* and $Nik^{-/-}$ mice have a block in p100 processing (Xiao et al., 2001).

Since IKK2 and NEMO are not required for the alternative pathway, IKK1 homodimers that are not bound to NEMO are believed to exist *in vivo*, but have not been formally identified (Senftleben et al., 2001a). However, the presence of these IKK1 homodimers is supported by the low affinity IKK1 possesses for NEMO,

relative to IKK2: NEMO affinity (May et al., 2002). Once activated, IKK1 has been shown to target two serine residues, Ser867 and 870, on p100 for phosphorylation (Senftleben et al., 2001a). Introduction of mutation at these sites blocks phosphorylation and p100 processing. Subsequent K48–linked polyubiquitination of p100 and partial proteolysis proceed in a similar manner to the canonical pathway, mediated by the E3 ligase βTrCP–SCF complex and 26S proteasome, respectively (Senftleben et al., 2001a, Xiao et al., 2001, Fong and Sun, 2002).

1.1.6.4 K63 ubiquitination and deubiquitination

Ubiquitination of some proteins, catalysed by specific E3 ligase complexes, does not result in proteasomal degradation, but can lead to changes in their conformation, subcellular localization or catalytic activity. This observation led to the discovery of a novel K63–linked ubiquitination mechanism. This process was first described in the interleukin (IL)–1–induced pathway leading to activation of NF–κB, where TRAF6 was identified as a K63–specific E3 ligase of the TAK1 associated protein, TAB2 (Deng et al., 2000). Several targets of K63–linked ubiquitination in the NF–κB pathway have since been characterised, including RIP1, NEMO and the E3 ligases TRAF2 and TRAF6 (Kanayama et al., 2004), Although this is a new field, a number of studies demonstrate that ubiquitin chains are necessary for NF–κB activation. For example, K63–linked chains mediate RIP:NEMO interactions following TNFα stimulation, thus promoting recruitment of the IKK complex to the receptor, and subsequent activation (Ea et al., 2006, Wu et al., 2006a).

The significance of K63–linked polyubiquitination is illustrated by the identification of various deubiquitinases (DUBs), capable of removing the K63–linked polyubiquitin chains. These include cylindromatosis (CYLD), which can inactivate TRAF2, TRAF6 and NEMO (Kovalenko et al., 2003), and A20 which deubiquitinates TRAF2, TRAF6 and RIP1 (Wertz et al., 2004). Consequently, these DUBs can downregulate NF–κB activating pathways, which is of particular importance since excessive NF–κB activity is highly deleterious. Indeed, A20 and CYLD are required for protection against autoimmunity and intestinal inflammation (reviewed in (Liu et al., 2005)).

1.1.7 Processing of p105

Processing, as described above, is a rare mechanism of partial proteolysis, which results in the removal of the C–terminus of p100 and p105. However, the mechanisms of p100 and p105 processing are very different. Whereas p100 processing is a signal–induced event and requires IKK1, processing of p105 occurs constitutively (Coux and Goldberg, 1998). Thus, processing of p105 regulates cellular levels of p50, and results in similar levels of p105 and p50 in most cell types.

The differences between p105 and p100 processing also extend to distinct ubiquitination and proteolytic events. Since ubiquitination occurs on adjacent lysine residues of p105 that are distinct from those recognized by βTrCP, an

alternative E3 ligase has been suggested to promote proteolysis of p105 by the 26S proteasome (Orian et al., 1999, Orian et al., 2000, Cohen S). However, a ubiquitin-independent processing of p105 by the 20S proteasome has also been suggested (Moorthy et al., 2006). This model suggests a co–translational processing of p105, which occurs during random pauses in ribosomal translation (Lin et al., 1998, Lin et al., 2000). Consistent with this idea, a p60 truncated form of p105 can be processed to p50 (Fan and Maniatis, 1991). In contrast, several groups have observed a precursor/product relationship between p105 and p50 in pulse–chase labelling experiments (Mercurio et al., 1993, Belich et al., 1999).

The processing of both p105 and p100 requires the presence of a glycine–rich region (GRR), located between the RHD and ankyrin repeats (Orian et al., 1999, Lin and Ghosh, 1996). This is postulated to protect the N-terminal p50 and p52 fragments from degradation by acting as a stop signal for the 26S proteasome after degradation proceeding from the C-terminus. However, an alternative model suggests that the 26S proteasome may remove the C-terminal by an endoproteolyitc cleavage, and thus the GRR could act as a cleavage site (Lee et al., 2001).

1.1.8 Signal–induced degradation of p105

Binding of NF–κB dimers appears to inhibit processing of p105, perhaps by interfering with entry into the proteasome (Cohen S , Harhaj et al., 1996b). In this case, complete degradation of p105, induced by IKK, then becomes the

predominant form of p105 proteolysis. Mechanistically, p105 degradation is analogous to the classical pathway of IκBα degradation, involving phosphorylation by the IKK1/IKK2/NEMO complex and degradation triggered by the SCF–βTrCP E3 ligase complex (Heissmeyer et al., 1999). Phosphorylation of IκBα and p105 share similar kinetics, although the lower efficiency of p105 ubiquitination may slow down p105 proteolysis (Lang et al., 2003, Salmeron et al., 2001). Two serine residues, 927 and 932, have been identified as targets of phosphorylation by IKK2. These sites are directly recognized by the βTrCP ligase and are necessary for degradation of p105 (Lang et al., 2003, Salmeron et al., 2001). Ser927 and Ser932 are found in a conserved motif of the p105 proline–glutamic acid–serine–threonine–rich (PEST) region, homologous to the IKK target sequence in IκBα, suggesting that the location and spacing of these residues is important for βTrCP recognition (Lang et al., 2003, Salmeron et al., 2001, Orian et al., 1999, Heissmeyer et al., 1999). However, little is known about the upstream events leading to this signal–induced p105 proteolysis, such as the involvement of TRAFs and MAP3Ks (Figure 1.3).

1.1.8.1 Function

The main result of IKK–induced proteolysis appears to be the complete degradation of p105, as demonstrated by overexpression of IKK2 in HEK–293 cells (Heissmeyer et al., 2001). Furthermore, TNF or LPS–induced proteolysis of p105 does not affect p50 levels in a variety of cell–types ((Harhaj et al., 1996b), suggesting that IKK–induced phosphorylation of p105 is not involved in regulating

processing of p105 to p50. Although the consequence of signal–induced proteolysis of p105 is unclear, p105 has been found to form a stochiometric complex with the MAP–3K tumour progression locus–2 (TPL–2) (Belich et al., 1999).

1.1.8.2 Tumour progression locus–2 (TPL–2)

The serine–threonine MAP–3K TPL–2 was first identified as a highly expressed protein in a particular human cancer, thus is also known as Cot (cancer Osaka thyroid) (Miyoshi et al., 1991). Analysis of TPL–2 deficient *Map3k8*$^{-/-}$ mice has demonstrated its importance in the extracellular–signal–regulated kinase (ERK) MAPK pathway in innate and adaptive immune cells. TNFα or lipopolysaccharide (LPS) stimulation of TPL–2 deficient macrophages results in a specific block in ERK activity and reduced synthesis of and TNFα (Dumitru et al., 2000, Eliopoulos et al., 2003). As a result, TPL–2– deficient *Map3k8*$^{-/-}$ mice are resistant to LPS–induced septic shock (Dumitru et al., 2000). Furthermore, in *Map3k8*$^{-/-}$ B cells, signalling to ERK is defective in response to CD40 ligation, whilst IgE synthesis is impaired in response to CD40 and IL–4 antibodies (Eliopoulos et al., 2003).

Interestingly, the C–terminal portion of p105 associates with high affinity to TPL–2 *via* two distinct interactions (Belich et al., 1999, Beinke S, 2003). A region of p105 located N–terminal to the ankyrin repeats interacts with the C–terminus of TPL–2, whilst the p105 death domain (DD) binds the kinase domain of TPL–2. The TPL–2–p105 interaction is very strong, such that all detectabe TPL–2 is found

associated to p105 in unstimulated macrophages (Belich et al., 1999, Lang et al., 2004). However, only a small fraction of total cellular p105 is associated with TPL–2 (Lang et al., 2004). There appear to be two functions of TPL–2 and p105 binding. Firstly, association to p105 is necessary to maintain TPL–2 stability (Beinke S, 2003, Waterfield et al., 2003). Accordingly, cells deficient of p105 express very low basal levels of TPL–2 protein, although *Map3k8* mRNA levels are normal (Beinke S, 2003, Waterfield et al., 2003). Secondly, binding to p105 prevents TPL–2 from phosphorylating its physiological substrates MEK–1/2, blocking TPL–2/MEK interaction rather than inhibiting TPL–2 catalytic activity (Waterfield et al., 2003, Beinke S, 2003). Consequently, IKK–induced degradation of p105 is necessary for activation of TPL–2/MAPK pathways.

1.1.8.3 A20–binding inhibitor of NF–κB–2 (ABIN–2)

Proteomic approaches to identify novel p105–binding proteins discovered A20–binding inhibitor of NF–κB–2 (ABIN–2) (Lang et al., 2004, Bouwmeester et al., 2004). The function of ABIN–2 is unclear. However, ABIN–2 interacts with A20 (Van Huffel et al., 2001), an inducible protein that is involved in termination of NF–κB signals following TNFα stimulation (Lee et al., 2000). Subsequently, A20 has been characterised as a DUB, removing K63–linked ubiquitin chains on RIP and promoting its K48–linked ubiquitination, targeting RIP for proteasomal degradation (Wertz et al., 2004). Since overexpression of ABIN–2 leads to a block in TNFα mediated NF–κB activation, ABIN–2 may also be involved in the negative regulation of NF–κB. Interestingly, a K63–ubiquitin binding domain in ABIN–2 has

been identified, similar to that found in NEMO (Wagner et al., 2008). Furthermore, deletion of this region results in loss of ABIN–2 -inhibition of NF–κB activation in overexpression assays (Wagner et al., 2008). However, *Abin2*$^{-/-}$ mice showed no signs of impaired NF–κB activity (Papoutsopoulou et al., 2006).

In bone–marrow derived macrophages (BMDM), the majority of ABIN–2 is found complexed with p105 and TPL–2, in an interaction necessary for ABIN–2 stability (Waterfield et al., 2003, Lang et al., 2004, Beinke S, 2003). Furthermore, RNA interference and genetic studies have demonstrated that ABIN–2 is required for the stability of TPL–2 protein (Waterfield et al., 2003, Lang et al., 2004, Beinke S, 2003). However, the function of IKK–induced proteolysis of p105 to release ABIN–2 remains unclear.

1.1.9.3 IKK–induced p105 proteolysis and NF–κB activation

The C–terminal region of p105 contains seven ankyrin repeats, which have implicated p105 as an inhibitor of NF–κB (Rice et al., 1992, Hatada et al., 1992). Experiments in transfected cell lines have demonstrated that p105 can associate with RelA, c–Rel and p50 subunits *in vitro* (Rice et al., 1992, Mercurio et al., 1992, Mercurio et al., 1993). Furthermore, binding studies using recombinant proteins have shown that p105 preferentially binds p50 homodimers (Liou et al., 1992). In accordance with this, *Nfkb1*$^{\Delta C/\Delta C}$ mice, which are unable to express p105 protein but retain expression of p50, have high levels of constitutive nuclear p50 homodimers, since these cannot be retained by IκBα (Ishikawa et al., 1998).

Therefore, p105 has widely been considered as an inhibitor of p50 homodimers. However, levels of total p50 in *Nfkb1*$^{\Delta C/\Delta C}$ mice were markedly above physiological levels, and levels of TPL–2 and ABIN–2 were presumably low due to instability of these proteins in the absence of p105 (Liou et al., 1992). Therefore the contribution of IKK–induced p105 proteolysis to NF–κB activation remains unclear.

1.2 Activation of NF–κB in T cells

1.2.1 Immune responses

Mammals are continuously exposed to pathogens that can cause them harm. Therefore, mechanisms exist to prevent pathogen–induced injury. The major role of the immune system is to recognize pathogens that have invaded the host, prevent their spread and clear them from the body. Two branches of the immune system are responsible for this defence, the innate and adaptive. NF–κB has a critical role in regulation of both of these responses.

Innate response The innate immune system is regarded as the first line of defense encountered by pathogens, providing a rapid response. Cells of the innate immune system typically derive from the myeloid lineage and include monocytes, macrophages, dendritic cells (DCs), mast cells, neutrophils, basophils and eosinophils, but also encompass the natural killer (NK), natural killer T (NKT) and γδ T cells of the lymphoid lineage. These cells express a number of invariant receptors to maintain host defenses against foreign agents. Amongst these,

macrophages and DCs utilize specific receptors named pattern–recognition receptors (PRRs) to recognize pathogen–associated molecular patterns (PAMPS), common to many micro–organisms (reviewed in (Medzhitov and Janeway, 2000)). Such a mechanism allows innate immune responses to a large number of microbes. A number of PRRs have been identified, which act as sensors in the innate immune system, and the best–characterised of these are the Toll–like receptors (TLRs) (reviewed in (Medzhitov and Janeway, 2000, Schnare et al., 2001)). At present, thirteen members of the TLR family have been described in mice, with the natural ligands for most identified. These include TLR4, which recognizes the cell wall polysaccharide lipopolysaccharide (LPS) found on Gram negative bacteria, and TLR9, which binds, unmethylated CpG abundant in bacterial DNA. Engagement of TLRs activates macrophages and DCs to produce pro–inflammatory cytokines and enhances antigen presentation to naive T cells by expression of costimulatory molecules (Kopp and Medzhitov, 1999).

Induction of NF–κB in innate immune cells is vital for regulating genes that both aid recruitment of immune cells to the site of an infection, and which promote killing of microbes, through phagocytosis or release of cytotoxic molecules (degranulation). NF–κB target genes include pro–inflammatory cytokines e.g. TNFα, IL–1β and IL–6 in macrophages; chemokines including regulated upon activation, normal T–cell expressed and secreted (RANTES) by endothelial cells; adhesion molecules such as vascular cell adhesion molecule–1 (VCAM–1) on epithelial cells; and enzymes that generate reactive intermediates e.g. inducible nitric oxide synthase (iNOS)

(Mukaida et al., 1990, Marui et al., 1993, Ledebur and Parks, 1995, Beinke and Ley, 2004).

Adaptive response In contrast to the innate immune system, the adaptive immune system is only found in vertebrates and is characterized by specificity and memory. Two facets of the adaptive immune system exist; a humoural response through B cell production of antibodies, and a cell–mediated protection provided by T cells. Activation of the adaptive immune response is vital for elimination of microbes that evade innate immune cell killing. To achieve this, T cells, which often trigger the adaptive immune response, must be presented with their cognate antigen on major histocompatibility (MHC) proteins at cell surfaces. Once they have identified foreign antigen, T and B lymphocytes undergo clonal expansion and generate specific responses to eliminate particular pathogens or pathogen–infected cells.

Macrophages and DCs can act as antigen presenting cells (APCs), presenting microbial antigen on MHC class II proteins to activate $CD4^+$ T cells, as well as expressing costimulatory molecules necessary for T cell activation. Following pathogen recognition, this presentation is enhanced in an NF–κB–dependent upregulation of MHC proteins on APCs. Activation of NF–κB also upregulates the costimulatory molecules B7.1(CD80) and B7.2 (CD86) on their cell surface (Medzhitov and Janeway, 1997). Furthermore, NF–κB signalling has a vital role in the function of lymphocytes, regulating survival, proliferation, B cell class switching

to enable the production of antibodies with different effector functions, and production of cytokines by T cells (Li and Verma, 2002, Beinke and Ley, 2004).

NF–κB clearly plays an important role in many aspects of the innate and adaptive immune responses. However, this study is primarily concerned with the impact of IKK–induced proteolysis in T lymphocytes. Therefore, the following sections will focus on current knowledge on the regulation and function of NF–κB in T cells.

1.2.2 Antigen presentation

Peripheral T cell activation is dependent on the ability of the T cell to recognise antigenic peptides. This is achieved through recognition of cognate antigens as a processed peptide fragment presented on MHC class I or class II molecules to the T cell receptor (TCR). MHC molecules are encoded as large cluster of polymorphic genes in the host genome. During antigen presentation, ingested antigens are spliced into peptides in the cytosol and re–expressed on the cell surface linked to MHC proteins. Whilst cytoplasmic proteins, including those derived from viruses and some intracellular bacteria, are degraded to peptides by the proteasome and transported into the endoplasmic reticulum (ER) where they bind MHC class I molecules, peptide fragments from antigen that have been internalized by APCs are processed by the phagosome–endosome pathway and bind MHC class II molecules (Ramachandra et al., 1999). CD8 and CD4 accessory molecules on T cells bind MHC molecules class I and class II respectively, though at distinct sites

to the TCR, which increases the affinity of interaction between the T cell and the APC (Konig et al., 1992, Sun et al., 1995).

Cell surface expression of the MHC class I:peptide complex occurs in most cell types and is recognized by $CD8^+$ T cells in a peptide specific manner, triggering destruction of cells infected with intracellular pathogens, such as viruses, and cancerous cells (York and Rock, 1996). In contrast, antigens re–expressed on MHC class II molecules on professional APCs such as B cells, macrophages and DCs, interact with $CD4^+$ T cells and promotes, through cytokine release, differentiation of $CD4^+$ T helper subsets (*detailed in Section 1.4.2).* Therefore, expression of CD4 or CD8 accessory molecules on T cells defines MHC restriction as well as effector function.

1.2.3 Antigen recognition by T cells

1.2.3.1 T cell receptor

Activation of T cells results from engagement of the multimeric TCR complex with antigenic peptides in the context of appropriate MHC. The ligand binding subunit of the TCR is composed of a heterodimer of the variable α and β chains. Associated with TCR$\alpha\beta$ are the invariant CD3γ,δ and ε chains that together with TCRζ, are responsible for transmitting signals into the T cell interior (Davis et al., 1998).

1.2.3.2 Costimulatory molecules

For productive activation, a T cell must receive a second signal generated from costimulatory and accessory molecules, which are induced on APCs during an immune response, usually by products of pathogens (Bretscher, 1999). Without costimulation, T cells become anergic, unable to produce the autocrine growth factor IL–2 and proliferate. This two–signal mechanism is thought to be important for self–tolerance, preventing inappropriate responses to self, since self–peptides would not usually be presented with costimulatory molecules (Kane et al., 2002). Strikingly, most of these costimulatory molecules can activate NF–κB transcription factors.

1.2.4 TCR activation events

1.2.4.1 Immunological Synapse

TCR and costimulatory receptor engagement induces rearrangement of membrane proteins at the T cell–APC contact site, resulting in the formation of an immunological synapse (IS) (Monks et al., 1998). This change brings the TCR and co–receptors together on T cell surfaces, concentrating tyrosine phosphorylation events into a central zone. Adhesion receptors and molecules are forced to the periphery, generating distributions known as supramolecular activation clusters (SMAC). These consist of central (cSMAC) regions, where the TCR, associated signalling proteins and APC plasma membrane are in close proximity, and peripheral (pSMAC) regions, which stabilize the interaction between the T cell and APC (Monks et al., 1998, Nel, 2002). These contact sites are enriched for lipid

rafts, which act as platforms for the assembly of the signalling complex and are enriched for proteins involved in the immediate stages of IS formation (Kabouridis et al., 1997, Zhang et al., 1998b, Drevot et al., 2002, Fragoso et al., 2003, Arcaro et al., 2001).

The aggregation of the IS was initially proposed as a mechanism required for TCR signal transduction. However, later work demonstrated that T cell signaling occurs before IS formation (Lee et al., 2002). Therefore, the current view is that segregation and redistribution of signalling molecules in the IS contributes to amplification and maintenance of receptor signals, and this may be important for directing T cell effector functions through cytokine signalling (Davis and van der Merwe, 2001).

1.2.4.2 TCR signalling

Signals resulting from ligation of the TCR are dependent on the activation of TCR proximal signalling proteins and adaptors (summarised in Figure 1.4). Tyrosine phosphorylation events are rapidly induced *via* the SH2 region containing (Src) family protein tyrosine kinases $p56^{Lck}$ (Lck) and $p59^{Fyn}$ (Fyn), through the dephosphorylation of a negative regulatory C terminal tyrosine residue by the CD45 dephosphatase (McNeill et al., 2007, Pingel et al., 1999, Seavitt et al., 1999). Lck is associated with CD4 or CD8 co–receptors, whereas Fyn is associated with the TCR itself (Barber et al., 1989, Veillette et al., 1988). Clustering of the TCR and co–receptors bring Lck into close proximity with the CD3 complex, where both Lck and Fyn can phosphorylate the conserved immunoreceptor tyrosine activation

motifs (ITAMs) on each of the CD3 chains and the three ITAMs on TCRζ (Straus and Weiss, 1992, van Oers et al., 1993). Phosphorylated ITAMS serve as recruitment motifs, recognised by tandem SH2 domains of the ζ chain–associated protein kinase of 70kDa (ZAP–70) (Wange et al., 1993, Isakov et al., 1995). Recruitment of its substrate at the TCR promotes phosphorylation by Lck, Fyn (Mege et al., 1996, Fusaki et al., 1996, Chan et al., 1995) and/or autophosphorylation of ZAP–70 (Brdicka et al., 2005, Neumeister et al., 1995), activating its kinase function. Activated ZAP–70 can then phosphorylate a variety of cytoplasmic substrates, including the adaptor proteins SH2–domain–containing leukocyte protein of 76 kDa (SLP–76) and the transmembrane linker for activation of T cells (LAT) (Bubeck Wardenburg et al., 1996, Zhang et al., 1998a).

SLP–76 and LAT are important for the formation of multi-protein signalling complexes in the lipid raft, which trigger a number of downstream signalling pathways, including activation of phospholipase C γ (PLCγ) (Yablonski et al., 2001), protein kinase C (PKC) (Sun et al., 2000a, Baier-Bitterlich et al., 1996) and the small GTPase, Ras (Morrison and Cutler, 1997, Buday et al., 1994, Zhang et al., 2000). These pathways together result in the activation of activator protein 1 (AP–1), Ca^{2+}-dependent nucleating factor of activated T cells (NFAT), and NF–κB transcription factors, inducing expression of genes required for T cell activation and proliferation (Nel, 2002).

1.2.5 Early events in TCR–induced NF–κB activation

Recent genetic and biochemical experiments has elucidated the signalling pathway by which ligation of TCR activates IKK and NF–κB. The current model suggests that TCR signalling activates PKCθ, which acts upstream of a complex of oligomerized proteins to promote NEMO polyubiquitination and subsequent IKK activation (see Figure 1.4).

1.2.5.1 Protein kinase C θ

PKCθ is a diacyl glycerol (DAG)–dependent Ca^{2+}–independent isoform of PKC. Knowledge of PKCθ function in T cells has been obtained from analysis of two different *Pkcθ*$^{-/-}$ mice. Analysis of one of these *Pkcθ*$^{-/-}$ mouse strains indicates that PKCθ is required for TCR–induced NF–κB activation and IL–2 induction in mature T cells (Sun et al., 2000a). However, these results are inconsistent with the second PKCθ–deficient mouse created, in which IL–2 production was impaired due to an abrogation of NFAT activation rather than NF–κB (Pfeifhofer et al., 2003). The reason for this discrepancy is unclear, but may be due to differences in the generation of these mice. Nevertheless, a large body of biochemical and pharmacological studies support a role for PKCθ in activating NF–κB after TCR ligation (Schulze-Luehrmann and Ghosh, 2006).

The steps leading to PKCθ activation are still to be defined. However, the recruitment of PKCθ to lipid rafts has been shown to be vital for the IKK–induced canonical pathway of NF–κB activation (Bi et al., 2001, Li et al., 2005). Several

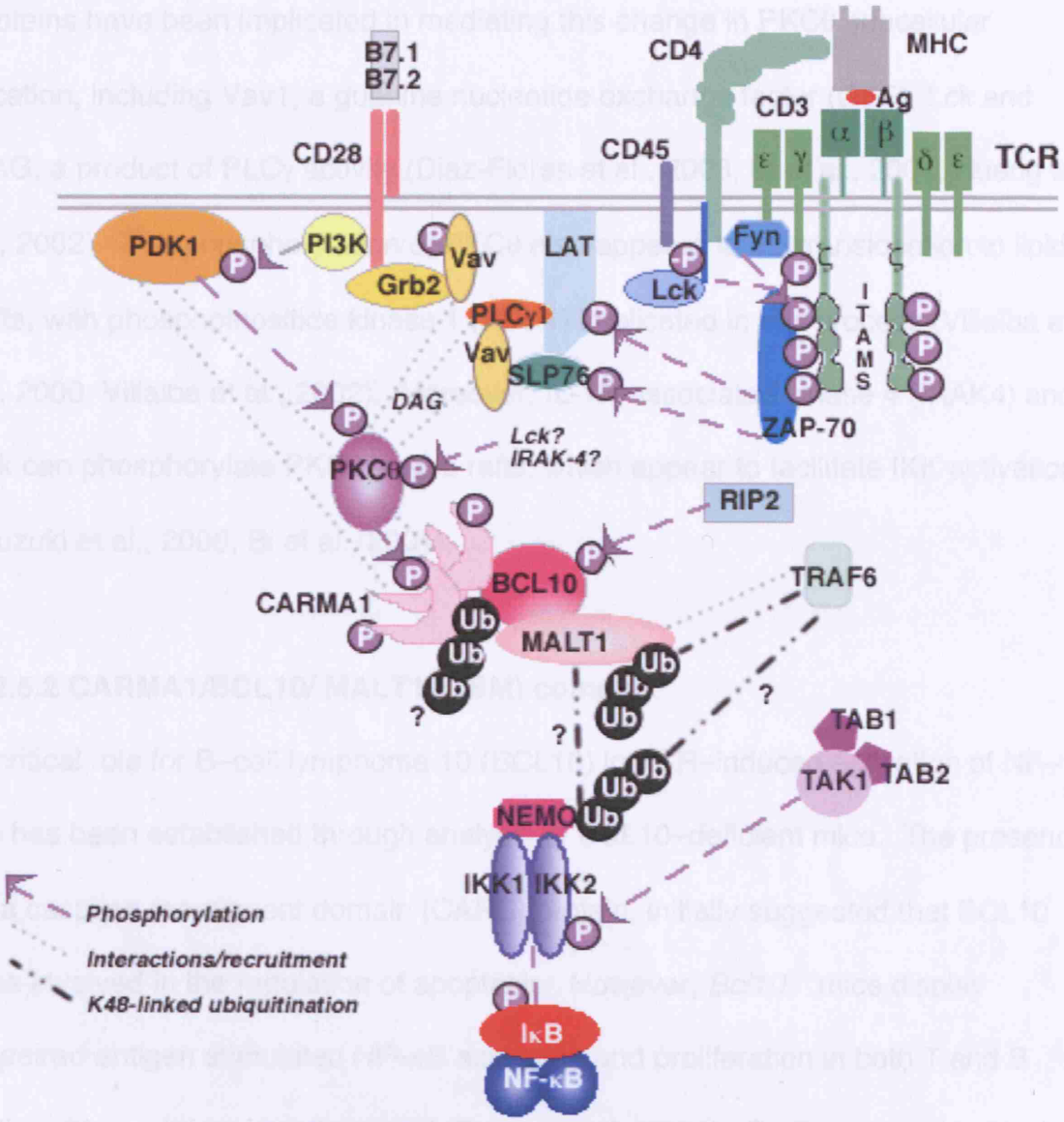


Figure 1.4 Model of TCR and CD28-induced NF-κB activation.

Binding of TCR to peptide-MHC and costimulation through CD28-B7 interaction leads to activation of several signal transducers, including the Src and Syk family tyrosine kinases Lck and Zap-70, the adaptor protein LAT and the LAT-associated proteins SLP76 and Vav. PLCγ1 and PI-3K activation also occurs downstream of TCR and CD28, which recruit PKCθ and CARMA1 to lipid rafts. Here, phosphorylation (P) of PKCθ by PDK1 leads to CARMA1 activation and oligomerization, inducing the formation of CARMA1-BCL10-MALT1 complexes. RIP-2-mediated phosphorylation of BCL10 and K-63 linked ubiquitination (Ub) of BCL10, MALT1 (*via* TRAF 6) and NEMO (by TRAF6 or MALT1) are important for the activation of NF-κB. Phosphorylation of IKK by the MAP3K TAK1 is also necessary for activation NF-κB after TCR ligation.

Abbreviations: BCL10, B cell-lymphoma 10; CARMA1, CARD (caspase recruitment domain)-MAGUK1 (membrane-associated guanylate kinase kinase 1); IκB, inhibitor of NF-κB; IKK, inhibitor of NF-κB kinase; IRAK4 (IL-1R-associated kinase 4); LAT, linker of activated T cells; MALT1, mucosa-associated lymphoid tissue 1; MAP3K, mitogen activated kinase kinase kinase; MHC, major histocompatibility complex; NF-κB, nuclear factor κB; PDK1, phosphoinositide dependent kinase 1; PI-3K, phosphoinositide-3 kinase; PKC, protein kinase C; PLC, phospholipase C; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; TAK1, TGFβ-activated kinase 1; TCR, T-cell receptor; TRAF6, TNF (tumour necrosis factor) receptor associated factor 6; ZAP-70, ζ-associated protein of 70-kDa.

proteins have been implicated in mediating this change in PKCθ subcellular location, including Vav1, a guanine nucleotide exchange factor (GEF), Lck and DAG, a product of PLCγ activity (Diaz-Flores et al., 2003, Bi et al., 2001, Huang et al., 2002). The phosphorylation of PKCθ also appears to aid translocation to lipid rafts, with phosphoinositide kinase 1 (PDK1) implicated in this process (Villalba et al., 2000, Villalba et al., 2002). Moreover, IL-1R-associated kinase 4 (IRAK4) and Lck can phosphorylate PKCθ in lipid rafts, which appear to facilitate IKK activation (Suzuki et al., 2006, Bi et al., 2001).

1.2.5.2 CARMA1/BCL10/ MALT1 (CBM) complex

A critical role for B–cell lymphoma 10 (BCL10) in TCR–induced activation of NF–κB has been established through analysis of BCL10–deficient mice. The presence of a caspase recruitment domain (CARD) protein, initially suggested that BCL10 was involved in the regulation of apoptosis. However, *Bcl10*$^{-/-}$ mice display impaired antigen stimulated NF–κB activation and proliferation in both T and B cells, with no defect in cell survival (Ruland et al., 2001). Furthermore, this block in proliferation cannot be rescued by treatment with phorbol 12–myristate 13–acetate (PMA), suggesting that BCL10 acts downstream of PMA–sensitive PKCs such as PKCθ (Ruland et al., 2001). Various proteins have since been found to interact with BCL10. Of these, the CARD–membrane–associated guanylate kinase 1 (MAGUK1), known as CARMA1, and mucosal-associated lymphoid tissue 1 (MALT1) proteins have been identified as key components of an IKK–activating complex in T cells.

CARMA1, also known as CARD11, is a lymphocyte–specific member of the MAGUK family of proteins. Studies in cell lines first identified CARMA1 in TCR-mediated activation of the IKK complex, with overexpression of CARMA1 resulting in BCL10 phosphorylation and NF–κB activation (Gaide et al., 2001). Furthermore, this overexpression in *Bcl10*$^{-/-}$ T cells could not restore the block in NF–κB activation, suggesting a function for CARMA1 upstream of BCL10 (Ruland et al., 2001). Similarly, PKCθ overexpression fails to induce NF–κB activity in cells where CARMA1 is knocked–down (Wang et al., 2002), proposing CARMA1 as a link between PKCθ and BCL10 in TCR-induced NF–κB activation. Consistently, T cells from *Carma1*$^{-/-}$ mice exhibit a block in NF–κB activation following TCR stimulation (Egawa et al., 2003, Hara et al., 2003).

Similar to the *bcl10* gene, chromosomal rearrangement of the membrane–associated lymphoid tissue 1 gene (*malt1)* is implicated in MALT lymphomas. This raised the possibility that both genes are involved in the same signalling pathway, resulting in lymphoma formation when dysregulated. Indeed, yeast–2–hybrid and co–immunoprecipitation assays in T cells confirmed an interaction between the paracaspase MALT1, and BCL10. Moreover, generation of *Malt1*$^{-/-}$ mice by two groups recreated a T cell phenotype similar to that of CARMA1 and BCL10–deficient mice, demonstrating a requirement of MALT1 for TCR–induced activation of NF–κB (Ruefli-Brasse et al., 2003, Ruland et al., 2001)

1.2.5.3 Activation of IKK by CBM complex

The mechanism by which the CARMA1/BCL10/ MALT1 complex (CBM) activates the IKK complex, and subsequently NF–κB, is not completely understood. However, recent studies have provided some insights. MAGUK proteins in other cell types recruit signalling complexes to the plasma membrane (Dimitratos et al., 1999). Therefore, after TCR stimulation, CARMA1 has been proposed to translocate to the cell membrane where PKCθ catalyses its phosphorylation. This activation step can trigger CARMA1 oligomerization, which aids recruitment of the BCL10/MALT1 complex *via* CARD-CARD interactions (Schulze-Luehrmann and Ghosh, 2006, Gaide et al., 2001). CARMA1 also appears to phosphorylate BCL10, although RIP2 has also been implicated in this process (Gaide et al., 2001, Ruefli-Brasse et al., 2003, Ruefli-Brasse et al., 2004).

K63–linked ubiquitination appears to be a vital mechanism in TCR–induced NF–κB activation, with two independent experiments demonstrating its requirement for IKK activation by BCL10 and MALT1 (Zhou et al., 2004, Sun et al., 2004b). Both studies demonstrate that NEMO is a target for ubiquitination, but propose different mechanisms for this modification. One study provides evidence to suggest that MALT1 can act as an ubiquitin E3 enzyme, despite a lack of any known E3 domains (Zhou et al., 2004). In contrast, the second study indicates that TRAF6, upon MALT1 binding, can catalyse K63 ubiquitination of NEMO (Sun et al., 2004b). Interestingly, TRAF6 itself can associate with MALT1 after TCR stimulation and mediates K63–linked ubiquitination of MALT1 (Oeckinghaus et al., 2007).

Intriguingly, both this modification and the inducible proteolytic activity of MALT1 are necessary for optimal TCR–mediated NF–κB activation. Furthermore, K63–linked ubiquitination of BCL10 in response to T cell ligation is required for interaction of the CBM complex with NEMO (Wu and Ashwell, 2008).

The recruitment of the IKK complex to lipid rafts is also an essential step for NF–κB activation. Some evidence exists that this occurs through Vav interaction with IKK1, targeting IKK1 to the membrane and activating it in response to TCR/CD28 stimulation (Piccolella et al., 2003). RNAi–mediated silencing and reconstitution experiments have suggested a role for TAK1 as the kinase that activates IKK after TCR signalling. To support this hypothesis, thymocytes from *Lck–Cre Tak1*$^{FL/FL}$ mice, demonstrate that TAK1 is essential for TCR–mediated activation of NF–κB (Sato et al., 2005).

1.2.6 CD28 costimulation

Costimulation of naïve T cells is required to prevent T cell anergy and subsequent activation induced cell death (AICD) (Schmitz et al., 2003). Signalling through co–receptors can complement or modify signals provided through the antigen receptor. It has been suggested that ligation of CD80 and CD86 molecules, expressed on APCs, to the constitutively expressed CD28 receptor (Azuma et al., 1993, Jenkins et al., 1991, Shahinian et al., 1993) provides the most important second signal for prevention of anergy, IL–2 production, proliferation and survival of T cells (Kane et al., 2002). Furthermore, CD28 costimulation can act in a number of ways to lower

the threshold of activation in naïve $CD4^+$ T cells. These include enhancing the assembly of signalling components at the TCR synapse, particularly PKCθ (Huang et al., 2002), activating NF–κB and JNK pathways and increasing/stabilizing IL–2 mRNA (Lindstein et al., 1989, Jenkins et al., 1991, Nel, 2002). Consistent with its importance in T cell activation, *Cd28*$^{-/-}$ mice generate immune responses much less efficiently than wild–type mice (Shahinian et al., 1993, Kane et al., 2002).

1.2.6.1 NF–κB in CD28 costimulation

NF–κB activation has been shown to be a key event in CD28 costimulation (Khoshnan et al., 1999, Harhaj et al., 1996a), with the CD28RE of IL–2 containing binding sites for c–Rel-, p50- and RelA- containing dimers (Ghosh et al., 1993). CD28 contributes to NF–κB activation through two signalling pathways. The first has been proposed to occur in a phosphoinositide-3 kinase (PI3K)–dependent manner, since mutating the cytoplasmic PI3K/CD28 interaction site blocks the recruitment of PKCθ to the c–SMAC region of the IS and impairs NF–κB nuclear translocation and IL–2 gene transcription (Huang et al., 2002). An important downstream effector of PI3K is PDK1, which phosphorylates and activates Akt. PDK1 appears to regulate the activation of NF–κB through signal–dependent recruitment of both PKCθ and CARMA1 to lipid rafts (Lee et al., 2005). Furthermore, Akt has been reported to induce the phosphorylation of BCL10, leading to NF–κB activation, although this is contentious (Narayan et al., 2006). Other kinases have also been suggested in the CD28–mediated activation of NF–κB, including RIP2 and MEKK1 (Ruefli-Brasse et al., 2004, Tao et al., 2002). A

second PI-3K-independent pathway of CD28 costimulation has been proposed to contribute to NF–κB activation. This results in sustained tyrosine phosphorylation and membrane localization of Vav, which enhances PLCγ generation of DAG. Consequently, PKCθ is recruited to the cell membrane and activated, leading to NF–κB activation (Villalba et al., 2002).

1.2.7 IL–2 in T cells

Naïve T cells exist as small resting cells in the G_0 phase, outside of the cell cycle. Upon activation, T cells re–enter the cell cycle, proliferate and differentiate into effector cells. Whilst TCR–stimulation is required for activated T cells to leave G_0 and enter G_1, cell cycle progression through G1 to S–phase appears to be mediated by production of IL–2 and IL–2R ligation (Cantrell and Smith, 1983). IL–2 is maiinly produced by T cells, and acts in an autocrine/paracrine fashion to promote T cell proliferation. Paradoxically, mice deficient in IL–2 or IL–2R components share a common autoimmune phenotype with an accumulation of activated T lymphocytes (reviewed in (Kim et al., 2006)). These findings are consistent with the ability of IL–2 to stimulate survival and functions of natural regulatory T cells, a sub-population of T cells that can suppress immune responses *(discussed in Section 1.3.2)* (Malek et al., 2002, Furtado et al., 2002, de la Rosa et al., 2004, Thornton et al., 2004). Furthermore, IL–2 stimulation has been suggested to promote the Fas–mediated apoptotic death of $CD4^+$ T cells (Refaeli et al., 1998, Kneitz et al., 1995). Therefore, IL–2 can act as a positive and negative regulator of immune activation.

IL–2R The IL–2R is comprised of three distinct polypeptides; α (CD25) (Leonard et al., 1982), β (CD122) (Teshigawara et al., 1987) and γ (CD132, common γ chain) (Takeshita et al., 1992). On resting naïve T cells, the IL–2Rβ and γ chains are expressed, forming a functional receptor with intermediate affinity for IL–2 (K_d=10^{-9}M). TCR–induced activation results in upregulation of IL–2Rα (CD25), which forms a trimeric high affinity receptor (K_d=10^{-11}M) with the β and γ chains (Kim et al., 2006). Since the IL2-Rα chain has a very short cytoplasmic domain, it does not transmit intracellular signals (Hatakeyama et al., 1986). In contrast, the β and γ chains play the predominate role in transducing intracellular signals. Neither of these chains has intrinsic enzymatic function, but they associate with the intracellular tyrosine kinases Janus–associated kinase (JAK)-1 and JAK3, respectively (Miyazaki et al., 1994, Russell et al., 1994). Ligand binding results in the activation of JAK1 and JAK3, which phosphorylate multiple tyrosine residues found in the cytoplasmic tail of the IL2Rβ chain that serve as docking sites for SH2-domain-containing adaptor or effector molecules (Sharon et al., 1989). From here, three signalling pathways have been identified: Ras/Raf/MAPK; JAK/STAT (signal transducers and activators of transcription) and PI–3K/Akt signalling pathways (Smith, 2004). Evidence points to the STAT5a/b molecules as being the primary regulators of lymphocyte development as well as being critical for promoting cell cycle progression of mature lymphocytes, such as by regulating activation of cyclin D2 (Moriggl et al., 1999b, Moriggl et al., 1999a, Yao et al., 2006). Although PI–3K/Akt signalling has been suggested to modulate

NF–κB activation, the role of NF–κB activation following IL–2R ligation in T cells is unclear (Zheng et al., 2003, Narayan et al., 2006).

1.2.7.1 Regulation of IL–2 and IL–2R

The IL–2 promoter contains binding sites for several transcription factors activated by TCR stimulation, including NFAT, AP–1 and NF–κB (Garrity et al., 1994). Binding at the IL–2 promoter is cooperative, with occupation of all elements in the IL–2 promoter/enhancer required for optimal transcription (Rothenberg and Ward, 1996). Furthermore, CD28 costimulation can enhance transcription of IL–2 due to the presence of a CD28 response element (CD28RE) within the IL–2 promoter (Fraser et al., 1991). This is a combinatorial response element that requires NF–κB and AP–1 binding for full activation (Khoshnan et al., 1999). Therefore, there are two distinct NF–κB binding sites in the promoter of IL–2. Notably, CD28 signalling also serves to markedly increase IL–2 post–transcriptionally by stabilizing IL–2 mRNA (Lindstein et al., 1989).

Expression of the IL–2Rα chain is tightly regulated at the transcriptional level, by both signals from the TCR and the IL-2R itself. Indeed, IL–2 stimulation has been shown to increase and prolong expression of IL–2Rα, acting as a positive feedback regulator for its own high affinity receptor (Hatakeyama et al., 1989, Plaetinck et al., 1990). Interestingly, TCR–induced NF–κB activity is involved in upregulation of both IL–2 and IL–2Rα, with upregulation of IL–2Rα particularly dependent on p50 and RelA subunits (Costello et al., 1993). CD28 costimulation also induces a long–

lasting activation of NF–κB, resulting in stable association of NF–κB at the IL–2Rα promoter and sustained expression of IL–2Rα (Kahn-Perles et al., 1997, Algarte et al., 1995).

1.2.8 NF–κB and apoptosis

Apoptosis plays a crucial role during the development and function of an effective immune system. Indeed, regulated cell death is vital for selection of thymocytes *(discussed in section 1.3)*, and for the maintenance of T cell homeostasis after clonal expansion. T cell survival is influenced by signals through the TCR, co-stimulatory receptors including CD28, adhesion molecules, pro- and anti-apoptotic molecules and cytokine receptors. A number of apoptotic pathways have been described (Krammer et al., 2007), and a role for NF–κB has been speculated in several of these. The best characterised of these, known as the intrinsic pathway, involves regulation of the balance between pro- and anti- apoptotic members of the B cell lymphoma-2 (Bcl-2) family. Antigen–receptor and CD28 costimulation of T cells promotes upregulation of the NF–κB-target gene Bcl–xL, which can prevent AICD in restimulated cells (Chen et al., 2000). This function of NF–κB appears to specifically involve RelA and p50 subunits (Chen et al., 2000, Khoshnan et al., 2000). Activation of the JAK/STAT pathway downstream of cytokine receptors such as IL-2 and IL-7 also induces changes in the expression or trafficking of the Bcl-2 proteins, including upregulating expression of the Bcl–2 gene, and therefore enhancing the survival of T cells (von Freeden-Jeffry et al., 1997, Khaled et al., 1999). A role for NF–κB downstream of cytokine signalling is unclear, although

there are reports that CD28 and IL-2 mediated activation of Akt is important for cell survival (Ahmed et al., 1997, Kelly et al., 2002), and a controversial role for Akt in activating NF–κB has been suggested (Narayan et al., 2006). Furthermore, NF–κB has been implicated in other apoptotic pathways, since inhibition of NF–κB results in accumulation of reactive oxygen species which promote apoptosis, and an upregulation of the pro-apoptotic molecule p73 is observed after TCR stimulation (Krammer et al., 2007).

1.3 NF–κB in T lymphocyte development

A number of genetically modified mouse strains have revealed an essential role for NF–κB in the development, survival and effector functions of T cells. In addition, NF–κB has been shown to be required for the generation of certain T cell sub–populations, as summarised in Table 1.1 and Figure 1.5 (Siebenlist et al., 2005).

1.3.1 Thymic development

Cellular components of the immune systems are generated from haematopoietic stem cells (HSCs) in the adult bone marrow (BM) or fetal liver, which give rise to the myeloid and lymphoid compartments. NK, T and B lymphocyte precursors are all produced from common lymphoid progenitors (CLPs). However, the maturation

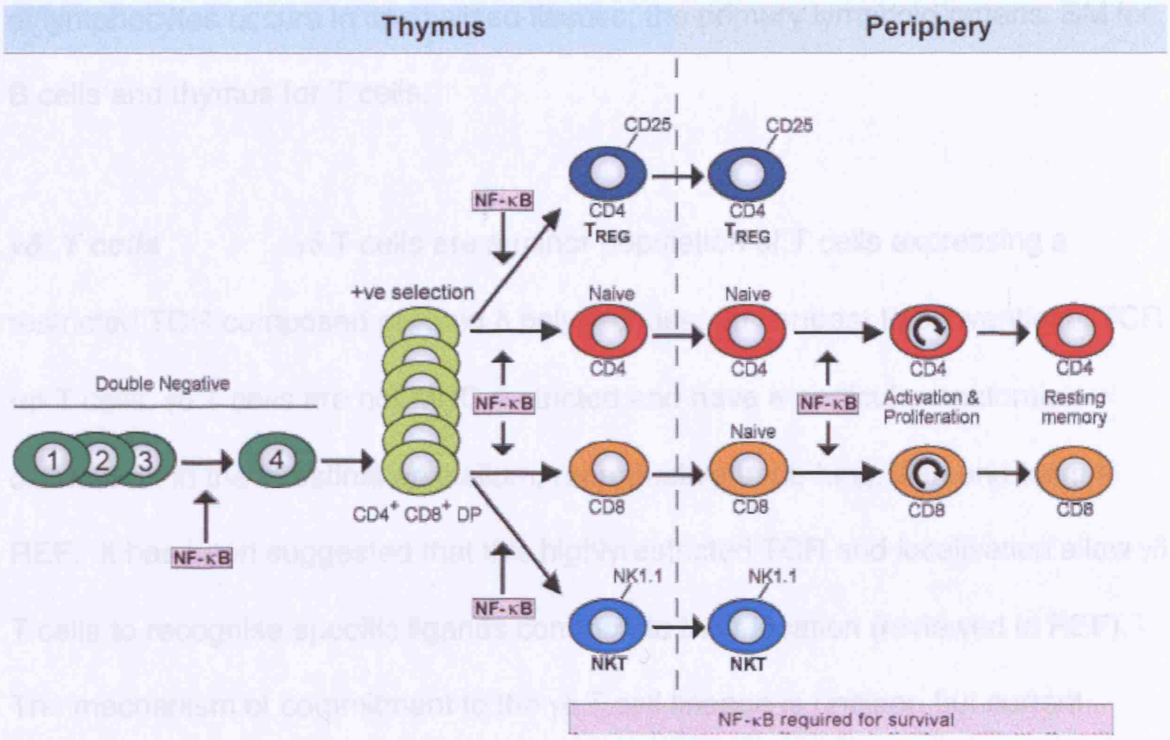


Figure 1.5 Role of NF-κB in T cell development.

Analysis of a number of NF-κB-deficient mice has demonstrated a role for NF-κB transcription factors at various stages of conventional αβ T cell thymic development. Survival of peripheral T cells and thymocytes, particularly DN4, CD4SP and CD8SP cells, require TCR and/or TCR-independent activation of NF-κB. A role for NF-κB activity in the development of the T_{reg} and NKT cell sub-populations has also been demonstrated.
Abbreviations: DP, double positive; NF-κB, nuclear factor κB; NKT, natural killer T cells; SP, single positive, T_{reg}, regulatory T cells.

Adapted from Siebenlist et al., Nat Rev. ***5,*** *435-445 (2005)*

of lymphocytes occurs in specialized tissues, the primary lymphoid organs, BM for B cells and thymus for T cells.

γδ T cells γδ T cells are a minor population of T cells expressing a restricted TCR composed of γ and δ polypeptides. In contrast to conventional TCR αβ T cells, γδ T cells are not MHC restricted and have a particular anatomical distribution in the intestinal epithelium, reproductive tract, lung, skin and tongue REF. It has been suggested that this highlyrestricted TCR and localisation allow γδ T cells to recognise specific ligands common to their location (reviewed in REF). The mechanism of commitment to the γδ T cell lineage is unclear, but current evidence indicates that they arise form a common precursor to αβ T cells (REF). Subsequently, γδ T cell development appears distinct to that of αβ T cells, independent of pre-TCR signalling and the double positive (DP) stage of differentiation (REF). However, the focus of this section will be the development of TCR αβ T cells.

1.3.1.1 Double negative (DN) stages

DN1 and DN2 thymocytes Some progenitor cells migrate in the bloodstream, from the BM to the thymus. Here, they begin their development into αβ T cells as $CD4^-CD8^-$ double negative (DN) cells in the cortex. Four DN stages have been observed, distinguished by expression of the cell surface glycoprotein CD44 (phagocyte glycoprotein 1) and CD25 as follows: DN1 $CD44^+$ $CD25^-$; DN2 $CD44^+$ $CD25^+$, DN3 $CD44^-$ $CD25^+$; DN4 $CD44^-CD25^-$ (Godfrey et al., 1993,

Pearse et al., 1989). During the DN1and DN2 stages, both α and β chains of the TCR are in the germ–line configuration.

The role of NF–κB at the early stages of T cell development is unclear but has been suggested to involve protection against apoptosis. Adoptive transfer of hepatic stem cells from *Ikk2*$^{-/-}$ and *Rela*$^{-/-}$ mice reveal that these cells are unable to generate lymphocytes, in contrast to WT stem cells (Senftleben et al., 2001b, Beg and Baltimore, 1996). However, lymphopoiesis can occur when hepatic stem cells of *Ikk2*$^{-/-}$ mice crossed with either *Tnfa*$^{-/-}$ or *Tnfr1*$^{-/-}$ mice are transferred (Alcamo et al., 2002, Doi et al., 1999, Grossmann et al., 1999, Grossmann et al., 2000, Horwitz et al., 1997). These findings demonstrate a need for NF–κB–induced activation of anti–apoptotic genes such as Bcl–xL in protecting progenitor T cells against the detrimental effects of excessive TNFα.

Pre–TCR signalling in DN3 thymocytes Maturation of DN2 cells leads to a downregulation of CD44, forming $CD44^{-}CD25^{+}$ DN3 cells that can undergo TCRβ chain rearrangements (Godfrey et al., 1994, Livak et al., 1999, Petrie et al., 1995). The TCRβ gene is made up of four gene segments that encode V (variable), D (diversity) and J (joining), C (constant) regions. To generate a productive β chain, T cells must rearrange the DNA segments by somatic recombination. This process generates the diversity among TCR, and is mediated by the recombination–activation genes (Rag1 and Rag2), the lymphocyte–specific components of the V(D)J recombinase complex (Yancopoulos et al., 1986). Thymocytes which fail to

successfully rearrange the β chain die by apoptosis (Falk et al., 2001), whilst those that achieve functional rearrangement can associate with an invariant pre –T cell α chain (Groettrup and von Boehmer, 1993) and CD3 complex, forming a pre –TCR (von Boehmer and Fehling, 1997).

Signalling through the pre–TCR is required for prevention of programmed cell death (Penit et al., 1995) and the downregulation of CD25 for transition from DN3 to DN4, in a mechanism known as β selection. Potentiation of this signalling cascade requires Lck or Fyn kinases (Molina et al., 1992, Appleby et al., 1992), Zap–70 and spleen tyrosine kinase (Syk), although genetic analysis reveals there may be some redundancy between these kinases (Cheng et al., 1995, Cheng et al., 1997, Negishi et al., 1995). NF–κB also seems to play an important role at this stage, as mice expressing a non–degradable "super–repressor" IκBα in their T cells, partially blocking NF–κB activation, have a reduction in thymic DN4 cells compared to WT mice. This impaired progression from DN3 to DN4 was attributed to an increased susceptibility of DN3 thymocytes to apoptosis (Voll et al., 2000).

1.3.1.2 Double positive (DP) stage

Pre–TCR signalling serves to select thymocytes that have successfully rearranged the β chain, leading to arrest of further β chain rearrangements (allelic exclusion) in immature double positive (DP) thymocytes. Expression of a functional pre–TCR also induces upregulation of both CD4 and CD8 co–receptors, generating $CD4^+CD8^+$ DP thymocytes that can enter the cell cycle and undergo clonal

expansion (Vasseur et al., 2001, Hoffman et al., 1996). These cells make up the majority of thymocytes. Once DP thymocytes stop dividing, rearrangement of the α chain to form the mature TCR is enhanced (Janeway, 2005). However, the expression of a functional TCR is insufficient for generation of mature T cells, and thymocytes must undergo a further checkpoint in which the strength of TCR ligation/signalling is determined.

1.3.1.3 Thymocyte selection

Selection occurs to aid maturation of only those T cells that recognize antigenic peptide in the context of self–MHC molecules, and to prevent autoimmunity by removing cells with strong affinity to self–peptide (sp) MHC complexes (Kisielow et al., 1988b, Teh et al., 1988). To this end, an affinity/avidity model has been established. This proposes a positive selection mechanism, whereby pro–survival signals are delivered to only those DP thymocytes whose MHC–restricted TCR are capable of forming low affinity interactions with cortical epithelial cells expressing spMHC. DP cells without sufficient affinity for ligand do not receive this signal and die by neglect (Huesmann et al., 1991, Bevan, 1997, McGargill et al., 2000, Jenkinson et al., 1994). Thymocytes that have successfully undergone positive selection migrate to the medulla, where potentially autoreactive thymocytes are eliminated, by the process of negative selection. Here, thymocytes expressing TCR which bind spMHC expressed on bone–marrow derived APC or medullary epithelial cells with a high affinity are rapidly deleted from the T cell repertoire by apoptotic cell death (Brocker et al., 1997, Marrack et al., 1988, Hoffmann et al.,

1992, Degermann et al., 1994, Kisielow et al., 1988a).

A role for NF–κB in selection has been proposed, based on the increased DP population and reduced single positive (SP) populations in T cells expressing IκBα "super–repressor" (Hettmann et al., 1999, Hettmann and Leiden, 2000). However, more detailed analysis has provided conflicting data, where NF–κB seems to have a function in both positive and negative selection, although the precise role remains to be determined as discussed below.

NF–κB in positive selection A clear anti–apoptotic function for NF–κB during positive selection exists, consistent with the established role for NF–κB in regulating pro–survival genes. For example, TCR transgenes that are selected for on certain backgrounds fail to produce DP cells when co–expressed with the T–cell specific IκBα super–repressor (Mora et al., 2001b).

NF–κB in negative selection The role of NF–κB in negative selection still remains to be clarified, but has been studied in H–Y (male antigen)–restricted TCR–transgenic male mice. These mice have a reduced DP subset due to increased negative selection compared to WT mice. However, partially inhibiting NF–κB activation in T cells of these mice through expression of IκBα super–repressor under the control of the Lck promoter, can rescue DP numbers (Mora et al., 2001b). Although this and several other studies (Kim et al., 1999, Kim et al., 2002) suggest a pro–apoptotic role for NF–κB in negative selection, there is also

some conflicting evidence that inhibition of NF–κB needs to occur for negative selection (Fiorini et al., 2002).

These results have lead to the general view that when NF–κB activation is impaired, the strength of TCR signal is reduced, either dampening strong positive selection signals so that a positively–selected cell does not survive or reducing the strength of a negative signal, such that it is not enough to induce apoptosis (Siebenlist et al., 2005).

1.3.1.4 Single positive thymocytes

Only a small fraction of DP thymocytes successfully undergo selection. DP thymocytes that have been selected downregulate the CD4 and CD8 co–receptor, but will subsequently upregulate either CD4 or CD8 to produce single positive (SP) $CD4^+$ and $CD8^+$ thymocytes. Commitment to the CD4 or CD8 lineage is vitally important, since these populations play different effector roles that are critical for the clearance of infection and host immunity. The mechanisms that determine this decision are not clear, and are the subject of ongoing debate. Two hypotheses were originally proposed. Firstly, an instructive model suggests that qualitatively different signals are provided by MHC class I and class II interactions with CD8 and CD4 co–receptors, respectively, which dictates lineage commitment (Borgulya et al., 1991, Robey et al., 1990). In contrast, the stochastic model hypothesises that DP thymocytes undergo a random lineage commitment, and that cells expressing inappropriate co–receptor expression are subsequently deleted.

However, work with transgenic mice produced results incompatible with either model, and a revised instructive "strength of signal" model has now been established (Borgulya et al., 1991, Basson and Zamoyska, 2000). This proposes that a quantitative difference in signal strength may determine the differentiation to $CD4^+$ or $CD8^+$ SP cells (Itano et al., 1996, Matechak et al., 1996). A stronger signal is believed to be provided by TCR recognition of class II MHC proteins, based on the higher affinity with which CD4 co–receptor binds to Lck, compared to CD8 (Ravichandran and Burakoff, 1994). Accordingly, replacement of the CD8α intracellular domain with that of CD4, re–directs class I–restricted thymocyte development to the CD4 lineage (Itano et al., 1996). Furthermore, the duration of TCR signal may also influence commitment, with longer exposure to TCR ligands directing thymocytes to theCD8 lineage (Liu and Bosselut, 2004, Yasutomo et al., 2000). Moreover, emerging work examining how quantitative differences in TCR signalling are transmitted has identified transcription factors, such as Thelper–inducing PO/Kruppel factor (Th–POK), which are essential for CD4 lineage commitment (He et al., 2005, Sun et al., 2000b).

NF–κB in SP T cells Whether NF–κB plays a role in CD4/CD8 lineage commitment is unclear, however, survival of SP thymocytes requires NF–κB activation. $CD4^+$ and $CD8^+$ SP thymocytes show increased sensitivity to apoptosis in various NF–κB–deficient mice, particularly affecting $CD8^+$ SP T cells. This has been attributed to the greater dependence $CD8^+$ SP T cells possess for TCR:spMHC interactions than $CD4^+$ T cells, since $CD8^+$ SP thymocytes have

higher levels of NF–κB activity, but conversely lower expression levels of the NF–κB–target Bcl–2 than $CD4^+$ SP thymocytes (Polic et al., 2001). For example, mice with a T cell–specific conditional deletion of IKK2 (*CD4–Cre/Ikk2$^{FL/D}$*), which still retain NF–κB activity through compensatory IKK1 activity, have reduced fractions of thymic and peripheral $CD4^+$ and $CD8^+$ T cells, most striking in the $CD8^+$ T cell pool (Schmidt-Supprian et al., 2003). A similar phenotype is seen in transgenic mice in which T cell NF–κB activation is partially blocked by transgenic expression of a "super–repressor" IκBα (Boothby et al., 1997). However, a complete block in NF–κB activation in mice expressing either a kinase–dead form of IKK2 or lacking NEMO in their T cells have no peripheral T cells, apparently due to increased susceptibility of SP thymocytes to apoptosis (Schmidt-Supprian et al., 2003). This pathway appears to signal in a TCR–independent manner, as blocking TCR–induced activation of NF–κB through deletion of BCL10, does not affect the generation of thymic SP T cells (Schmidt-Supprian et al., 2003). This suggests that signalling through an alternative receptor to the TCR may activate NF–κB to promote T cell survival.

1.3.2 $CD4^+CD25^+$ natural regulatory T (T_{reg}) cells

Despite thymic selection, some autoreactive lymphocytes fail to be deleted and reach the periphery, necessitating further mechanisms to prevent autoimmunity. Specialised T cells that are committed to maintenance of immune homeostasis have been identified as key players in peripheral tolerance (Kronenberg and Rudensky, 2005), including $CD4^+CD25^+$ regulatory T cells (T_{reg}). Interestingly,

several mutant mouse strains with impaired NF–κB activation in their T cells display a reduction in this self–reactive T cell population (summarised in Table 1.1).

1.3.2.1 Properties of T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells constitute 5–10% of the peripheral $CD4^+$ T cell population and are the best characterised of the regulatory T cells. These regulatory properties were first observed in experiments examining the autoimmunity seen upon transfer of $CD4^+CD25^-$ naïve T cells into athymic mice (Powrie et al., 1993, Sakaguchi et al., 1995). The co–transfer of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ cells provided protection from these symptoms, identifying this subset as capable of suppressing immune responses and inhibiting development of autoimmunity (Sakaguchi et al., 1995). Subsequently, $CD4^+CD25^+$ T_{reg} cells have been demonstrated to suppress responses to viral, bacterial and protozoal infections, as well as protective anti-tumoural immunity (Sakaguchi, 2003, Wang et al., 2004). The mechanisms by which T_{reg} cell function is controlled is unclear, although a role for cytokines, costimulatory molecules on APCs and TLRs on T_{reg} cells have been speculated (reviewed in (Sutmuller et al., 2006)).

Characterisation of $CD4^+CD25^+$ T_{reg} cells *in vitro* has demonstrated their ability to suppress the proliferative capacity and IL-2 production of $CD4^+CD25^-$ and $CD8^+$T cells, consistent with the ability of T_{reg} cells to modulate immune responses (Shevach, 2002, Kronenberg and Rudensky, 2005, Thornton A.M., 1998). However, disparate features between T_{reg} cells *in vivo* and *in vitro* have been

described. For example, T_{reg} cells were originally described as anergic, due to their inability to produce IL–2 and proliferate *in vitro* after TCR stimulation (Takahashi et al., 1998). This anergic state was found to be overcome *in vitro* by the addition of IL–2, whereby the *in vitro* suppressive function of T_{reg} cells was also speculated to be lost (Thornton A.M., 1998), although later work has shown that IL-2 mRNA levels in responder cells were still suppressed (Thornton et al., 2004). In contrast, *in vivo* proliferation of T_{reg} cells has been demonstrated after transfer of $CD4^+CD25^+$ T cells into lymphopoenic hosts, dependent on MHC–class II interactions, and in response to antigenic stimuli (Gavin et al., 2002, Walker et al., 2003, Fisson et al., 2003, Yamazaki et al., 2003). Notably, T_{reg} cells that are able to proliferate *in vivo* can still exert regulatory function *in vitro* (Gavin et al., 2002). The discrepancy between *in vitro* and *in vivo* proliferation may be explained by the finding that stimulation of T_{reg} cells with DCs rather than the usual splenocytes can induce T_{reg} cells to proliferate *in vitro* (Yamazaki et al., 2003).

1.3.2.2 Mechanisms of suppression

The suppressor function of T_{reg} cells *in vitro* appear to bare little resemblance to that *in vivo* , and therefore, the mechanisms of suppression are still poorly defined. Studies examining the *in vitro* suppressive function suggest a dependence on TCR stimulation, but independent of secretion of cytokines IL–10 and TGFβ, which are produced by activated T_{reg} cells, although these cytokines are considered to be important for T_{reg} cell function *in vivo* ((Liu et al., 2003, Thornton A.M., 1998, Asseman et al., 1999). A role for the membrane–bound TGFβ found on T_{reg} cells

has also been proposed, since cell–cell contact is required for *in vitro* T_{reg} cell-mediated suppression (Thornton A.M., 1998), which is inhibited by antibodies to TGFβ (Nakamura et al., 2001, Shevach, 2002, Kronenberg and Rudensky, 2005). However, conflicting results regarding the role of TGFβ have been generated both *in vitro* and *in vivo* (Piccirillo et al., 2002, Kullberg et al., 2005), including recent analysis of *Tgfb1*$^{-/-}$ mice which indicates that TGFβ binding in T_{reg} cells is required for thymic expansion and maintenance of peripheral Foxp3^{+} (discussed below) cells, rather than having direct effector function (Marie et al., 2005). Several other features of T_{reg} cells have been implicated in their function, including their constitutive expression of CD25. Since expression of CD25 (IL–2Rα) allows assembly of a high affinity IL–2R, this was proposed to provide T_{reg} cells with a competitive advantage over bystander T cells (Barthlott et al., 2005). However, this seems unlikely, since even in the presence of exogenous IL–2, naïve T cell IL–2 mRNA levels were found to be suppressed by the presence of T_{reg} cells (Thornton A.M., 1998). Nevertheless, IL-2 appears to be required for suppressive activity, since two groups have shown that neutralization of IL-2 can prevent T_{reg} suppressor function *in vitro* (Thornton et al., 2004, de la Rosa et al., 2004). Expression of cytotoxic T–lymphocyte antigen–4 (CTLA–4), an alternative inhibitory ligand for CD80/86 in T_{reg} cells, has also been suggested to contribute to their regulatory properties (Read et al., 2000). Indeed, transfer of CD4^{+}CD25^{+} T_{reg} cells with monoclonal CTLA–4 antibodies does not protect against colitis induced by naïve T cells (Read et al., 2000). Taken together, the mechanisms by which T_{reg}

cells exert suppression of immune responses, and how activity of T_{reg} cells is modulated still remain unclear.

1.3.2.3 Identification of T_{reg} cells

A hallmark of T_{reg} cells is their constitutive expression of CD25 (Sakaguchi et al., 1995), although cell surface molecules such as CTLA-4, glucocorticoid–induced T–lymphocyte receptor (GITR) and the αE integrin CD103 are also expressed at higher levels on $CD4^+CD25^+$ T cells compared to $CD4^+CD25^-$ T cells (McHugh et al., 2002, Gavin et al., 2002). However, these molecules are also upregulated in naive $CD4^+CD25^-$ T cells following TCR stimulation and therefore cannot serve as definitive T_{reg} markers.

Foxp3 The identification of T_{reg} cells has been facilitated by the characterisation of the X–chromosome–encoded forkhead box protein P3 (Foxp3) as a T_{reg} lineage–specific transcription factor (Hori et al., 2003, Fontenot et al., 2003). Using mice expressing an enhanced green fluorescence protein (EGFP) cDNA knocked into the Foxp3 gene, cells possessing suppressor activity correlated with expression of Foxp3, irrespective of CD25 expression (Fontenot et al., 2005b). Furthermore, forced expression of Foxp3 is sufficient to induce suppressor activity in murine $CD4^+CD25^-$ cells (Fontenot et al., 2003). Therefore, Foxp3 is believed to program suppressor function of T_{reg} cells. Significantly, a mutation of *Foxp3* that generates a non–functional Foxp3 protein results in an absence of T_{reg} cells. This is the cause of an X–linked recessive inflammatory disease in *Scurfy* mutant mice (Brunkow et al., 2001). A similar mutation in humans results in defective T_{reg}

function and an X–linked autoimmune lymphoproliferative disorder termed immune dysregulation, polyendocrinopathy, enteropathy, X–linked (IPEX) (Bennett *et al.*, 2001). A T–cell specific deletion of Foxp3 expression gives rise to a lymphoproliferative autoimmune disease similar to that seen in Foxp3–deficient mice, consistent with the idea that Foxp3 expression in T cells is required and sufficient to generate T_{reg} cells (Fontenot et al., 2005b).

Recently, several studies have demonstrated that Foxp3 can bind a consensus fork–head motif upstream of many Foxp3 target genes, or regulate genes in cooperation with NFAT (Marson et al., 2007, Zheng and Rudensky, 2007, Wu *et* al., 2006b). Furthermore, a complex interaction between environmental factors and Foxp3 in promoting T_{reg} differentiation has been revealed, since Foxp3 protein regulates distinct gene subsets in thymic T_{reg} compared to peripheral T_{reg} cells (Gavin et al., 2007, Zheng and Rudensky, 2007).

1.3.2.4 Development of T_{reg} cells

TCR:spMHC Thymectomy at day 3 after birth results in low/undetectable numbers of T_{reg} cells, suggesting that these cells are generated in the thymus (Sakaguchi et al., 1995). Development of T_{reg} cells also requires interaction with spMHC class II molecules expressed on cortical epithelial cells for their selection (Bensinger et al., 2001). Several studies of TCR transgenic mice have established the idea that T_{reg} cell development is promoted in $CD4^+$ T cells expressing TCR with increased avidity for spMHC (Jordan et al., 2001, Apostolou et al., 2002). Indeed, T cells expressing a high–affinity TCR for influenza haemagglutinin can

develop into T_{reg} cells in mice expressing haemagglutinin, whilst T cells bearing a receptor with low affinity transgenic TCR for haemagglutinin cannot. However, an alternative model has been proposed, based on the finding that TCR expressed by naive and T_{reg} cells share some overlap, but not all T cells expressing a shared TCR become T_{reg} cells. This proposes that Foxp3 expression, or some limiting thymic signal, spares cells with high avidity TCR from deletion, and that these subsequently develop into T_{reg} cells (Liston and Rudensky, 2007).

IL–2 Mice deficient in IL–2, IL–2Rα or IL–2Rβ succumb to an aggressive, lymphoproliferative autoimmune syndrome (Papiernik et al., 1998, Malek et al., 2002, Almeida et al., 2002), and show reduced frequencies of $CD4^+CD25^+$ T_{reg} cells in the thymus, spleen and LN. Therefore, IL–2 has been speculated to be necessary for the generation of T_{reg} cells. However, early studies relied on CD25 expression for the identification of these cells, which can be upregulated by IL–2 stimulation (Hatakeyama et al., 1989, Plaetinck et al., 1990) and therefore the role of IL-2 in T_{reg} cell generation has been controversial. Indeed, transfer of thymic and splenic *Il2*$^{-/-}$ $CD4^+$ T cells into myelin basic protein (MBP)–TCR transgenic mice, a model for experimental allergic encephalomyelitis (EAE), showed that these cells still retain suppressive activity (Furtado et al., 2002), and since recipient–derived IL–2 can drive expansion of *Il2*$^{-/-}$ T_{reg} cells (Almeida et al., 2002), these results suggest that T_{reg} cell differentiation is independent of IL–2. The use of Foxp3 has provided a more reliable marker for T_{reg} cells, and has generated evidence supporting an IL–2–independent development of T_{reg} cells. For example, the generation of *Il2*$^{-/-}$ and *Il2ra*$^{-/-}$ mice expressing EGFP–Foxp3 protein revealed

that thymic and peripheral Foxp3$^+$ T_{reg} cells are produced (Fontenot et al., 2005a). Although these cells were somewhat reduced in number, they displayed suppressive properties *in vivo*. Similar conclusions were independently reached using a transgenic TCR system and analysis of intracellular Foxp3 protein (D'Cruz and Klein, 2005). Moreover, two studies using mixed BM chimeras and adoptive transfers suggest that Foxp3$^+$ T_{reg} cells deficient in IL–2 signalling have reduced competitive fitness and capacity to survive compared to WT cells (D'Cruz and Klein, 2005, Fontenot et al., 2005a). This conclusion is supported by the impaired expression of genes involved in metabolic pathways and cell survival in T_{reg} cells from *Il2ra*$^{-/-}$ mice (Fontenot et al., 2005a). Therefore, these findings seemed to rule out a requirement for IL–2 in Foxp3$^+$ T_{reg} cell differentiation, but rather a role in their maintenance.

CD28 Development and peripheral maintenance of T_{reg} cells have been proposed to require CD28 costimulation, as evident by the drastic reduction in thymic and peripheral Foxp3$^+$ cells in *Cd28*$^{-/-}$ mice, as well as mice treated with B7 antibodies (Tang et al., 2003, Tai et al., 2005). Although IL–2 production by CD28–deficient Foxp3$^-$ cells is reduced compared to WT Foxp3$^-$ cells, IL–2 from WT cells in mixed BM chimeras cannot rescue *Cd28*$^{-/-}$ T_{reg} cell numbers, suggesting that IL–2 generation is unlikely to be the sole cause of the T_{reg} defect in *Cd28*$^{-/-}$ mice (Tai et al., 2005). Therefore, CD28 appears to play an additional, as-yet-unidentified role in T_{reg} cell development.

STAT5 The IL–2R and IL–15R share the IL–2Rβ and IL–2Rγ (common-γ)

chains, and have been implicated in T_{reg} cell differentiation, since *Il2rg*$^{-/-}$–EGFP–Foxp3 mice and *Il2rb*$^{-/-}$ IL-2Rβ transgenic mice (Suzuki et al., 1995, Malek et al., 2000, Malek et al., 2002) generate dramatically reduced numbers of Foxp3^{+} T_{reg} cells compared to WT mice (Fontenot et al., 2005b). Recent studies reveal a redundant role for IL–2 and IL–15 in T_{reg} cell development (Burchill et al., 2007, Burchill et al., 2008, Yao et al., 2007), with Foxp3^{+} T_{reg} cells absent in the thymus and periphery of *Il2*$^{-/-}$*Il15*$^{-/-}$ mice (Burchill et al., 2007). Consistent with these findings, mice with a T–cell specific deletion of STAT5, which is activated by IL–2R and IL–15R signalling, show a block in T_{reg} cell development (Burchill et al., 2007). A critical role for STAT5 in T_{reg} cell generation was confirmed by the restoration of Foxp3^{+} T_{reg} differentiation *via* expression of a constitutively active STAT5b transgene in *Cd28*$^{-/-}$, *Il2rg*$^{-/-}$ *and Il2rb*$^{-/-}$ bone marrow cells (Burchill et al., 2008, Burchill et al., 2007). Furthermore, several groups have suggested a possible mechanism through which STAT5 induces T_{reg} cell differentiation, by demonstrating it's binding to the Foxp3 promoter, and thus identifying it as a possible transcriptional regulator (Burchill et al., 2007, Yao et al., 2007, Zorn et al., 2006).

1.3.2.5 NF–κB in T_{reg} cells

The requirement for a strong TCR signal and CD28 ligation suggest that the signals involved in selection of T_{reg} cells are distinct to those for naïve T cells (Liston and Rudensky, 2007). The involvement of NF–κB in this process has been demonstrated by a reduction in Foxp3^{+} T_{reg} cells in a number of NF–κB–deficient mice, including those with a T cell–specific deletion of IKK2 (Schmidt-Supprian et

al., 2003) (see Table 1.1). Furthermore, since T_{reg} cell generation is blocked in *Bcl10*$^{-/-}$mice, and significantly impaired in *Pkcθ*$^{-/-}$mice, it appears that NF–κB activation through the TCR is important for this process (Schmidt-Supprian et al., 2004a).

1.3.3 Natural killer T cells

Natural killer T (NKT) cells have been described as a bridge between innate and adaptive immunity, displaying features of T lymphocytes but activated with the rapidity of the innate immune system (Seino and Taniguchi, 2004, Kronenberg and Rudensky, 2005, Kronenberg and Gapin, 2002). Accordingly, murine NKT cells express an invariant TCR, are either $CD4^+CD8^-$ or $CD4^-CD8^-$ DN but express certain NK cell markers (Seino and Taniguchi, 2004). Strikingly, NKT cells, like T_{reg} cells, are believed to be a self–reactive and may regulate autoimmune responses. Consistent with this idea, the surface phenotype of NKT cells resembles that of activated T cells ($CD44^{hi}$ $CD62L^{lo}$ $CD69^+$) in germ-free mice (Park et al., 2000).

1.3.3.1 Activation of NKT cells

Most murine $NK1.1^+$ T cells have been found to express an invariant TCR Vα14–Jα18 rearrangement which recognize glycolipids presented by the atypical MHC class I–like CD1d molecule (Sidobre et al., 2002, Hammond et al., 1999). A high affinity is particularly shown for the glycosphingolipid α–galactosylceramide (αGal–Cer), a property that has been used to identify CD1d–restricted NKT cells (Bendelac et al., 1997, Matsuda et al., 2000). Upon activation, these CD1d–

(Lisbonne et al., 2003, Akbari et al., 2003). Therefore, the modulation of cytokines produced by Vα14i NKT cells appears to be crucial for their role in autoimmune responses.

1.3.3.2 NKT cell development

Vα14i NKT cells are found in a tissue–specific manner and at different frequencies, the significance of which is not known (Hammond et al., 1999). Murine NKT cells are found at the highest levels in the liver (10–40% of liver lymphocytes) and in lower fractions (1%) in thymus, bone marrow, spleen, lymph node, and blood (Emoto et al., 1999). This distribution, and the presence of NKT cells in nude mice initially led to the idea that NKT cells were generated extrathymically (Kikly and Dennert, 1992, Sato et al., 1995). However, several studies have now established that Vα14i NKT cells develop in the thymus from T cell progenitors (Coles and Raulet, 2000, Hammond et al., 1998), and commitment to the NKT lineage occurs at the DP stage of T cell development (Benlagha et al., 2005, Bezbradica et al., 2005), although Vα14i NKT cells undergo positive selection events that are distinct to conventional T cells (Bezbradica et al., 2005). For example, selection of Vα14i NKT cells is mediated by bone marrow–derived $CD4^+CD8^+$ cortical thymocytes expressing CD1d molecules presenting self–lipids, rather than epithelial cells (Coles and Raulet, 2000, Gapin et al., 2001). Furthermore, a higher–affinity TCR:self–lipid/MHC interaction is required for Vα14i NKT cell differentiation than conventional αβ T cells, in a similar manner to that of T_{reg} cells (Bendelac et al., 1997, Bendelac, 1995, Kronenberg and Gapin, 2002). Although poorly understood,

the signalling requirements for Vα14i NKT cell development are also different to those for conventional T cells, requiring IL–15, the T–box expressed in T cells (T–bet) transcription factor and the Src family kinase Fyn (Kronenberg and Rudensky, 2005).

1.3.3.3 NF–κB in NKT cells

A role for NF–κB signalling appears to be important in Vα14i NKT cell development. Although conventional T cell development is normal in RelB and NIK–knockout mice, Vα14i NKT cell development is impaired due to non–haematopoietic cell defects (Sivakumar et al., 2003, Elewaut et al., 2003). Furthermore, a T cell–intrinsic requirement for NF–κB activation in NKT cell development has been demonstrated in transgenic mice expressing a T cell specific IκBα "super–repressor" (Sivakumar et al., 2003, Schmidt-Supprian et al., 2004a). Consistent with this, T cell deficiency of IKK2 profoundly affects Vα14i NKT cell differentiation, resulting in their complete absence in thymus and peripheral organs (Schmidt-Supprian et al., 2004a). These findings have suggested that RelB expression in stromal cells is important for differentiation of NKT, whilst classical NF–κB activity in T cells is important for expansion of thymic NKT cells (Sivakumar et al., 2003). Interestingly, *Bcl10*$^{-/-}$ mice, which lack NF–κB activity downstream of the TCR, show a defect in peripheral Vα14i NKT cells, but normal thymic numbers (Schmidt-Supprian et al., 2004a, Sivakumar et al., 2003). These conflicting results may indicate that NF–κB activation occurs through a different receptor to the TCR during Vα14i NKT cell development.

1.4. NF–κB in peripheral T cell function

1.4.1 Mouse models

Analysis of the role NF–κB plays in mature T cell function has been hindered by the absence of peripheral T cells in mice that lack NF–κB activity in their T cells, including mice expressing a kinase–dead form of IKK2 or a deletion of NEMO in their T cells (Schmidt-Supprian et al., 2003). However, T-cell specific knockout strains with a partial block in NF–κB activity, or which lack particular NF–κB subunits, have revealed essential but redundant roles for NF–κB in mature T cells. A particular role in T cell survival, growth, proliferation and effector function has been inferred (summarised in Table 1.1).

The canonical NF–κB pathway in peripheral T cells has been studied extensively using a transgenic mouse expressing a T cell–restricted super–repressor of IκBα (Boothby et al., 1997). Splenic $CD4^+$ T cells from these mice have a proliferation defect in response to anti–CD3 and anti–CD28 costimulation. Although IL–2 production is also impaired, addition of exogenous IL–2 had no impact on the proliferation of these cells. Defects in proliferation have also been observed in other NF–κB–deficient mutant mouse strains, including *Rela*$^{-/-}$ $CD4^+$ T cells from stem cell chimeras, which cannot be rescued by pharmacological stimulation (PMA/ionomycin) or IL–2. In contrast, T cells from *crel*$^{-/-}$ mice show a marked block in proliferation defect that is overcome by addition of exogenous IL–2 or

CD28 costimulation (Kontgen et al., 1995, Zheng et al., 2001). This result is consistent with an NF–κB–dependent upregulation of IL–2, and suggests a non–redundant role for c–Rel in this event. Furthermore, analysis of *crel*$^{-/-}$ T cells reveals that c–Rel can regulate chromatin remodeling across the IL–2 promoter (Rao et al., 2003, Chen X, 2005).

The functional redundancy between NF–κB transcription factors is demonstrated by double subunit knockouts, such as *Nfkb1*$^{-/-}$*crel*$^{-/-}$ CD4^{+} T cells and CD4^{+} T cells expressing IκBα "super–repressor", which display compound phenotypes. In accordance with a role for NF–κB in regulating apoptosis, *Nfkb1*$^{-/-}$*crel*$^{-/-}$ CD4^{+} T cells and T cells expressing the IκBα super–repressor are more susceptible to TCR–induced cell death than WT cells. In *Nfkb1*$^{-/-}$*crel*$^{-/-}$ CD4^{+} T cells, this is due to an inability to upregulate expression of the anti–apoptotic Bcl–xL gene (Zheng et al., 2003). In addition to a survival defect, *Nfkb1*$^{-/-}$*crel*$^{-/-}$ CD4^{+} T cells have a TCR–induced defect in cell cycle progression and proliferation, which is partially caused by a defect in IL–2 production (Zheng et al., 2003). On the other hand, the cause of the proliferation defect in T cells from *crel*$^{-/-}$ *Rela*$^{-/-}$ radiation chimeras is their inability to upregulate c–myc and enter the G1 phase of the cell cycle (Grumont et al., 2004). Therefore, NF–κB is involved in a number of cellular functions in CD4^{+} T cells.

Table 1.1 Knockout and transgenic mouse strains of NF-κB subunits and NF-κB signalling proteins

1.4.2 $CD4^+$ T helper cells

The T helper (Th) cell hypothesis arose from research suggesting that upon encounter with antigen, naïve murine $CD4^+$ T cells can differentiate into specialised subsets, with differing cytokine production profiles (Mosmann et al., 1986). Two subsets were initially proposed to be generated, Th1 and Th2, depending on the types of co–stimulatory molecules expressed and cytokines secreted by APCs, particularly DCs (Constant and Bottomly, 1997).

1.4.2.1 Th1 and Th2 subsets

The production of IL–12 by DCs promotes development of Th1 cells, whilst IL–4 from activated T cells and NK cells, can drive differentiation of Th2 cells (reviewed in (O'Garra, 1998, Glimcher and Murphy, 2000)). Since the cytokine profiles of Th1 and Th2 cells are distinct, this divergence provides an insight into how the immune system responds selectively to different microbes, thereby directing the quality of the immune response by targeting specific cells (O'Garra, 1998, Farrar et al., 2002). Th1 cells secrete pro–inflammatory cytokines such as interferon (IFN)γ, lymphotoxin, TNFα and IL–2. These cytokines attract and augment the microbicidal capacity of monocytes and macrophages, and promote cytotoxic $CD8^+$ T cell development. Th2 cells produce the signature cytokine IL–4, but also IL–5, IL-6, IL–10 and IL–13, which serve to activate proliferation and drive differentiation of B cells into antibody secreting plasma cells, modulate Th2 cell development and suppress inflammatory responses. Consequently, Th1 cells are important for

cellular immunity, in responses to intracellular pathogens and viruses, whereas Th2 cells induce humoural immunity against extracellular pathogens such as multicellular parasites (Constant and Bottomly, 1997).

Differentiation of Th1 and Th2 cells is mediated by STAT-induced activation of several key transcription factors. Differentiation of Th1 cells critically relies on IL–12 induction of STAT4, STAT1 and the T-box transcription factor T–bet, which potentiates IFNγ production, whilst downregulating Th2 cytokine expression (Szabo et al., 2000, Mullen et al., 2001). For Th2 differentiation, STAT6–induced activation of GATA–3 is necessary, upregulating expression of IL–4 and Th2 cytokine genes, whilst suppressing factors which promote Th1 differentiation (Zheng and Flavell, 1997, Ouyang et al., 1998, Ouyang et al., 2000). As a result of this transcriptional regulation, particular immune responses are dominated by either a Th1 or Th2 subsets.

1.4.2.2 Th17 cells

Recently, a novel $CD4^+$ T cell helper subset, Th17, has been described, which has been suggested to regulate inflammatory responses of non–lymphoid tissues (Harrington et al., 2005). Th17 cells are characterized by their production of IL–17A, but can also produce IL–21, IL–22 and IL–17F (reviewed in (Ouyang et al., 2008)). IL-17 stimulates the secretion of pro-inflammatory cytokines by epithelial, endothelial and stromal cells, which can recruit neutrophils and macrophages to the site of infection (Kolls and Linden, 2004). Thus, Th17 cells may play a protective role in immunity. Consistent with this, in the absence of IL-17, mice

succumb to the bacterial pathogen *Klebsiella pneumoniae* (Happel et al., 2005, Happel et al., 2003). Importantly, the local tissue inflammation triggered by Th17 cells has also been implicated in the pathogenesis of autoimmune diseases such as collagen–induced arthritis and EAE (Komiyama et al., 2006).

Differentiation of Th17 cells *in vitro* and *in vivo* requires TGFβ and IL–6 signalling (Veldhoen et al., 2006, Bettelli et al., 2006, Mangan et al., 2006), from which STAT3 and the Th17 lineage–specific transcription factors retinoic acid receptor (RAR)–related orphan receptor (ROR) family, RORγt and RORα are activated (Ivanov et al., 2006). Furthermore, several other cytokines are believed to maintain Th17 cells, including IL–21, which acts in an autocrine manner, and IL–23 and IL–1 produced by myeloid cells (Nurieva et al., 2007, Korn et al., 2007, Veldhoen et al., 2006). Conversely, IL–4, IFNγ, IL–27 and IL–2 can inhibit Th17 differentiation (Laurence et al., 2007, Batten et al., 2006, Stumhofer et al., 2006).

1.4.2.3 NF–κB in $CD4^+$ T cell helper function

The differentiation of $CD4^+$ T cells into Th1 and Th2 cells appears to require NF–κB activation. Indeed, $CD4^+$ T cells from *Nfkb1*$^{-/-}$ mice are unable to differentiate into Th2 cells and secrete IL–4 and IL–13 due to an inability to upregulate GATA–3 expression, even under Th2–inducing stimuli (Das et al., 2001, Artis et al., 2002). In contrast, expression of T–bet does not require NF–κB1, and production of IFNγ in *Nfkb1*$^{-/-}$ $CD4^+$ T cells is normal *in vitro*. However, *Nfkb1*$^{-/-}$ mice are more susceptible to *Leishmania major* compared to WT mice (Artis et al., 2002). A

similar defect in Th1–mediated clearance of *Toxoplasma gondii* occurs in *crel*$^{-/-}$ mice (Mason et al., 2004). In both of these cases, clonal expansion of Th1 cells is impaired, suggesting NF–κB is required for optimal proliferation of Th1 cells. NF–κB also seems to play a role in Th17 differentiation, although this remains to be characterised. However, impairment of NF–κB activation in *Nfkb1*$^{-/-}$ and *crel*$^{-/-}$ mice has been seen to provide protection against the Th17–dependent neuronal autoimmune disease EAE (Hilliard et al., 2002, Hilliard et al., 1999). Furthermore, T–cell specific deletion of IKK2 virtually prevents all incidence of MOG (Myelin oligodendrocyte– glycoprotein)– induced EAE (Greve et al., 2007).

1.4.3 Memory T cells

The acquisition of memory is a hallmark of the adaptive immune system. Upon exposure to antigen, specific naïve T lymphocytes undergo clonal expansion and differentiation to become effector cells. After clearance of antigen, a contraction phase follows, where most effector cells die by apoptosis. Those that remain are long–lived antigen–specific memory T (T_{mem}) cells. These cells form the basis of the rapid and specific responses of the adaptive innate response, due in part to the increased precursor frequency of antigen specific T cells in immune mice compared to naive animals (Bousso et al., 1999, Kaech et al., 2002b). Furthermore, accumulation of lipid rafts with higher proportion of phosphorylated proteins (Kersh et al., 2003) and increased expression of genes that promote cell cycle progression (Latner et al., 2004, Veiga-Fernandes and Rocha, 2004) are found in T_{mem} cells compared to naïve T cells. Together, this is believed to make

T_{mem} cells more sensitive than naïve T cells to antigenic stimulation, allowing more rapid entry into the cell cycle and cell division when antigen is re–encountered.

Central and effector memory Memory $CD4^+$ and $CD8^+$ T cells are a heterogeneous population, and two broad subsets have been identified, based on their surface phenotype and effector functions. Effector memory T cells (T_{EM}) cells are characterized as $CCR7^-$ and largely $CD62L^-$, and migrate to inflamed non-lymphoid tissues, displaying immediate effector functions. In contrast, central memory T cells (T_{CM}) cells express the lymphoid-homing receptors CD62L and CCR7, but have little constitutive effector function, producing mainly IL-2 (Sallusto et al., 1999). However, factors influencing the generation and maintenance of these populations are unclear (reviewed in (Sallusto et al., 2004)).

1.4.3.1 Generation of T_{mem} cells

Initial activation of naïve T cells occurs in response to TCR engagement and innate immune cell costimulation. The subsequent expansion and contraction of antigen–specific T cells requires cellular proliferation and survival signals, resulting in the generation of T_{mem} cells. However, the pathway that leads to formation of these cells is unclear. Although many studies indicate that memory cells are generated directly from differentiated effector cells (Garcia et al., 1999, Kaech et al., 2002a), some groups propose that memory cells are generated independent of an intermediate effector stage (Lauvau et al., 2001, Manjunath et al., 2001). However, more recently an "intersecting model" of T_{mem} generation has been put forward, which suggests that T_{mem} cells can develop at various stages of effector

differentiation (Moulton and Farber, 2006, Harrington et al., 2008).

Memory–phenotype cells In the absence of immunization, a small population of T cells resembling memory T cells (T_{mem}–phenotype cells) are found, which expand in size with age (Linton and Dorshkind, 2004). The generation of these T_{mem}–phenotype cells is widely believed to occur in response to environmental antigens, such as to resident microbes, although some may also arise from homeostatic proliferation after recognition of spMHC ligands in conjunction with IL–7 signals, as in lymphopoenic conditions (Viret et al., 1999, Ernst et al., 1999, Goldrath and Bevan, 1999, Kieper and Jameson, 1999).

1.4.3.2 Memory T cell homeostasis

The naïve and memory T cell pools are maintained at relatively fixed sizes, important to ensure the diversity of the T cell repertoire, whilst allowing re–encounter with antigen to elicit a rapid response. Initially, these populations were thought to be regulated by independent homeostatic mechanisms. Most studies indicate that naïve T cells require constant spMHC:TCR interaction and IL–7 for their long–term survival and homeostatic proliferation (Takeda et al., 1996b, Seddon et al., 2000, Tan et al., 2001, Kirberg et al., 1997, Tanchot et al., 1997). In contrast, T_{mem} cells can persist in the absence of MHC molecules or after TCR ablation, suggesting that their survival does not depend on continual stimulation by spMHC:TCR molecules (Swain et al., 1999, Murali-Krishna et al., 1999, Kassiotis et al., 2002, Tanchot et al., 1997). Therefore, a role for cytokines in T_{mem} cell homeostasis was inferred. However, understanding the homeostasis of T_{mem} cells

has been complicated by differential requirements, depending on the memory-subset and the manner in which the cells are generated (reviewed in (Surh et al., 2006).

CD8⁺ memory A number of studies indicate that homeostatic proliferation and/or long–term persistence of viral–specific $CD8^+$ T_{mem} cells and $CD8^+$ T_{mem}–phenotype cells are dependent on IL–15 (Ku et al., 2000, Schluns et al., 2002, Goldrath et al., 2002, Kaech et al., 2003, Tan et al., 2002). Accordingly, $CD8^+$ T_{mem}–phenotype cells are absent in *Il15*$^{-/-}$or *Il15r*$^{-/-}$mice (Kennedy et al., 2000, Lodolce et al., 1998) and do not undergo homeostatic proliferation or survive after transfer into *Il15*$^{-/-}$ hosts (Judge et al., 2002). Interestingly, survival of a subset of $CD8^+$ T_{mem}–phenotype cells ($CD122^{hi}$), is supported by IL–7 (Schluns et al., 2002, Kondrack et al., 2003) whilst $CD122^{lo}$ $CD8^+$ T_{mem}–phenotype cell survival require MHC interactions (Boyman et al., 2006). Furthermore, an emerging role for $CD4^+$ T helper cells in persistence of $CD8^+$ memory has been described, although it is unclear whether a distinct subset of $CD4^+$ effector cells mediates these functions (Bourgeois et al., 2002, Janssen et al., 2003, Shedlock and Shen, 2003, Sun et al., 2004a, Sun and Bevan, 2003).

CD4⁺ memory The requirements for $CD4^+$ T_{mem} cell generation and maintenance are less clear than for $CD8^+$ T_{mem} cells, partly due to the heterogeneity of this population. Early studies in *Il2rg*$^{-/-}$and *Il15*$^{-/-}$mice suggested that γ–chain cytokines and IL–15 were not necessary for survival or homeostatic proliferation of $CD4^+$ T_{mem}–phenotype cells (Lantz et al., 2000, Judge et al., 2002).

However, basal proliferation and survival of antigen–specific $CD4^+$ T_{mem} cells have since been shown to be influenced by levels of IL–15 (Lenz et al., 2004), and a requirement for IL–7 signals in the proliferation and survival of $CD4^+$ T_{mem}–phenotype cells in the absence of TCR signals has also been demonstrated (Seddon et al., 2003). Moreover, $CD4^+$ effector cell transition to T_{mem} cells is impaired upon transfer into *Il7*$^{-/-}$ mice, indicating that antigen–specific $CD4^+$ T_{mem} cells require IL–7 in the presence of intact TCR signalling (Li et al., 2003, Kondrack et al., 2003). Interestingly, ablation of TCR signals has been reported to impair the basal proliferation of $CD4^+$ T_{mem}–phenotype cells *in vivo* (Polic et al., 2001), whilst expansion and functional activity of antigen–specific $CD4^+$ T_{mem} cells is impaired in the absence of contact with MHC molecules (Kassiotis et al., 2002). Therefore, these recent studies indicate that the signals that regulate naïve and T_{mem} cell homeostasis may not be as distinct as was originally proposed and that IL-7 and TCR signals may be required for both subsets (Caserta and Zamoyska, 2007). Furthermore, biochemical analysis has started to reveal points of convergence of TCR and cytokine signalling pathways in $CD4^+$ T_{mem} cells (Riou et al., 2007).

1.4.3.3 Phenotypic identification

Changes in surface markers upon T cell activation, particularly L–selectin (CD62L), CD44 and CD45, are significant for function of T_{mem} cells. The expression of these markers can therefore provide a phenotypic means of distinguishing T_{mem} cells from naïve and effector T cells. CD62L is an adhesion molecule, whose expression is lost on $CD4^+$ T_{mem} cells as a result of proteolytic cleavage, whereas CD44 (H–CAM) is upregulated in T_{mem} cells (Bradley et al., 1994, Bradley et al., 1992, Cho et

al., 1999). These changes allow migration of T_{mem} cells from blood into tissues, instead of recirculation between lymphoid organs. In addition, splicing of exons encoding the extracellular domain of CD45 in $CD4^+$ T_{mem} cells results in expression of a distinct smaller isoform, CD45RO, rather than CD45RB that is expressed on naïve murine $CD4^+$ T cells (Rothstein et al., 1992, Clement et al., 1988). This change in CD45 isoform facilitates antigen recognition by the TCR. An important caveat to the use of markers to identify T_{mem} cells, is that many of these changes can also occur in activated T cells or cells undergoing homeostatic proliferation (Burkett et al., 2004).

1.4.3.4 NF–κB in memory cells

The importance of NF–κB in generation and/or survival of T_{mem}–phenotype cells has been demonstrated by genetic analysis. Mice deficient in both c–Rel and p50 reveal a marked reduction in splenic $CD4^+CD44^{hi}CD62L^-$ T cells (Zheng et al., 2003). Furthermore, a reduced pool of T_{mem}–phenotype cells is found in mice expressing a conditional T cell IKK2–deletion, with a more pronounced defect in $CD4^+CD44^{hi}$ cells than $CD8^+CD44^{hi}$ cells (Schmidt-Supprian et al., 2003).

1.5 Thesis Aims

Genetic studies have implicated the NF–κB family of transcription factors in the regulation of T cell development and function. The canonical NF–κB pathway has been most extensively studied using transgenic mice expressing a T cell–specific non–degradable form of IκBα. This mutation significantly reduces nuclear levels of

p50/RelA and p50/c–Rel heterodimers. However, the role of IKK–induced proteolysis of NF–κB1 p105 in T cell function is unknown. In this study, we specifically address this question through analysis of a knock–in mouse strain expressing a mutant p105, $p105^{SS/AA}$, which lack IKK–target serine residues, and is therefore resistant to signal–induced proteolysis (depicted in Figure 1.6).

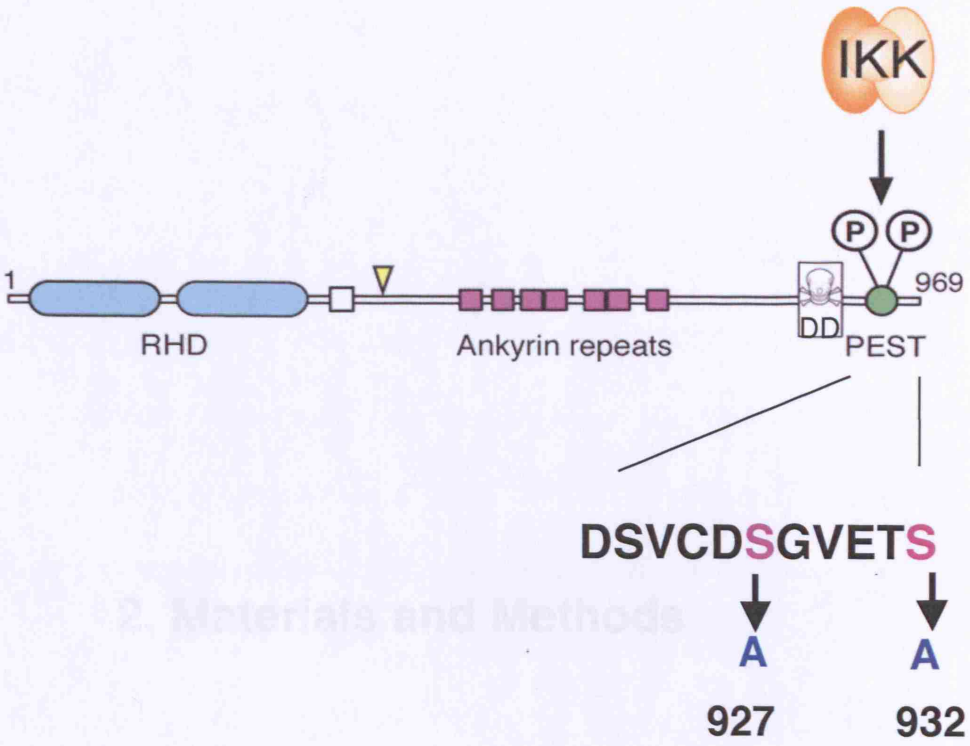


Figure 1.6 IKK-target sites on p105 and *Nfkb1*$^{SS/AA}$ mutation.
In activated cells, the kinases of the IKK complex, IKK1 and IKK2, can target p105 for phosphorylation at serine residues 927 and 932 of the PEST region. These phosphorylated sites are recognized by the βTRCP of the SCF E3 ligase complex, and are necessary for polyubiquitination and subsequent degradation of p105. In *Nfkb1*$^{SS/AA}$ mice, serine 927 and serine 932 IKK-target sites are mutated to alanine, thus blocking IKK-induced p105 proteolysis. Abbreviations: βTRCP, β-transducin repeat containing protein; IKK, inhibitor of NF-κB kinase; SCF, Skp1/Cul 1/F-box.

2. Materials and Methods

2. Materials and Methods

2.1 Mice

2.1.1 *Nfkb1*$^{SS/AA}$ mice

Nfkb1 "knock-in" *Nfkb1*$^{SS/AA}$ mice were generated by M. Belich (NIMR, Ley laboratory). A targeting vector of *Nfkb1* exon 24, in which Ser927Ala and Ser932Ala mutations were introduced, was used for recombination with endogenous *Nfkb1* alleles. The expressed p105^{S927A,S932A} protein is processed to p50 as normal, but is resistant to signal-induced proteolysis (Lang et al., 2003, Salmeron et al., 2001). Mice generated were on a mixed C57BL/6 x 129S8 background. Experiments were carried out on homozygous *Nfkb1*$^{SS/AA}$ mice that had been backcrossed four times with129S8 congenic mice (referred to as *Nfkb1*$^{SS/AA}$ 129S8mice) aged 8-12 weeks old. Age and sex-matched wild-type (WT) 129S8 mice were used as controls. Some experiments required *Nfkb1*$^{SS/AA}$ mice on a C57BL/6 background, in which case *Nfkb1*$^{SS/AA}$ mice that had been backcrossed for at least five generations with C57BL/6 mice were used, referred to as mixed background mice. For these experiments, WT littermates were used as controls. 129S8 and mixed background *Nfkb1*$^{SS/AA}$ mice were generated at expected Mendelian ratios. No gross abnormalities were observed, except that the majority of *Nfkb1*$^{SS/AA}$ mice either lacked, or had smaller inguinal lymph nodes (LN) than that of WT mice. Interestingly, *Nfkb1*$^{-/-}$ mice also lack inguinal LN and previous studies have demonstrated the importance of the alternative pathway in secondary lymphoid architecture (Caamano et al., 1998, Lo et al., 2006).

2.1.2 Other mouse strains

Map3k8$^{-/-}$ mice, on a C57BL/6 background, were a kind gift from P.N. Tsichlis (Tufts University, Boston). H2^{a} *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*(C5*$^{-/-}$*)* mice were generously provided by B. Stockinger (NIMR, London), and kept on irradiated drinking water. C57/BL6 *Rag1*$^{-/-}$ mice and C57/BL6 CD45.1 congenic mice were generated at the NIMR. All mice were housed in a pathogen-free environment. Animals used in experiments were killed by a Schedule One method, according to Home Office regulations.

2.2 Cell isolation

2.2.1 LN and spleen

Spleen and LN (axillary, brachial, mesenteric, cervical and lumbar nodes) of experimental mice were dissected and cell suspensions prepared by pressing lymphoid organs through 70 μm cell strainers (BD Pharmingen) in air buffered Iscove's Modified Dulbecco's Media (AB-IMDM) containing 5 % (v/v) fetal calf serum (FCS; Labtech International). To remove erythrocytes, cells were centrifuged (1200 rpm, 5 min for this and all subsequent steps) and resuspended in ACK buffer (0.155 M ammonium chloride, 0.1 M ethylene diamine tetraacetic acid (EDTA), 0.01 M potassium bicarbonate in dH_20) for 3 min, or until the solution cleared, to a maximum of 5 min. Cells were washed in ice cold AB-IMDM to dilute ACK buffer, centrifuged, and resuspended in fresh AB-IMDM containing 5 % (v/v) FCS. Cells were kept on ice for all assays.

2.2.2 Peripheral blood

Blood was collected from tail veins of experimental mice, to a total of approximately 100 μl and mixed in 100 μl of heparin (Sigma). Red blood cells were lysed in 2 ml ACK buffer/sample and washed in AB-IMDM as described above.

2.3 $CD4^+$ T cell purification

2.3.1 $CD4^+CD25^-$ T cell magnetic depletion

$CD4^+CD25^-$ T cells were purified from splenic or LN cell suspensions by means of negative selection. B lymphocytes, $CD8^+$ cells, $CD25^+$ cells, NK cells, macrophages, monocytes and neutrophils were labelled for removal with a "cocktail" of biotinylated antibodies consisting of anti-B220, -CD19, -CD8, -CD25, -DX5, -MacI and Gr1 (ebiosciences), diluted 1:200 in AB-IMDM containing 5 % (v/v) FCS. Cell suspensions were incubated with antibodies on ice for 30 min. Stained cells were then washed in phosphate buffered solution (PBS), before being resuspended in 4 ml PBS containing 5 % (v/v) FCS and streptavidin-conjugated Dynalbeads (M280, Dynal Invitrogen) at a 1:1 ratio of cells to beads. Cells were incubated for 30 min at 4 °C with constant rotation. Depletion of bead-bound cells was then performed twice using a magnetic Dynal bead separator (Dynal, Invitrogen), with an intermediary wash in PBS containing 5 % (v/v) FCS. To culture, purified $CD4^+CD25^-$ T cells were washed and resuspended in complete IMDM media (10 % FCS, 2 mM L-Glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 50 μM β-mercaptoethanol). Depletion efficiency was determined by

fluorescence-activated cell sorting (FACS), and consistently found to be 90-95 % from LN and 75-80 % from spleen.

2.3.2 Cell sorting

Prior to cell sorting $CD4^+$ T cells were depleted as described above, but with two modifications. Firstly, cell suspensions of pooled LN and spleen were used to increase yield, whilst biotinylated-CD25 was excluded from the depleting antibody cocktail. Enriched $CD4^+$ T cells were washed in FACS staining buffer (Dulbecco's PBS supplemented with 5 % (v/v) FCS) and stained with APC-conjugated CD4 (CD4-APC), CD25-PE and CD44-FITC diluted, according to manufacturers guidelines, in 1 ml FACS staining buffer (PBS, 0.1 % (w/v) sodium azide). Cells were stained for 30 min at 4 °C in the dark to prevent antibody degradation. After incubation, cells were washed and resuspended in 4 ml IMDM and finally passed through 40 μM cell strainers (BD Pharmingen). Cell sorting was performed on a Moflow cell sorter (Dako Cytomation).

2.4 $CD4^+$ T cell *in vitro* stimulation

Stimulation of purified $CD4^+$ T cells was carried out in flat-bottom 96-well plates (Nunc) pre-coated overnight with monoclonal CD3ε antibody (3C-11;BD Biosciences) at 4 °C. Soluble CD28 antibody (37.51 BD Biosciences; 1 μg/ml) and recombinant murine IL-2 (Peprotech; 20 ng/ml) were used in indicated cultures. In some experiments, pharmacological stimulation with phorbol dibutyrate (PdBU; Sigma; 10 ng/ml) plus ionomycin (Sigma; 1 μg/ml) was carried out. Cells were

plated at $1x10^6$ cells/ml, counted using a Casy-1-cell counter (Scharfe System), to a total volume of 200 μl/well in 96-well plates. Cells were cultured in complete IMDM media for all experiments, and assays were carried out in triplicate.

2.5 Flow cytometry

2.5.1 Surface staining

Cells were either harvested from *in vitro* culture, or plated directly from cell suspensions for staining, at a density of $1x10^5$- $1x10^6$ cells/well into 96-well v-bottom plates (Sterilin). To block non-specific binding of antibody IgG to Fc-receptor, cells were pre-incubated for 10 min with 24G2 supernatant at 4 °C. Surface markers were stained with a mixture of appropriate fluorescence-labelled monoclonal antibodies and/or biotinylated antibodies diluted, as per manufacturer's guidelines, in FACS staining buffer. Antibodies used for flow cytometry are listed in Table 2.1. Cells were covered in foil and incubated with antibodies at 4 °C for 30 min. Stained cells were then washed with FACS buffer (PBS, 0.1 % (w/v) sodium azide), centrifuged and incubated with secondary antibody as required for a further 30 min at 4 °C in the dark. Prior to flow cytometric analysis, cells were resuspended in 100-300 μl FACS buffer cells and passed through 40 μM cell strainers.

2.5.2 Intracellular staining

For intracellular staining, surface proteins were stained as above, prior to fixation for 30 min on ice in the dark with 4 % (w/v) paraformaldehyde (Sigma) in PBS.

Cells were washed in FACS buffer, centrifuged and resuspended in 100 μl 0.1 % (v/v) NP-40 (Sigma) permeabilisation buffer for 10 min on ice. FACS buffer was added to stop the reaction and cells were pelleted by centrifugation at low speed (1000 rpm, 5 min). Cells were resuspended in 50 μl of fluorescent intracellular antibody, or corresponding IgG monoclonal isotype control at appropriate dilution, and incubated for 30 min on ice in the dark. After a further wash in FACS buffer, cells were resuspended in 100 μl FACS buffer for flow cytometric analysis. For intracellular Foxp3 staining, $1x10^6$ cells/stain an APC anti-mouse Foxp3 staining kit was used, according to manufacturer's instruction (ebiosciences).

Cells stained for surface and intracellular proteins were acquired on a FACSCalibur for 4-colour analysis or LSRII (BD Pharmingen) for 5-6-colour analysis. Data was analysed using the FlowJo v8.4 program (TreeStar, Ashland, OR), and is based on gating of live lymphocytes through characteristic forward and scatter profiles (referred to as live gates), with the exception of analysis for apoptosis assays.

2.5.3 Cd1d tetramer staining

Cell suspensions from thymus and spleen were plated in 96-well v-bottom plates (Sterilin) at a density of $1x10^6$ cells in 50 μl. After block of Fc receptors, cells were centrifuged and resuspended in 10 μl of CD1d tetramer loaded with αgalactosyl ceramide (αGal-Cer) and conjugated to APC, kindly provided by A. Bendelac (University of Chicago). Tetramer and cells were incubated with gentle agitation for 45 min at 37 °C in the dark. FACS antibodies were added directly to cell

suspensions and subsequent cell surface staining and flow cytometry was performed as detailed above (*Section 2.4.1*).

2.6 CFSE labelling

The fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) was used for proliferation assays and as a tracker dye *in vivo* (described in *Section 2.7*). To this end, $CD4^+$ or $CD4^+CD25^-$ T cells were first purified by magnetic depletion or cell sorting, as described in *Section 2.4*. Cells were washed and resuspended in pre-warmed Dulbecco's PBS (Invitrogen) to a concentration of $1x10^7$ cells in 0.5 ml. A further 0.5 ml of warm PBS containing CFSE, to a final concentration of 2-5 μM, was added to cell suspensions and mixed rapidly. Labelling was carried out for 10 min at room temperature, and followed by two washes in complete IMDM media.

2.6.1 Proliferation assays

For proliferation assays, cells were cultured in complete IMDM media under indicated activation conditions for 72 h. CFSE stably binds to intracellular amines, so that after each mitotic division, daughter cells contain half the amount of CFSE, emitting half the fluorescence. Measurement of CFSE content therefore permitted analysis of cell division. Prior to FACS analysis, cells were stained with 7-amino-actinomycin D (7-AAD; Sigma; 10 μg/ml), a dye able to bind double-stranded nucleic acids of membrane-compromised dead cells. 7-AAD positive cells were

therefore excluded from analysis, and proliferation assessed using the proliferation analysis tool of the FlowJo v8.4 program (TreeStar, Ashland, OR).

2.7 Cell survival assays

2.7.1 *In vitro* cell death assays

Assessment of cell viability and apoptosis were performed on purified $CD4^+CD25^-$ T cells stimulated with CD3 antibody (as in *Section 2.3* and *2.4*) or cultured in complete IMDM medium alone for 0, 24 and 48 h. Cells were then stained for surface expression of CD4 and washed in FACS buffer, as described in *Section 2.4*. Cells undergoing apoptosis express phosphatidylserine residues on their surfaces. To identify these, CD4-labelled cells were stained with Annexin V, which binds phosphatidylserine, using the Annexin V-FITC kit, according to manufacturers protocol (BD Biosciences). Cells were then washed in FACS buffer and resuspended in 100 μl FACS buffer containing 10 μg/ml 7-AAD for 5 min at room temperature, for assessment of a later phase of apoptosis. Cell numbers were quantified by the addition of a known number of fluorescent TruCount beads (Cytognos) into cell suspensions prior to flow cytometric analysis. Apoptosis of total gated $CD4^+$ T cells were then presented as a fraction of input cells.

2.7.2 Analysis of $CD4^+$ T cell survival *in vivo*

Total $CD4^+$ T cells from spleens of sex-matched WT or *Nfkb1*$^{SS/AA}$ C57BL/6 x 129S8 mixed background mice were isolated by magnetic depletion (*Section 2.3.1*) and labelled with 5 μM CFSE tracker dye as described above (*Section 2.6*).

Labelled cells were counted and adjusted to $5x10^6$ depleted $CD4^+$ T cells in 200 μl FCS-free IMDM. Cells were passed through 40 μM cell strainers before transfer *via* tail vein injection into replete WT C57BL/6 mice. On day 1, and for each subsequent week for 6 weeks, blood samples were collected (*Section2.2.2*) and the percentage of transferred $CD4^+CFSE^+$ T cells analysed by flow cytometry.

2.8 BrdU assays

2.8.1 *In vitro* assay of cell cycle progression

$CD4^+CD25^-$ T cells were depleted from LN of WT and *Nfkb1*$^{SS/AA}$ mice (*Section 2.3.1)*, and plated at $1x10^6$ cells/ml to a final volume of 200 μl/well in a 96-well plate, as detailed in *Section 2.2.1*. Stimulation was carried out for 24 h using monoclonal antibodies (*Section 2.4*). During the final 16 h, cultures were supplemented with 20 μl of 5-bromo-2-deoxyuridine (BrdU; Sigma) dissolved in complete IMDM to a final concentration of 10 μM. Cells were harvested, stained for surface markers and fixed in 100 μl of 4 % (w/v) paraformaldehyde in PBS. To neutralise fixative, 100 μl of 50 mM ammonium chloride was added. Washed cells were then permeabilised in 100 μl of 0.1 % (v/v) NP-40 for 10 min on ice. After two washes with PBS (in the absence of sodium azide), cells were resuspended in 10 μl solution of BrdU antibody conjugated with FITC containing DNase (BD biosciences). Intracellular BrdU staining was performed for 1 h at room temperature in the dark, before cells were washed in FACS buffer, resuspended in 100 μl and analysed by flow cytometry.

2.8.2 *In vivo* BrdU turnover assay

WT and *Nfkb1*$^{SS/AA}$ mice were administered 1.2 mg BrdU (Sigma) by intraperitoneal injection and drinking water supplemented with 0.8 mg/ml BrdU. After 12 d, mice were culled and cell suspensions prepared from spleen and LN. Intracellular BrdU staining was carried out as above and the percentage of BrdU stained $CD4^+CD44^{lo}$ naïve, $CD4^+CD44^{hi}$ memory and $CD4^+CD25^+$regulatory cells determined by flow cytometry (Tough and Sprent, 1994).

2.9 Generation of bone marrow-derived dendritic cells (BMDC)

Bone marrow (BM) was flushed from tibia and femur of experimental mice using a 23-guage needle, and collected in a 5 ml syringe. For hypotonic lysis of red blood cells, 1 ml ACK buffer/mouse was added to BM suspensions, after which cells were washed with ice cold IMDM. Cells were centrifuged and resuspended for determination of cell number, using a haemocytometer. $5x10^6$ BM cells were plated on p90 plates (Sterilin) in 10 ml complete IMDM supplemented with GM-CSF (10 ng/ml; kindly provided by A. O'Garra). Cells were cultured for 4 d, after which a further 10 ml of BMDC media was added to each plate. On d7, non-adherent cells were harvested, counted on a haemocytometer and plated for experiments (Inaba et al., 1992).

2.10 BMDC stimulation of sorted naïve cells

WT and *Nfkb1*$^{SS/AA}$ $CD4^+$ naïve T cells were isolated by cell sorting (*Section 2.3.2*), and labelled with 2 μM CFSE (*Section 2.6*), whilst BMDCs were generated from

syngeneic129S8 mice (*Section 2.9*). Cells were plated in 96-well flat-bottom plates (Nunc) at $0.2x10^6$ naïve $CD4^+$ T cells/well and $2x10^5$ BMDC/well, with the addition of 1 μg/ml soluble anti-CD3 to a total volume of 200 μl. After 72 h, cells were stained for surface molecules (*Section 2.5.1)* and resuspended in 10μg/ml 7AAD in FACS buffer *(Section 2.7.1)* for flow cytometric analysis. Proliferation was assessed based on CFSE dilution of live $CD4^+$ T cells.

2.11 Allogeneic proliferation assays

2.11.1 Mixed lymphocyte reaction (MLR)

BMDCs from syngeneic129S8 (H-2^b) and allogeneic Balb/C (H-2^d) mice were generated as described above (*Section 2.9*), and cultured at a ratio of 1:10 with CFSE-labelled depleted LN $CD4^+CD25^-$ T cells (*Section 2.3.1* and *2.6*). Cells were plated at a density of $1x10^5$ BMDC and $1x10^6$ $CD4^+CD25^-$ T cells/well in a 96-well flat bottom plate, to a volume of 200 μl. Cells were stained for surface markers and 10μg/ml 7-AAD after 96-120 h in culture, as above (*Section 2.5.1*), and proliferation of viable 7-AAD^- $CD4^+$ cells analysed by flow cytometry.

2.11.2 Measurement of *in vivo* allogeneic response

Splenic $CD4^+CD25^-$ T cells from WT and *Nfkb1*$^{SS/AA}$ 129sv mice (H-2^b) were enriched by negative selection and labelled with 2-5 μM CFSE as described above (*Section 2.3.1* and *2.6*). Cells were washed with FCS-free IMDM, resuspended and strained through a 40 μM cell strainer. $5x10^6$ CFSE-labelled $CD4^+CD25^-$ T cells in 200 μl IMDM were then transferred by intravenous injection into host H-2^a

Rag2$^{-/-}$*gc*$^{-/-}$(*C5*$^{-/-}$) mice (Kassiotis et al., 2003). Experimental mice were culled and spleens removed on d1, d4 and d7 post-transfer. To assess allogeneic proliferation of transferred CD4^{+} T cells, splenic cellularity was measured using a Casy-1 cell counter. Subsequently, splenocytes were stained for surface markers and CFSE content assessed by flow cytometry (*Section 2.5.1*).

2.12 Radiation Bone Marrow Chimeras

BM suspensions from mixed background (C57BL/6 x 129S8) *Nfkb1*$^{SS/AA}$ or WT littermate mice were isolated as above (*Section 2.9*) and removed of T cells using TCRβ-bio antibody (H57-97; BD bioscience) and magnetic depletion, as previously described (*Section 2.3.1*). To generate single bone marrow chimeras, a total of 5x10^{6} BM cells in 200 μl IMDM were transferred *via* tail vein injection into C57BL/6 *Rag1*$^{-/-}$ mice which had been irradiated 24 h previously (500 Rads / Caesium source). For competitive BM chimeras, T cell-depleted BM cells from congenic WT C57BL/6 CD45.1 mice were mixed at an excess of 4:1 with *Nfkb1*$^{SS/AA}$ or WT BM cells (CD45.2), or at a ratio of 1:1. These mixtures were then transferred by intravenous injection into irradiated C57BL/6 *Rag1*$^{-/-}$ mice in the same manner as above. Experimental mice were kept on irradiated water supplemented with 0.02 % (v/v)Baytril ® (Sigma) for 6-8 weeks. After this period, blood samples were checked for T cell reconstitution (*Section 2.2.2*). Mice were culled and removed of thymus, spleen and LN for FACS staining and analysis of T cell development by flow cytometry (*Section 2.5.1*).

2.13 *In vitro* suppression assay

Sorted WT $CD4^+CD44^{lo}CD25^-$ naïve T cells (*Section2.3.2*) were CFSE-labelled (*Section 2.6*) and cultured in the absence or presence of sorted $CD4^+CD44^{int}CD25^+$ regulatory T (T_{reg}) cells from WT or *Nfkb1*$^{SS/AA}$ mice. Responder naïve and T_{reg} cells were plated in 96-well plates at a ratio of 1:1, to a total of $2x10^5$ cells/well in 200 μl complete IMDM media. To stimulate, $1x10^4$ syngeneic BMDCs were generated from 129S8 mice (*Section 2.9*) and added to cultures with 1 μg/ml soluble anti-CD3. After 72 h, cells were surface stained and proliferation determined by CFSE flow cytometric analysis of viable ($7\text{-}AAD^-$) naïve $CD4^+$ T cells (*Section 2.4.1*).

2.14 Determination of protein concentration

$CD4^+CD25^-$ T cells were magnetically depleted from LN of WT and *Nfkb1*$^{SS/AA}$ mice (*Section2.3.1*), and stimulated with plate-bound CD3 antibody in 96-well plates for 24 h *(Section 2.4)*, as described above. Cells were harvested, washed with PBS, and lysed with 200μl of 1 % (v/v) NP-40 buffer (0.0 1M sodium bisphosphate (Sigma), 0.03 M sodium fluoride (Sigma), 0.001 M EDTA (Sigma), 0.05 M trishydroxymethylaminomethane (Tris;Sigma), 0.15 M sodium chloride (Sigma), 1mM vanadate (Sigma) and protease inhibitor (Roche)). Lysis was carried out for 10 min on ice, and cell debris removed by low-speed centrifugation (1100 rpm, 5 min). Protein concentration was determined using Bradford assay, whereby the amount of protein bound by Coomassie reagent (BioRad) was measured by

change in absorbance using a spectrophotometer, according to manufacturer's protocol.

2.15 Enzyme-linked immunosorbent assay (ELISA)

WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells were magnetically depleted (*Section 2.3.1*), and stimulated with plate-bound anti-CD3 (1 μg/ml) ± anti-CD28 (1 μg/ml) for 48 h, as described previously (*Section 2.4*). The amount of IL-2 in culture supernatants was determined using a commercial ELISA kit (ebioscience), according to the manufacturers' instructions.

2.16 Real-time quantitative PCR

CD4$^+$CD25$^-$ T cells were isolated from LN of WT and *Nfkb1*$^{SS/AA}$ mice (*Section 2.3.1*), and rested in complete medium for 1h at 5x10^6 cells/ml in 50 ml tubes (Corning). Cells were then plated in 24-well flat-bottom plates (Nunc) at 2x10^6 cells/well in a total volume of 1ml and stimulated with plate-bound anti-CD3 (5 μg/ml) ± soluble anti-CD28 (1 μg/ml) or no stimulus for 4 h. RNA from stimulated and unstimulated cells was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturers instructions and treated with DNase (Ambion). cDNA was produced by reverse transcription using oligo dT 14-18 (GE Healthcare and Invitrogen) and standard protocols. Expression of target genes was determined by real time PCR using an Applied Biosystems ABI Prism 7000 Sequence Detection System. Expression of *Il-2* mRNA was assayed using commercial FAM labelled probes (Applied Biosystems) and the Taqman Universal

PCR Master Mix (Applied Biosystems). *Il-2* mRNA amounts were normalized against hypoxanthine guanine phosphoribosyl transferase (*Hprt*) mRNA.

2.17 Calculation of absolute cell numbers

Absolute cell numbers from spleen, LN and thymus were calculated as follows:

Total organ cellularity as counted by Casy-1-cell counter	***= A***
Live lymphocyte gate (forward/side scatter)	***=B***
T cell subset gate	***=C***
Absolute number of T cell subset	***= A x B x C***

2.18 Statistical Analysis

For analysis of T cell development, the non-parametric Mann-Whitney test was applied. Student's t-test was used for analysis of *in vitro* assays, with sample sets of three or more. Average ±SEM are presented. Values of less than 0.05 or 0.005 were considered significant (* and ** respectively).

Marker	Antibody Clone	Conjugate	Supplier
B220	RA3-682	PE	BD biosciences
CD103	2E7	PE	ebiosciences
CD11b (Mac-1a)	M1 / 70	BIOTIN	ebiosciences
CD122 (IL-2Rb)	5H4	PE	BD biosciences
CD127 (IL-7Ra)	A7R34	PE	ebiosciences
CD132 (gc)	4G3	PE	BD biosciences
CD152 (CTLA-4)	UC10-4B9	PE	ebiosciences
CD19	MB19-1	FITC	ebiosciencess
CD2	RM2-5	PERCP	BD biosciences
CD24 (HSA)	MI / 69	FITC	BD biosciences
CD25 (IL-2Ra)	PC61.5	APC, BIOTIN	ebiosciencess
		FITC,PE	BD biosciences
CD28	37.51	PE, PURIFIED	BD biosciences
CD3	145.2C11	PE, PURIFIED	BD biosciences
CD4	RM45	APC, PE, PERCP	BD biosciences
		PE-TR	Catlab
CD44	IM7	FITC	BD biosciences
		APCCY7	Catlab
CD45.1	A20	PE	ebiosciencess
CD45.2	104	FITC	ebiosciencess
CD49b	DX5	BIOTIN	ebiosciences
CD5	53-7.3	BIOTIN	BD biosciences
CD62L	Mel-14	BIOTIN	ebiosciencess
CD69	H1-2F3	FITC	ebiosciencess
		PE	BD biosciences
CD8a	53-6.7	PERCP	BD biosciences
		APCCy7	Catlab
CD94	18d3	FITC	ebiosciences
FoxP3	FJK-16a	APC	ebiosciences
GITR	DTA-1	BIOTIN	ebiosciences
Gr1 n(Ly6G)	RB6-8C5	BIOTIN	ebiosciences
IL2	JES6-5H4	PE	BD biosciences
MHC-II (I-A / I-E)	M5 / 114.15.2	BIOTIN	ebiosciences
NK1.1	PK-136	PE	BD biosciences
Streptavidin	-	PERCP	ebiosciences
TCRb	H57-597	APC, FITC	BD biosciences
Thy-1	OX-7	PE	BD biosciences

Table 2.1 Antibodies used for FACS and $CD4^+$ T cell stimulation. Fluorescent conjugates are fluorescein isothio- cyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas Red (PE-TR), allophycocyanin (APC), allophycocyanin-Cyan 7 (APC-Cy7) and peridinin chlorophyll protein (PerCP).

Solutions	Components
Complete IMDM media	IMDM containing 10 % FCS (Labtech), L-Glutamine (Sigma;2 mM), Penicillin (Sigma;100 U/ml), Streptomycin (Sigma; 100 μg/ml), 2' β-mercaptoethanol (GIBCO;50 mM)
BMDC media	IMDM with 5 % (v/v) FCS (Labtech), L-Glutamine (Sigma; 2 mM), Penicillin (Sigma; 100 U/ml), Streptomycin (Sigma; 100 μg/ml), Hepes (Sigma;10 μM)
ACK buffer	dH20 containing 0.155 M ammonium chloride (Sigma), 0.1 M EDTA (Sigma), 0.01 M potassium bicarbonate (Sigma)
FACS buffer	PBS, 0.1% sodium azide
FACS staining buffer	PBS, 0.1 % sodium azide, 5 % FCS
NP-40 lysis buffer	dH20 containing 1 % (v/v) NP-40 (Sigma), sodium bisphosphate (Sigma; 0.01 M), sodium fluoride (Sigma; 0.03 M), EDTA (Sigma; 0.001 M), Tris (Sigma; 0.05 M), sodium chloride (BD biosciences; 0.15 M), 1mM vanadate (Sigma), Protease inhibitor (Roche)
Fixing solution	PBS, 4 % (v/v) parafomaldehyde (Sigma)
0.1 % NP-40 for intracellular staining	PBS, 0.1% (v/v) NP-40
Tail vein and intraperitoneal injection media	PBS (Gibco)

Table 2.2 List of media and buffers

3. Analysis of T cell development in *Nfkb1*$^{SS/AA}$ mice

3. Analysis of T cell development in *Nfkb1*$^{SS/AA}$ mice

3.1 T cell development is normal in the absence of IKK-induced p105 proteolysis

A role for NF-κB in T cell development has been established from analysis of mutant mice lacking Rel or IKK subunits or expressing IκBα resistant to signal-induced degradation (Siebenlist et al., 2005, Gerondakis et al., 1999). Partial inhibition of NF-κB activation can impair thymic development. This is exemplified in mice expressing a mutant IκBα that is resistant to degradation targeted to the T cell lineage, which show reduced numbers of DN4 and both thymic and peripheral CD8$^+$ SP T cells (Boothby et al., 1997, Mora et al., 2001b). In contrast, a complete block in NF-κB activity through the specific deletion of NEMO in T cells, results in a total lack of peripheral T cells due to apoptosis (Schmidt-Supprian et al., 2003). Therefore, development of *Nfkb1*$^{SS/AA}$ thymocytes and peripheral T cells was analysed.

3.1.1 DN cell numbers and expression of selection markers are normal in *Nfkb1*$^{SS/AA}$ mice

Transgenic mice expressing a signal-resistant form of IκBα have a partial block in NF-κB activity in their T cells. Detailed analysis of these mice reveals a role for NF-κB in regulating apoptosis during negative and positive selection (Mora et al., 2001b). To assess the significance of blocking IKK-induced p105 proteolysis in T cell development, maturation of *Nfkb1*$^{SS/AA}$ thymocytes was followed by flow

cytometry. Double negative (DN) cells were gated by exclusion of thymocytes positive for CD4, CD8, CD3, Gr-1, Mac-1, NK1.1 and B220. Using CD44 and CD25 expression as markers for DN subsets, DN stage I was defined as $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$ and DN4, $CD44^{-}CD25^{-}$. The percentages of these four early stages of maturation were comparable between WT and *Nfkb1*$^{SS/AA}$ mice (Figure 3.1A). In addition, absolute numbers of DN 1-4 cells were not statistically different between WT and *Nfkb1*$^{SS/AA}$ mice (Figure 3.1B).

CD5 expression is substantially upregulated after thymocytes receive the pre-TCR signal, and further in response to TCR engagement during positive/negative selection of DP thymocytes (Azzam et al., 1998, Azzam et al., 2001). These changes in surface CD5 expression reflect the functional role of CD5 in fine- tuning of the TCR repertoire by negatively regulating TCR signalling during thymocyte development (Tarakhovsky et al., 1995). Since CD5 levels on thymocytes can act as a marker for thymocyte maturation and selection, CD5 surface expression on DN, double positive (DP), $CD4^{+}$ single positive (SP) and $CD8^{+}$ SP *Nfkb1*$^{SS/AA}$ thymocytes was compared with corresponding WT subsets (Figure 3.1C). CD5 expression was found to be upregulated on both WT and *Nfkb1*$^{SS/AA}$ thymocytes, with the highest levels found on SP cells, in particular $CD4^{+}$ SP cells. Furthermore, expression overlays revealed that CD5 was upregulated on *Nfkb1*$^{SS/AA}$ thymocytes at all these developmental stages to a similar extent as WT thymocytes. Consistently, analysis of TCRβ expression suggested normal β chain rearrangements and selection in *Nfkb1*$^{SS/AA}$ thymocytes (Figure 3.1C).

3.1.2 Normal numbers of CD4 and CD8 subsets in *Nfkb1*$^{SS/AA}$ mice

Inhibition of NF-κB activation has previously been described to impair the development of SP T cells in the thymus, particularly affecting CD8$^+$ T cells (Schmidt-Supprian et al., 2003, Boothby et al., 1997, Siebenlist et al., 2005). Analysis of the fraction of thymic *Nfkb1*$^{SS/AA}$ CD4$^+$ SP and CD8$^+$ SP T cells, and also DN and DP compartments, showed no significant difference to WT controls (Figure 3.1D). Absolute numbers of *Nfkb1*$^{SS/AA}$ SP, DN and DP thymocytes were also comparable to WT subsets (Figure 3.1E). Consistently, peripheral CD4$^+$:CD8$^+$ T cell ratios were roughly 2:1 in both WT and *Nfkb1*$^{SS/AA}$ mice and absolute numbers of CD4 and CD8$^+$ T cells were unaffected by *Nfkb1*$^{SS/AA}$ mutation (Figure 3.1D and 3.1E).

Together, these results suggest that signal-induced proteolysis of p105 does not play a significant role in T cell maturation, thymocyte survival, or maintenance of CD4$^+$ and CD8$^+$ T cells in the periphery.

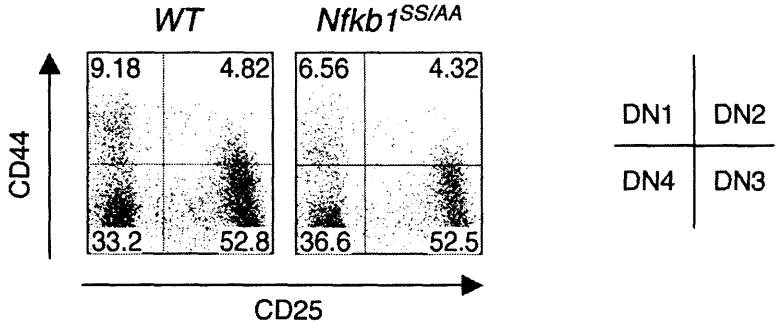
3.2 Generation of T cell sub-populations is impaired in *Nfkb1*$^{SS/AA}$ mice

3.2.1 IKK-induced p105 proteolysis is necessary for generation and/or maintenance of CD44^{hi} memory-phenotype (T_{mem}–phenotype) cells

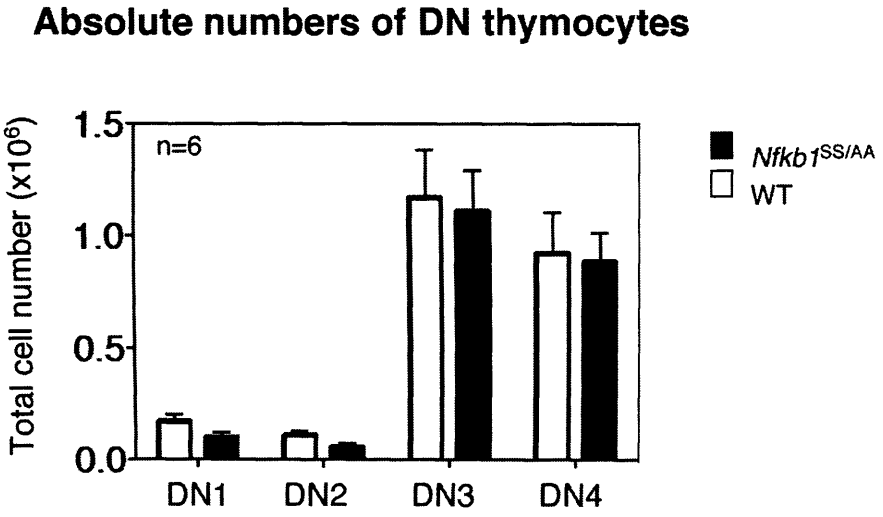
Upon activation, naïve T cells divide and differentiate into effector cells, with some of these antigen-experienced cells persisting in the T cell pool as memory T cells

(T_{mem}). T_{mem} cells upregulate CD44 to allow migration to non-lymphoid organs and $CD44^{hi}$ expression is commonly used as a marker for memory T cells (Sallusto et al., 2004). A population of T cells resembling memory T cells (T_{mem}–phenotype cells) is found in the absence of immunization (Surh et al., 2006). To investigate the effect of p105 mutation on the T_{mem}–phenotype cell subset, surface expression of CD44 was assessed. A three-fold reduction in the fraction of splenic $CD4^+$ T_{mem}–phenotype cells was observed in *Nfkb1*$^{SS/AA}$ mice compared to WT, whereas $CD8^+$ T_{mem}–phenotype cells were only reduced by two-fold (Figure 3.2A and 3.2B). These reductions were also reflected in reduced absolute numbers of $CD4^+$ and $CD8^+$ T_{mem}–phenotype cells in *Nfkb1*$^{SS/AA}$ mice compared to WT controls (Figure 3.2C). Decreases in $CD4^+$ T_{mem}–phenotype cell numbers were also observed in the LN, but were not statistically significant. The reduction in splenic memory-phenotype $CD4^+$ T cells was confirmed by analysing expression of CD45Rb and CD62L. Naïve cells express relatively high levels of CD62L and CD45RB, in contrast to memory T cells that express low levels of both CD45RB and CD62L (Budd et al., 1987, Lerner et al., 1989). Consistent with $CD44^{hi}$ memory T cells, the fractions of gated $CD4^+TCR^+$ cells that were $CD45Rb^{lo}$ or $CD62L^{lo}$ were reduced in spleens of *Nfkb1*$^{SS/AA}$ mice compared to WT mice (Figure 3.2D).

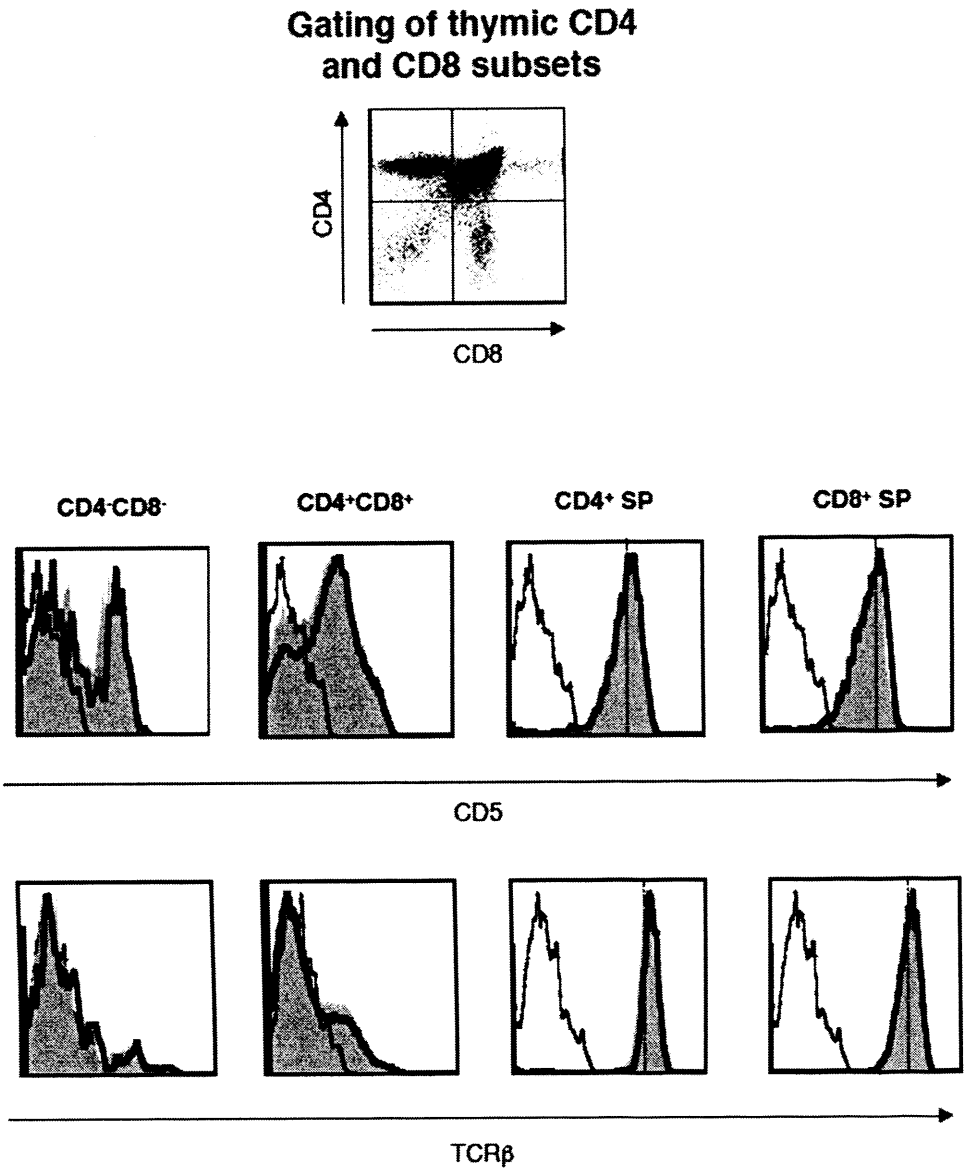
A


WT
Nfkb1SS/AA
9.18
4.82
33.2
52.8
6.56
4.32
36.6
52.5
CD44
CD25
DN1
DN2
DN4
DN3

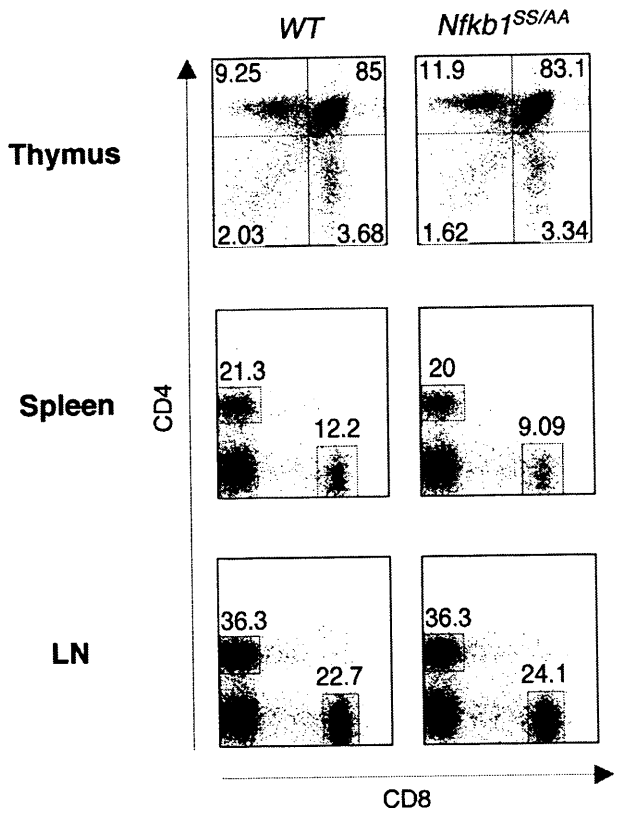
B


Absolute numbers of DN thymocytes
Total cell number (x10⁶)
1.5
1.0
0.5
0.0
n=6
DN1
DN2
DN3
DN4
Nfkb1SS/AA
WT

C


Gating of thymic CD4
and CD8 subsets
CD4
CD8
CD4-CD8-
CD4+CD8+
CD4+ SP
CD8+ SP
CD5
TCRβ

D


WT
Nfkb1SS/AA
Thymus
Spleen
LN
CD4
CD8
9.25
85
2.03
3.68
11.9
83.1
1.62
3.34
21.3
12.2
20
9.09
36.3
22.7
36.3
24.1

E

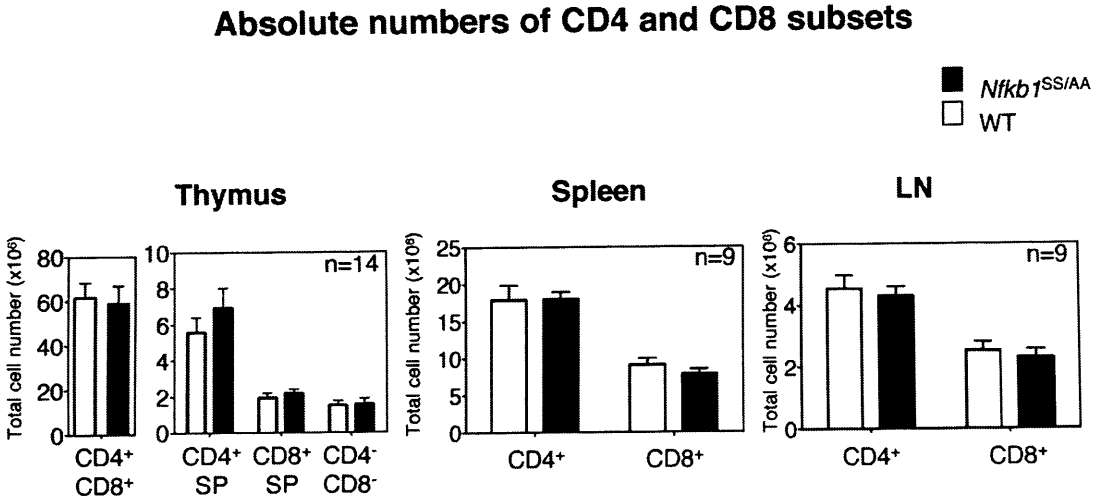

Absolute numbers of CD4 and CD8 subsets
Nfkb1SS/AA
WT
Thymus
Spleen
LN
Total cell number (x10⁶)
n=14
n=9
n=9
CD4+ CD8+
CD4+ SP
CD8+ SP
CD4- CD8-
CD4+
CD8+
CD4+
CD8+

Figure 3.1 Abrogation of IKK-induced p105 proteolysis does not affect conventional T cell development

T cell development in WT and *Nfkb1*$^{SS/AA}$ mice was analysed by flow cytometry.

(A) Surface staining for CD25 and CD44, as markers of indicated double negative (DN) stages. FACS plots show gated live cells, based on characteristic forward/side scatter profiles, and gated to exclude thymocytes expressing CD4, CD8, NK1.1, CD3, Gr-1, Mac-1 and B220. **(B)** Graphs, based on analysis in (A), represent absolute numbers of thymocytes in DN stages 1-4 in WT and *Nfkb1*$^{SS/AA}$ mice (average ± standard error of the mean (SEM); n=6). **(C)** Expression overlays of CD5 and TCRβ on WT (shaded gray) and *Nfkb1*$^{SS/AA}$ (bold line, no shade) thymocytes gated for live $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$ single positive (SP) or $CD8^+$ SP subsets. Isotype controls are also shown (thin line). **(D)** Cell suspensions from thymus, spleen and lymph nodes (LN) of WT and *Nfkb1*$^{SS/AA}$ mice were analysed for CD4 and CD8 surface expression, which are shown as FACS plots. Numbers in plots represent percentages of live-gated cells in corresponding quadrants. **(E)** Absolute cell numbers of thymic and peripheral CD4 and CD8 subsets, based on FACS analysis in (D) are shown as bar charts (average± SEM; number of mice analysed is indicated).

3.2.2 Generation of natural regulatory (T_{reg}) cells is reduced in *Nfkb1*$^{SS/AA}$ mice

3.2.2.1 $CD4^+CD25^+$ T_{reg} cells

The generation of $CD4^+CD25^+$ T_{reg} cells has been shown to be important for suppression of autoimmunity (Sakaguchi et al., 1995, Takahashi et al., 1998, Kronenberg and Rudensky, 2005), a disease state associated with dysregulated NF-κB signalling (Karin and Greten, 2005). Furthermore, generation of $CD4^+CD25^+$ T_{reg} cells is reduced in mice with impaired NF-κB activation (Schmidt-Supprian et al., 2004a, Zheng et al., 2003). T_{reg} cells constitutively express CD25 (IL-2Rα), and this marker is widely used to identify them. Therefore, surface staining for CD25 was carried out to identify $CD4^+CD25^+$ T_{reg} cells by flow cytometry. Roughly 10% of $CD4^+$ T cells in LN and spleen of WT mice were found to be positive for CD25, consistent with published data, and were also $CD44^{int}$ (Kronenberg and Rudensky, 2005, Shevach, 2002). However, analysis of *Nfkb1*$^{SS/AA}$ mice revealed a marked reduction in peripheral $CD4^+CD25^+$ T_{reg} cells, where only 2-3% of $CD4^+$ T cells were $CD25^+$ (Figure 3.2A). The absolute number of $CD4^+CD25^+$ T_{reg} cells in spleen and LNs was also markedly reduced by the introduction of p105 mutation (Figure 3.2E). Furthermore, generation of T_{reg} cells occurs in the thymus (Sakaguchi et al., 1995, Itoh et al., 1999), and flow cytometric analysis demonstrated that thymic $CD4^+CD25^+$ T_{reg} fractions and cell numbers were significantly reduced in *Nfkb1*$^{SS/AA}$ mice ($p \leq 0.005$) compared to WT mice (Figure 3.2A and 3.2E). Together, these results suggest that the reduction in

Nfkb1$^{SS/AA}$ $CD4^+CD25^+$ T_{reg} cells in the periphery was due to their impaired generation in the thymus.

3.2.2.2 $CD4^+Foxp3^+$ T_{reg} cells

CD25 is expressed on activated $CD4^+$ and $CD8^+$ T cells, and absent in some natural regulatory T cells (Fontenot et al., 2005b). Therefore, the use of CD25 as a sole marker for T_{reg} cells can be misleading. To better define the T_{reg} population, intracellular staining for Foxp3 was carried out. Foxp3 has been characterized as critical for T_{reg} function and is used as a highly specific marker for T_{reg} cells (Fontenot et al., 2003, Hori et al., 2003). This analysis revealed a significant reduction ($p \leq 0.005$) in the percentage and absolute numbers of $CD4^+Foxp3^+$ populations in *Nfkb1*$^{SS/AA}$ spleen, LN and thymus (Figure 3.2F and 3.2G). Furthermore, these results demonstrated that the reduction in $CD4^+CD25^+$ cells was not due to an inability of *Nfkb1*$^{SS/AA}$ T_{reg} cells to express CD25, since most $Foxp3^+$ cells were also $CD25^+$. Instead, these findings indicate a real reduction in the numbers of T_{reg} cells in *Nfkb1*$^{SS/AA}$ mice. Moreover, the thymic reductions observed suggest a requirement for agonist-induced p105 proteolysis for generation of T_{reg} cells rather than for peripheral maintenance.

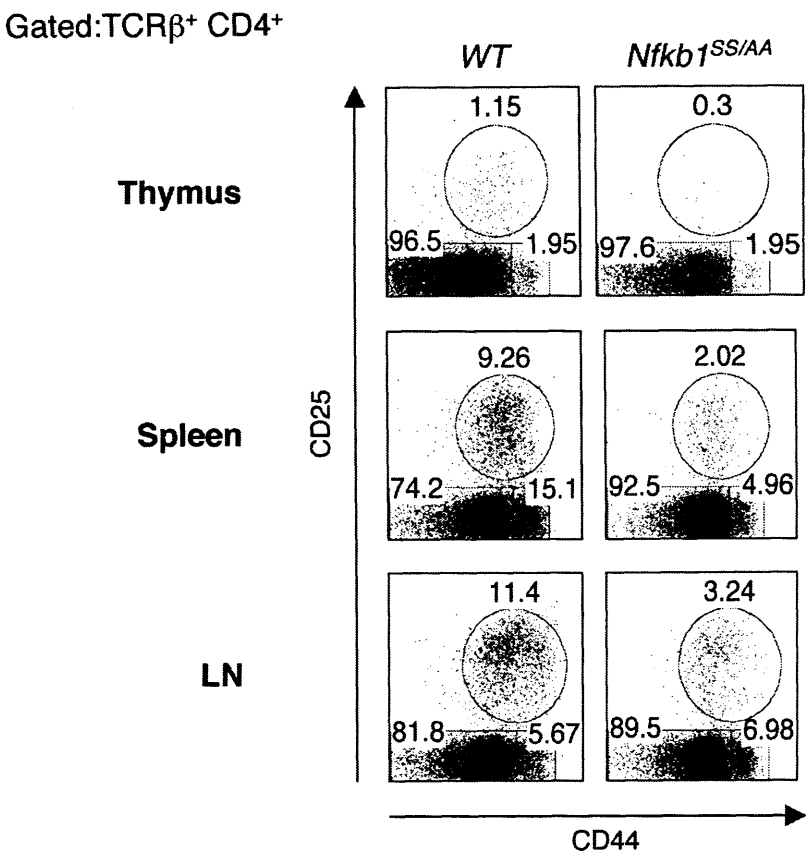
3.3 *Nfkb1*$^{SS/AA}$ mice have reduced numbers of thymic natural killer T (NKT) cell numbers

NKT T cells, a subset of lymphocytes generated in the thymus, are an important link between the innate and adaptive immune systems. NKT cells rapidly secrete

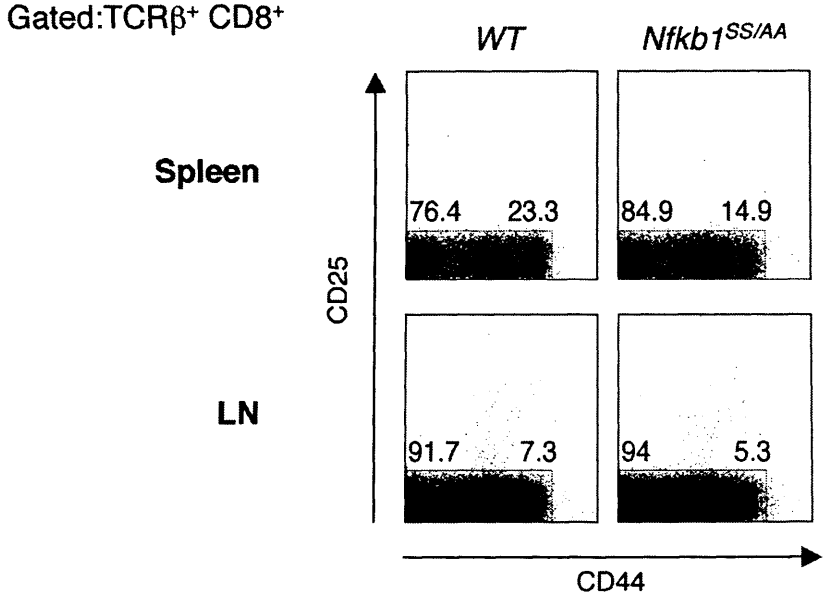
large amounts of cytokines such as IL-4, IL-10 and IFN-γ, suggesting a role in directing Th1 and Th2 differentiation. Additionally, impairment or deletion of NKT cells can enhance some autoimmune reactions and may impair tumour immunity (Kronenberg and Rudensky, 2005, Kronenberg and Gapin, 2002). Interestingly, NKT cell numbers are reduced in mice with impaired NF-κB activity in their T cells (Schmidt-Supprian et al., 2004a, Sivakumar et al., 2003). To examine this population in *Nfkb1*$^{SS/AA}$ mice, NK1.1^{+}TCRβ^{+} NKT cells were identified by flow cytometry. Since NK1.1 is not expressed on 129S8 mice, *Nfkb1*$^{SS/AA}$ 129S8 mice backcrossed at least five times on the C57BL/6 background were used for experiments (Carlyle et al., 1999). Analysis of thymic CD8^{-} HSA^{lo} gated cells revealed that the percentage and absolute number of NK1.1^{+}NKT cells were 3-fold lower in the thymus of *Nfkb1*$^{SS/AA}$ mice than WT mice. In contrast, normal fractions and numbers of NK1.1^{+}NKT cells were found in the spleen and lymph nodes (Figure 3.3A and 3.3B).

Expression of NK1.1 is not a definitive marker for NKT cells since NK cells and some CD8^{+} cells are NK1.1^{+}. Furthermore, NKT cells can themselves downregulate NK1.1 (Kronenberg and Rudensky, 2005, Assarsson et al., 2000). Most NKT cells express the Vα14i TCR, recognizing glycolipids on CD1d molecules (Hammond et al., 1999, Sidobre et al., 2002). This Vα14i subset of NKT cells have been identified by use of fluorescence-conjugated CD1d tetramers loaded with the glycolipid αGalCer (Bendelac, 1995, Bendelac et al., 1997, Benlagha et al., 2000). Similar to the reduction in NK1.1^{+}TCRβ^{+} cells, two-three

A

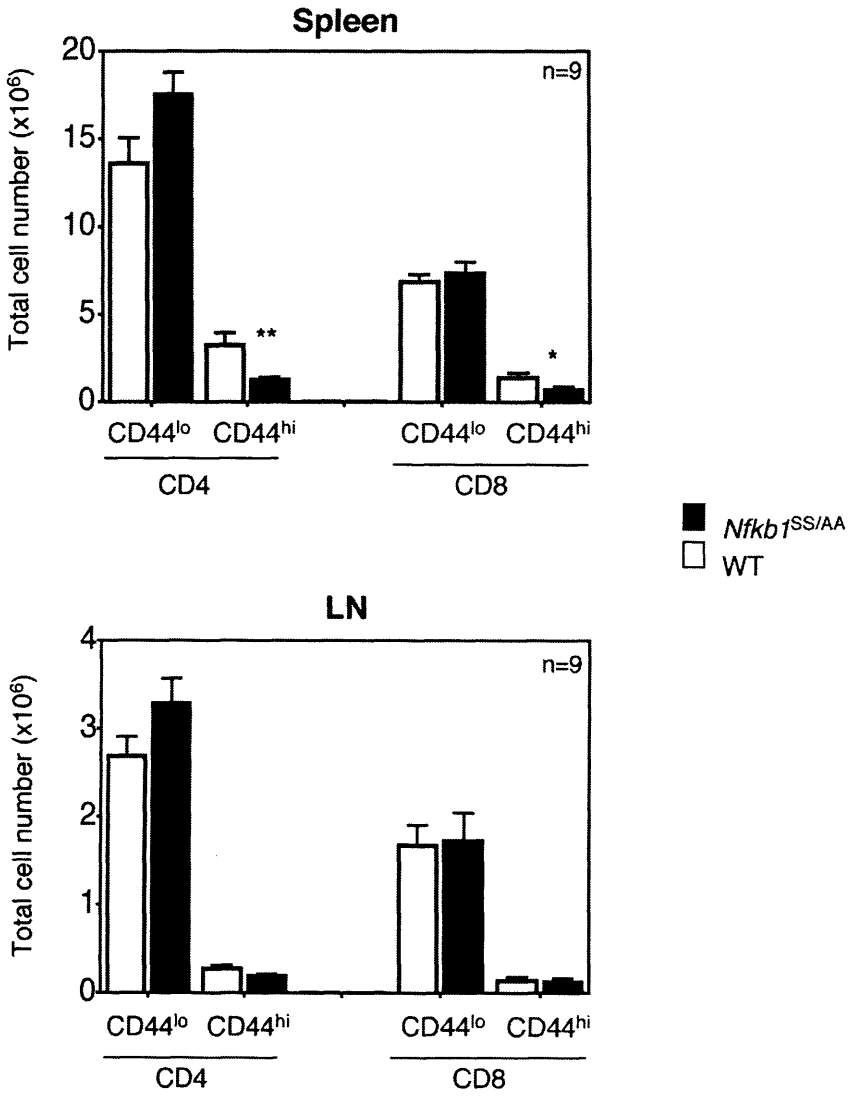

Gated:TCRβ+ CD4+
WT
Nfkb1SS/AA
Thymus
Spleen
LN
1.15
0.3
96.5
1.95
97.6
1.95
9.26
2.02
74.2
15.1
92.5
4.96
11.4
3.24
81.8
5.67
89.5
6.98
CD25
CD44

B


Gated:TCRβ+ CD8+
WT
Nfkb1SS/AA
Spleen
LN
76.4
23.3
84.9
14.9
91.7
7.3
94
5.3
CD25
CD44

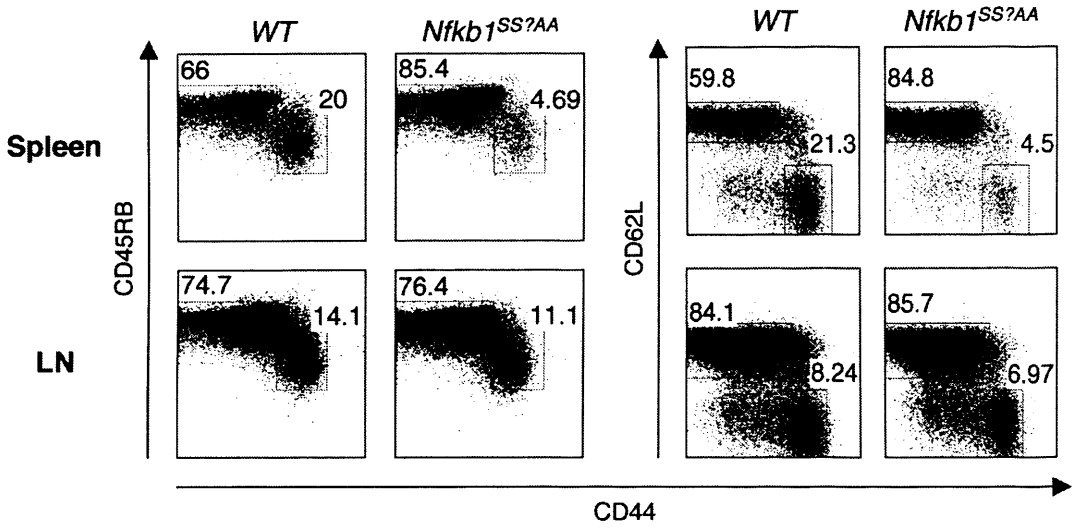
C

Absolute numbers of peripheral CD44^{hi} and CD44^{lo} populations



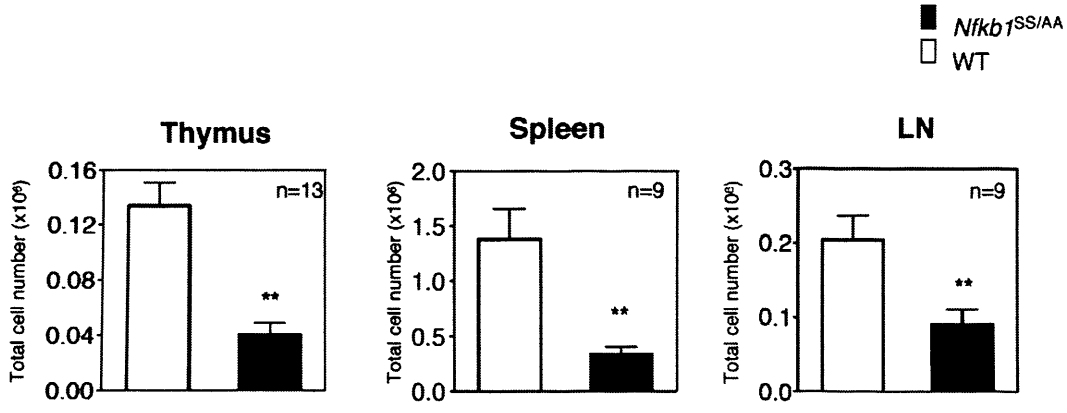
D

Gated:TCRβ+ CD4+

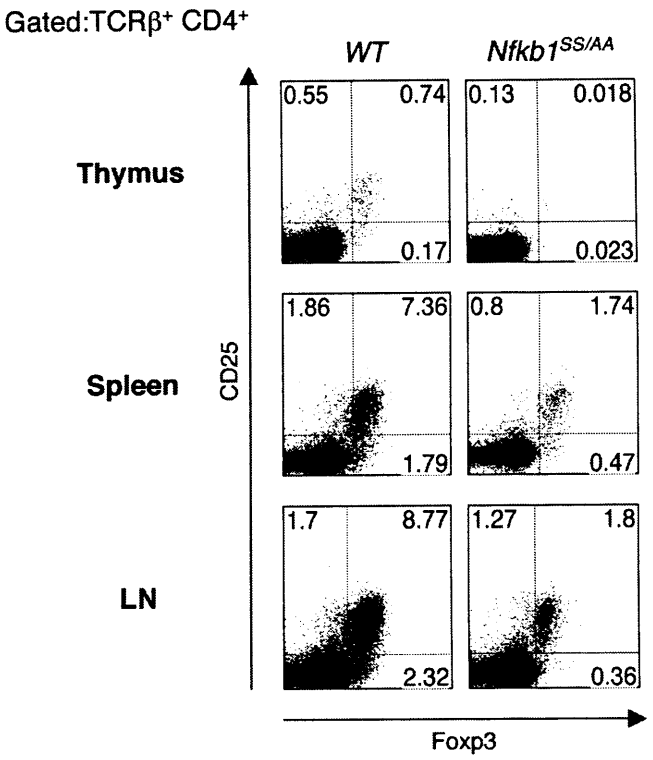


E

Absolute numbers of CD4+CD25+ T_{reg} cells



F


Gated:TCRβ+ CD4+
WT
Nfkb1SS/AA
Thymus
Spleen
LN
CD25
Foxp3
0.55
0.74
0.17
0.13
0.018
0.023
1.86
7.36
1.79
0.8
1.74
0.47
1.7
8.77
2.32
1.27
1.8
0.36

G

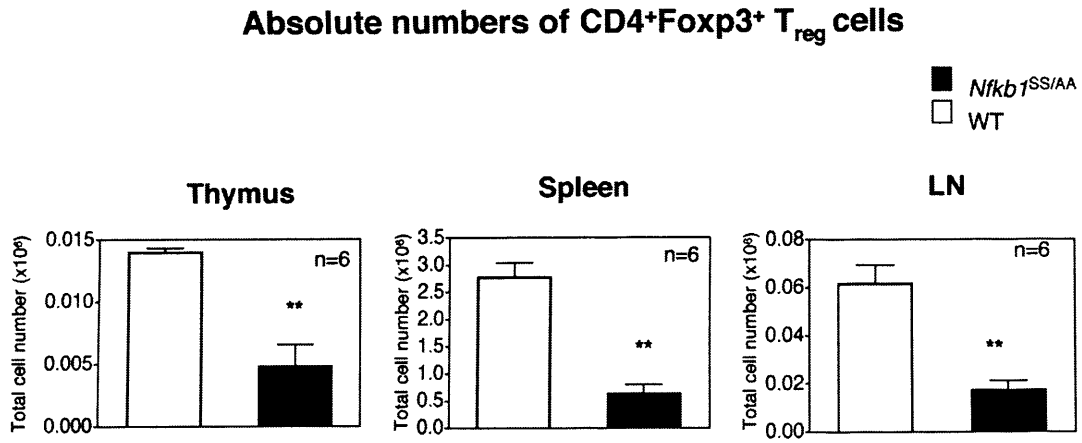
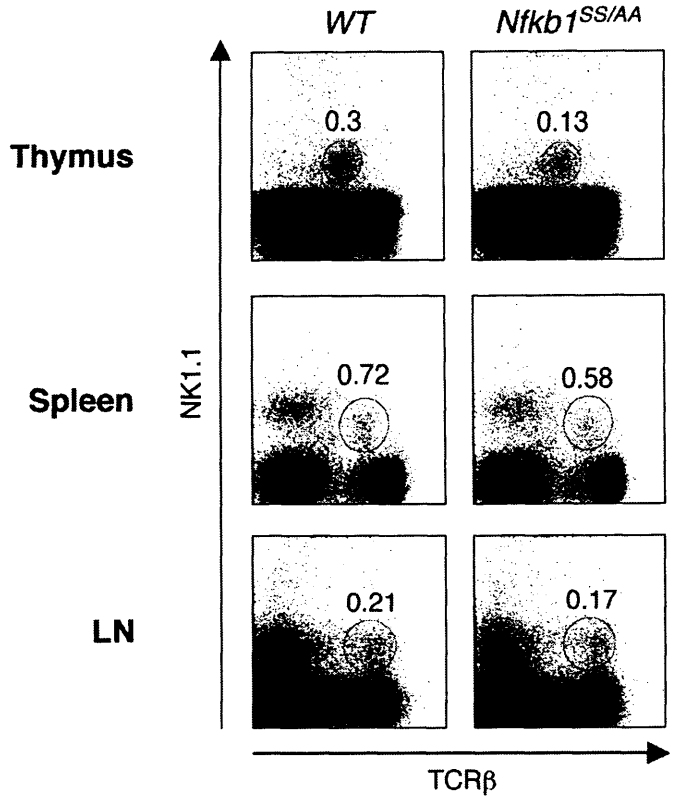

Absolute numbers of CD4+Foxp3+ Treg cells
Nfkb1SS/AA
WT
Thymus
Spleen
LN
Total cell number (x10⁶)
n=6
**
0.015
0.010
0.005
0.000
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.08
0.06
0.04
0.02
0.00

Figure 3.2 Reduced numbers of T_{reg} and splenic T_{mem}-phenotype cell sub-populations in *Nfkb1*$^{SS/AA}$ mice

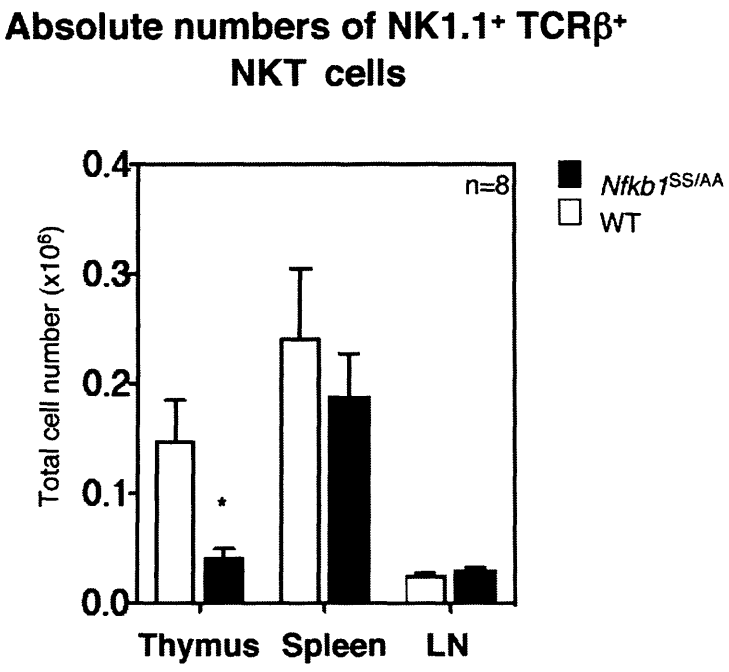
(A) Flow cytometric analysis of CD44 and CD25 surface expression on gated TCRβ$^+$ CD4$^+$ live cells from WT and *Nfkb1*$^{SS/AA}$ thymus, spleen and lymph node (LN). Numbers represent percentages of cells in corresponding gates. **(B)** Surface staining of cells from spleen and LN of WT and *Nfkb1*$^{SS/AA}$ mice with anti-CD44 and anti-CD25. FACS plots gated on TCRβ$^+$CD8$^+$ live cells are shown, with numbers indicating cell percentages in corresponding gates. **(C)** Bar charts represent absolute numbers of naïve (CD44^{lo}) and memory-phenotype (CD44^{hi}) cells from gated TCRβ$^+$ CD4$^+$ or TCRβ$^+$ CD8$^+$ live cells of indicated WT and *Nfkb1*$^{SS/AA}$ tissues (n=9). **(D)** Dot plots show surface expression of indicated markers on gated TCRβ$^+$CD4$^+$ live cells from spleen and LN of WT and *Nfkb1*$^{SS/AA}$ mice, as assessed by flow cytometry. Numbers in the outlined areas refer to the percentages of cells gated relative to total TCRβ$^+$CD4$^+$ T cells. **(E)** Graphs showing absolute numbers of WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^+$ T_{reg} cells in thymus and periphery, based on analysis of gated live TCRβ$^+$ cells in (A) (n=9). **(F)** Dot plot analysis of surface CD25 and intracellular Foxp3 expression in gated CD4$^+$TCRβ$^+$ live cells from indicated tissues of WT and *Nfkb1*$^{SS/AA}$ mice. Numbers in quadrants indicate percentage of cells in each gate. **(G)** Graphs represent absolute numbers of CD25$^+$Foxp3$^+$ cells, based on FACS analysis of gated CD4$^+$TCRβ$^+$ in (F). Graphs in this figure represent averages ± SEM, *, $p \leq 0.05$, **, $p \leq 0.005$; Mann-Whitney test.

fold fewer tetramer$^+$ cells were detected in the thymus of *Nfkb1*$^{SS/AA}$ mice from both C57BL/6x129S8 (N5) and 129S8 backgrounds than in WT thymus (Figure 3.3C). Numbers of tetramer$^+$ NKT cells in the periphery were similar between WT and *Nfkb1*$^{SS/AA}$ mice (Figure 3.3D).

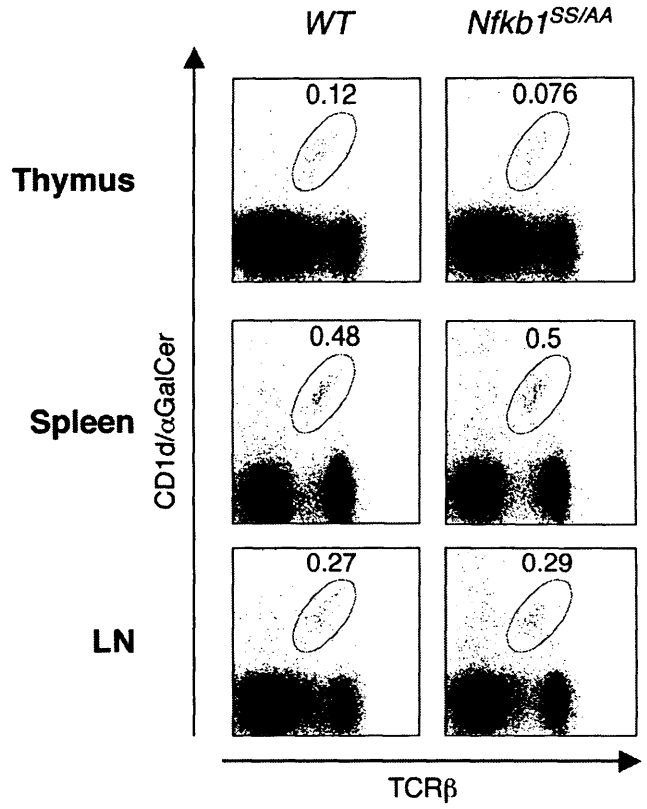
A


WT
Nfkb1SS/AA
Thymus
Spleen
LN
NK1.1
TCRβ
0.3
0.13
0.72
0.58
0.21
0.17

B


Absolute numbers of NK1.1+ TCRβ+ NKT cells
Total cell number (x10⁶)
0.4
0.3
0.2
0.1
0.0
n=8
*
Thymus
Spleen
LN
Nfkb1SS/AA
WT

C


WT
Nfkb1SS/AA
Thymus
Spleen
LN
0.12
0.076
0.48
0.5
0.27
0.29
CD1d/αGalCer
TCRβ

D

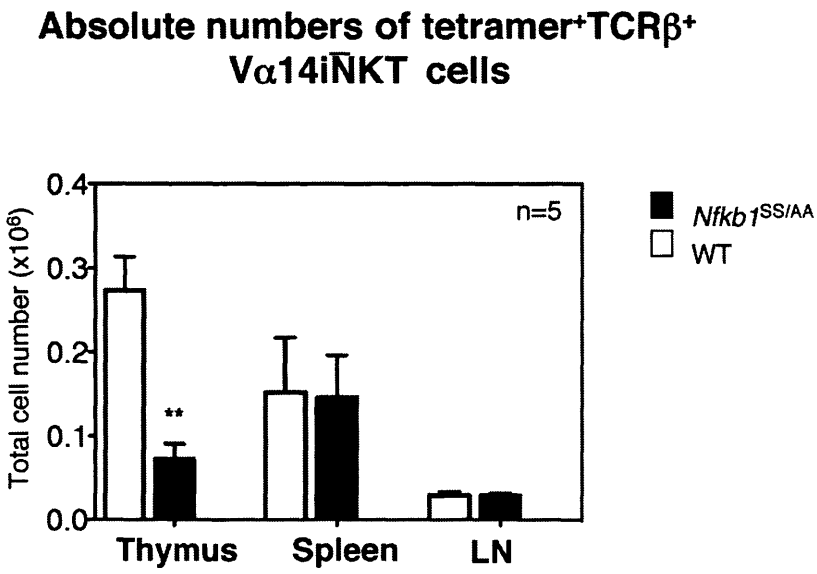

Absolute numbers of tetramer+TCRβ+
Vα14iNKT cells
Total cell number (x10⁶)
0.4
0.3
0.2
0.1
0.0
n=5
**
Thymus
Spleen
LN
Nfkb1SS/AA
WT

Figure 3.3 Decreased numbers of thymic NKT cells in mice expressing *Nfkb1*$^{SS/AA}$ mutation
(A) Flow cytometric analysis of NKT cells from mixed background (129S8, C57BL/6) WT and *Nfkb1*$^{SS/AA}$ mice, defined by TCRβ and NK1.1 staining of live gated cells. Fractions of TCRβ^{+}NK1.1^{+} cells from thymus, spleen and LN are indicated. **(B)** Absolute numbers (average± SEM) of NKT cells from (A) are represented graphically. Number of mice analysed is indicated. *,$p \leq 0.05$; Mann-Whitney test. **(C)** Analysis of VαiNKT cells in indicated WT and *Nfkb1*$^{SS/AA}$ tissues, based on staining with anti-TCRβ and α-galactosylceramide (α-GalCer)-loaded CD1d tetramers. Live cells were electronically gated, and percentages of TCRβ^{+} tetramer^{+} cells are represented on dot plots. **(D)** Absolute numbers of TCRβ^{+} tetramer^{+} cells, corresponding to analysis in (C), are shown as a graph of average ±SEM, *,$p \leq 0.05$; Mann-Whitney test.

3.4 *Nfkb1*$^{SS/AA}$ regulatory T cells are phenotypically normal and are suppressive *in vitro*

3.4.1 *Nfkb1*$^{SS/AA}$ mice do not develop features of colitis

The regulatory function of $CD4^+CD25^+$ T_{reg} cells *in vivo* can be observed by their ability to prevent colitis caused upon naive cell transfer into lymphopoenic mice (Sakaguchi et al., 1995). Furthermore, the suppressive role of T_{reg} cells is illustrated in certain mutant mice deficient in this T cell subset, including *Il-2*$^{-/-}$ and *il2ra*$^{-/-}$ mice. These mice develop autoimmunity, including the inflammatory bowel-disease colitis (Willerford et al., 1995, Sadlack et al., 1993). The key feature of colitis is a mucosal inflammation extending proximally from the rectum. Histopathological changes include epithelial cell hyperplasia, depletion of goblet cell mucin and infiltration of leukocytes into the *lamina propria* throughout the colon. Accumulation of DCs can also be observed (Leach et al., 1996). The reduction in numbers of $CD4^+CD25^+$ T_{reg} cells observed in *Nfkb1*$^{SS/AA}$ mice raised the possibility that these mice might be susceptible to autoimmune disease. However, analysis of histological sections stained with haemotoxylin and eosin (H and E; Histology department, NIMR), revealed a normal pathology in colons of *Nfkb1*$^{SS/AA}$ mice up to the age of 18months, with no signs of colitis (Figure 3.4A).

3.4.2 Markers of $CD4^+CD25^+$ T_{reg} cells are normal in *Nfkb1*$^{SS/AA}$ mice

Analysis of gene expression profiles from $CD4^+CD25^+$ T_{reg} cells has identified certain overexpressed molecules compared to $CD4^+CD25^-$ cells (Gavin et al., 2002, McHugh et al., 2002). Some of these markers may be involved in their

regulatory properties, such as $CD103^+$, which has been suggested to identify T_{reg} cells with more potent suppressive function than $CD103^-$ T_{reg} cells (Chen and Bromberg, 2006, Banz et al., 2003, Lehmann et al., 2002). To further characterise *Nfkb1*$^{SS/AA}$ T_{reg} cells, the expression of some of these molecules on gated $CD4^+CD25^+$ T_{reg} cells was assessed by flow cytometry. The expression levels of both CD103, the α_E chain of $\alpha_E\beta_7$ integrin, which interacts with E-cadherin, and the costimulatory molecule GITR, were similar between *Nfkb1*$^{SS/AA}$ and WT $CD4^+CD25^+$ T_{reg} cells (Figure 3.4B). Furthermore, intracellular CTLA-4 was highly expressed in $CD4^+CD25^+$ T_{reg} cells from both *Nfkb1*$^{SS/AA}$ and WT spleens, with no comparable difference in levels. Levels of CD69 and CD62L on *Nfkb1*$^{SS/AA}$ T_{reg} cells were also analysed, since T_{reg} cells from mice deficient in IL-2 signalling have an activated phenotype (Fontenot et al., 2005a). However, surface expression of these molecules was found to be normal (Figure 3.4B).

3.4.3 *Nfkb1*$^{SS/AA}$ $CD4^+CD25^+$ T_{reg} cells are able to suppress naïve $CD4^+$ T cell proliferation *in vitro*

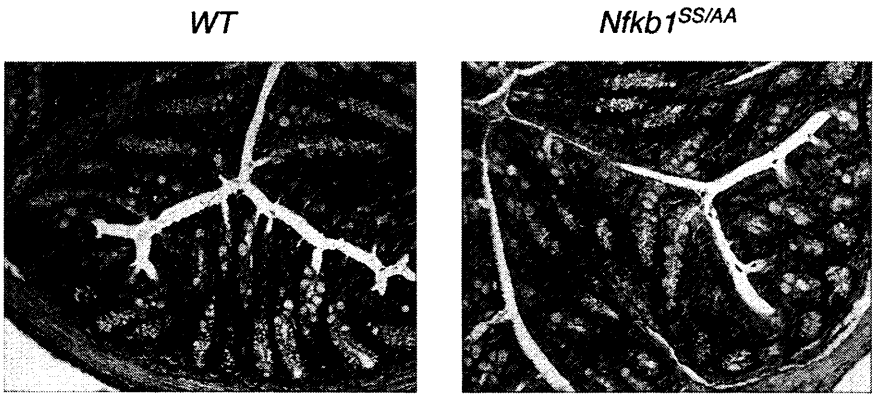
Regulatory T cells can suppress proliferation of bystander naïve T cells *in vitro* (Piccirillo and Shevach, 2001, Kronenberg and Rudensky, 2005). The effect of p105 mutation on this suppressive property of T_{reg} cells was analysed by culturing WT naïve $CD4^+$ T cells ($CD4^+CD44^{lo}CD25^-$) with *Nfkb1*$^{SS/AA}$ or WT T_{reg} cells ($CD4^+CD44^{int}CD25^+$). Naïve $CD4^+$ T cells and T_{reg} cells were sorted by FACS to ensure maximum purity (>98%) (Figure 3.4C). WT responder naïve cells, CFSE-labelled to assess proliferation, were stimulated with anti-CD3 and BMDCs in the presence or absence of an equivalent number of T_{reg} cells. Flow cytometric

analysis of CFSE dilution revealed that approximately 60% of WT naïve cells had divided when cultured without T_{reg} cells. Addition of WT T_{reg} cells reduced the percentage of WT naïve cells that had divided by 3-fold (Figure 3.4D). Culture with *Nfkb1*$^{SS/AA}$ T_{reg} cells also produced a similar decrease in proliferation of WT naïve $CD4^+$ cells. This suppression was not due to competition for factors or space (Barthlott et al., 2003), since addition of $T_{naïve}$ cells instead of T_{reg} cells did not inhibit proliferation of responder naïve T cells. These results therefore suggested that the ability of T_{reg} cells to suppress proliferation of naïve cells *in vitro* was not affected by p105 mutation.

The complement of this experiment was also carried out, whereby *Nfkb1*$^{SS/AA}$ naïve T cells were cultured with WT or *Nfkb1*$^{SS/AA}$ T_{reg} cells to assess whether *Nfkb1*$^{SS/AA}$ naïve cells were susceptible to *in vitro* suppression. Although the percentage of *Nfkb1*$^{SS/AA}$ naïve cells divided was low in cultures without T_{reg} cells, a two-fold reduction that was statistically significant was observed when WT or *Nfkb1*$^{SS/AA}$ T_{reg} cells were added. Again, both WT and *Nfkb1*$^{SS/AA}$ T_{reg} cells induced a reduction in proliferation of responder cells to a similar extent. Thus, *Nfkb1*$^{SS/AA}$ mutation did not affect the suppressive ability of T_{reg} cells or susceptibility to T_{reg} inhibition.

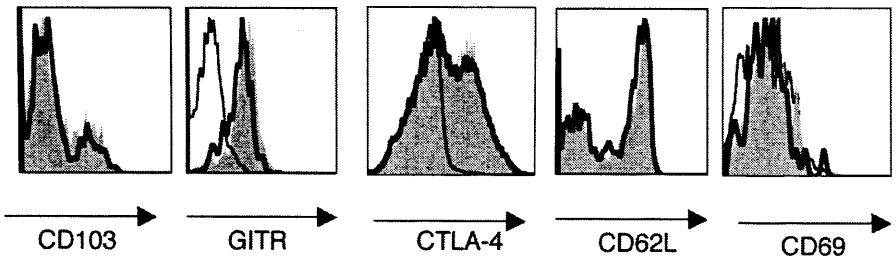
Taken together, these experiments suggest that although the numbers of $CD4^+CD25^+$ T_{reg} cells were significantly reduced in *Nfkb1*$^{SS/AA}$ mice, the phenotype of the cells was normal. Furthermore, the *in vitro* suppressive nature of the regulatory cells remained intact when p105 proteolysis was blocked.

A



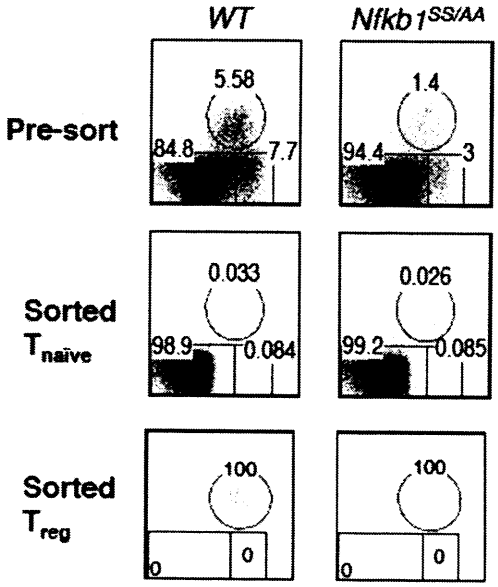
B

Gated:CD4+CD25+



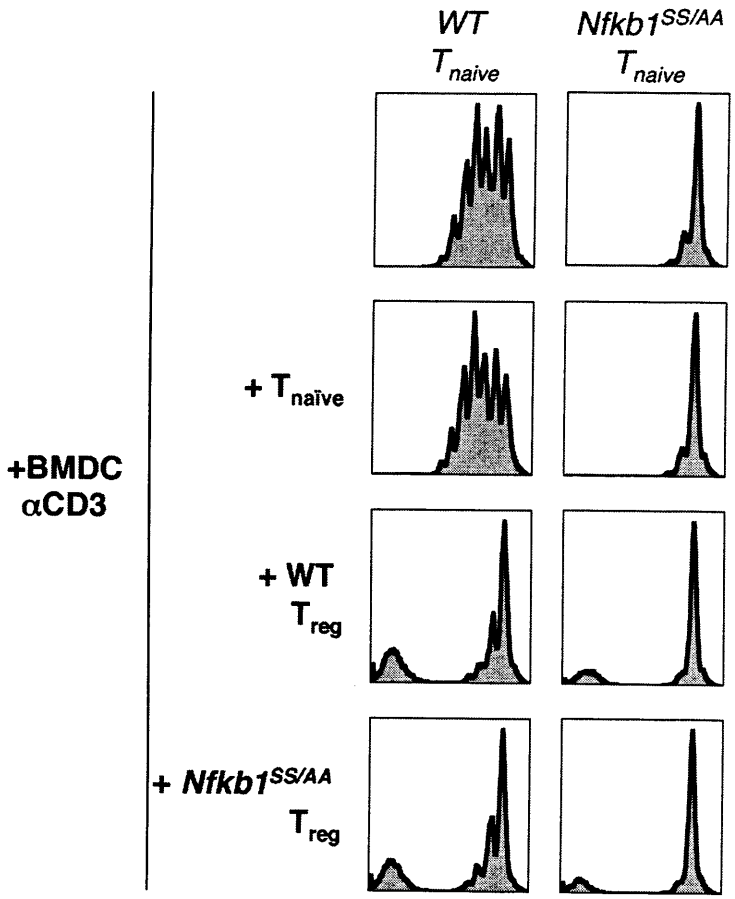
C

FACS analysis of sorted populations



D

Gated:TCRβ⁺CD4⁺
7AAD⁻



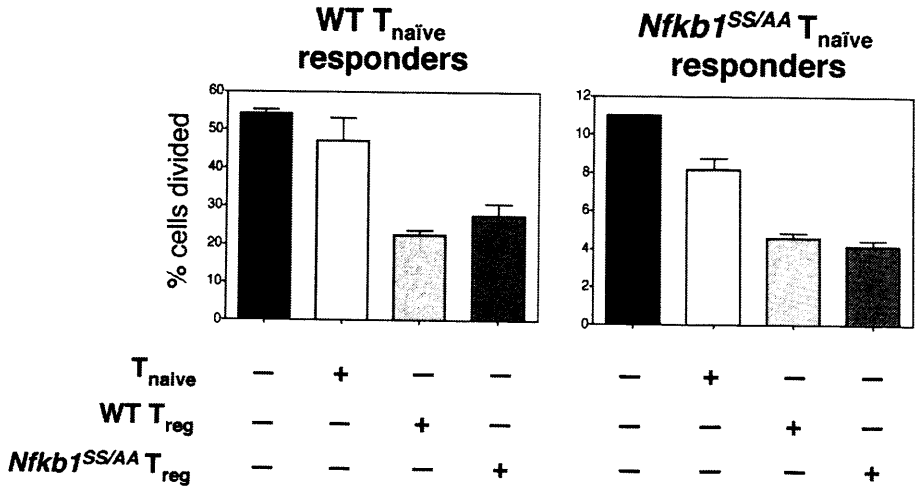


Figure 3.4 Analysis of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^+$ T$_{reg}$ cells

(A) Haematoxylin and eosin staining of sections (magnification x250) from colon of WT and *Nfkb1*$^{SS/AA}$ mice at 24 weeks of age. These data are representative of mice analysed between 12weeks – 18months of age (n=3). **(B)** Live gated CD4$^+$CD25$^+$ cells from spleens of WT and *Nfkb1*$^{SS/AA}$ mice were analysed for markers expressed on T$_{reg}$ cells by flow cytometry. Histogram overlays represent expression of indicated cell surface markers and intracellular levels of CTLA-4 from WT (grey shaded) and *Nfkb1*$^{SS/AA}$ (thick black lines) CD4$^+$CD25$^+$ T$_{reg}$ cells. Isotype controls are also shown (thin black line). Data are representative of three replicate experiments. **(C)** *In vitro* suppression assays were carried out using sorted populations of CD4$^+$ naïve (T$_{naive}$; CD44^{lo}CD25$^-$) T cells and CD4$^+$ T$_{reg}$ (CD44^{int}CD25$^+$) cells from WT and *Nfkb1*$^{SS/AA}$ mice, as indicated on dot plots. Numbers on plots represent percentages of gated live cells in associated gates. **(D)** To assess suppression, WT or *Nfkb1*$^{SS/AA}$ T$_{naive}$ cells were CFSE labeled and stimulated for 72 h with 1 μg/ml anti-CD3 on BMDCs, in the presence or absence of equivalent numbers of WT or *Nfkb1*$^{SS/AA}$ T$_{reg}$ cells. In indicated cultures, T$_{naive}$ cells were added instead of T$_{reg}$ cells. CFSE profiles of gated 7AAD$^-$ CD4$^+$ T cells are shown, with corresponding graphs of the percentage of cells divided (average±SEM). Experiments were carried out in triplicate, and data represent three independent experiments.

3.5 Haematopoietic intrinsic effects of *Nfkb1*$^{SS/AA}$ mutation on T_{reg} and $CD4^+$ T_{mem}-phenotype cell numbers

3.5.1 Development of thymic and peripheral $CD4^+$ and $CD8^+$ T cells is normal in *Nfkb1*$^{SS/AA}$ bone marrow (BM) chimeras

Stromal cells play a critical role in normal T cell maturation, providing critical cell-cell interactions and cytokines (Janeway, 2005). Therefore, the reduction in $CD4^+CD25^+$ T_{reg} and splenic $CD4^+$ and $CD8^+$ T_{mem} cells in *Nfkb1*$^{SS/AA}$ may have been secondary to the effect of p105 mutation in non-T cells and the environment in which the T cells developed. To explore these possibilities BM chimeras were generated. Here, WT or *Nfkb1*$^{SS/AA}$ BM cells were transferred into irradiated C57BL/6 *Rag1*$^{-/-}$mice to provide a WT environment for development of T cells. After 6-8 weeks, T cells arising from stem cells in BM had populated the thymus and peripheral organs of the host and were analysed by flow cytometry. The fractions of DN, DP, $CD4^+$ and $CD8^+$ SP thymic subsets were similar between chimeric mice generated from transfer of either WT or *Nfkb1*$^{SS/AA}$ BM, consistent with normal T cell development in intact *Nfkb1*$^{SS/AA}$ mice (Figure 3.5A). Additionally, the percentages of peripheral $CD4^+$ and $CD8^+$ T cells produced from WT and *Nfkb1*$^{SS/AA}$ BM were comparable.

3.5.2 T_{reg} and $CD4^+$ T_{mem}–phenotype cell numbers are reduced in *Nfkb1*$^{SS/AA}$ BM chimeras

Generation of T_{mem}–phenotype cells in chimeras was analysed by surface staining with anti-CD44. The fraction of splenic $CD4^+$ T_{mem}–phenotype cells arising from

Nfkb1$^{SS/AA}$ BM was significantly lower ($p \leq 0.05$) than those from WT BM (Figure 3.5B). Interestingly, although intact *Nfkb1*$^{SS/AA}$ mice displayed only a small defect in LN CD4$^+$ T_{mem}–phenotype cells that was not statistically significant, a more pronounced and significant reduction was observed in *Nfkb1*$^{SS/AA}$ BM chimeras compared to WT BM chimeras. Furthermore, a reduction in CD8$^+$ T_{mem}–phenotype cells from spleen but not LN was observed in *Nfkb1*$^{SS/AA}$ mice. However, chimeras generated from *Nfkb1*$^{SS/AA}$ BM showed normal proportions of CD8$^+$ T_{mem}–phenotype cells in spleen, in contrast to intact mice.

The fraction of CD4$^+$ T cells that were Foxp3$^+$CD25$^+$ was also analysed in chimeras. A three to four-fold reduction in these T_{reg} cells was observed in the thymus, spleen and LN of mice transferred with *Nfkb1*$^{SS/AA}$ BM relative to transfers with WT BM (Figure 3.5C). This decrease was comparable to the reduction in T_{reg} cells seen in intact *Nfkb1*$^{SS/AA}$ mice compared to WT mice.

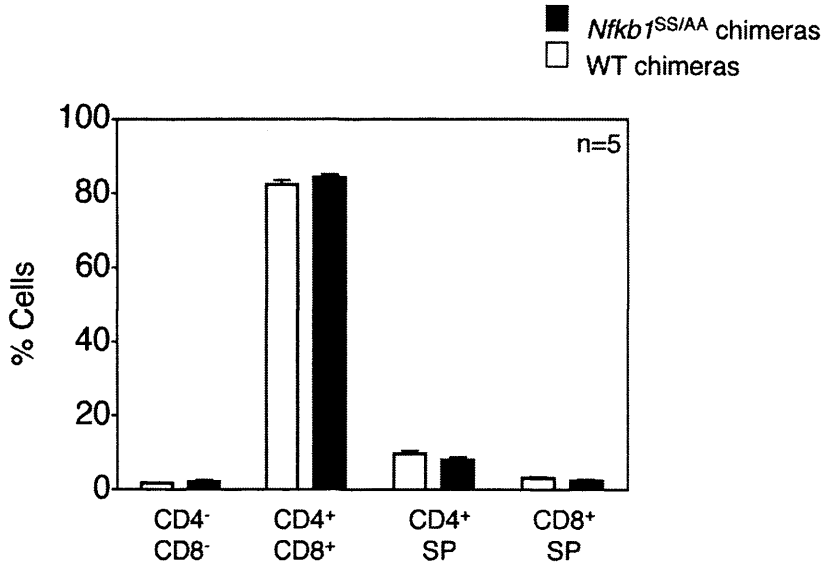
3.5.3 *Nfkb1*$^{SS/AA}$ chimeras have normal fractions of thymic NKT cells

The proportion of NKT cells that developed from *Nfkb1*$^{SS/AA}$ bone marrow was assessed using CD1d tetramer staining (Figure 3.4D). Although levels of thymic CD1d tetramer$^+$ cells were reduced in intact *Nfkb1*$^{SS/AA}$ mice, chimeras produced from *Nfkb1*$^{SS/AA}$ BM showed similar proportions of these cells in both spleen and thymus to WT BM chimeras.

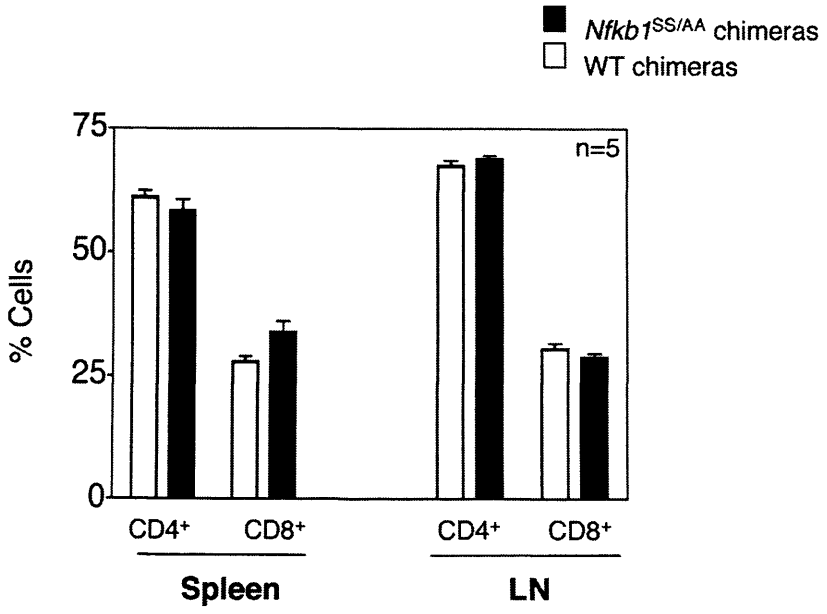
These findings from BM chimeras reveal that defects in haematopoietic cells contributed to the impaired generation of T_{reg} and CD4$^+$ T_{mem} cells in *Nfkb1*$^{SS/AA}$

A

Percentage of thymic CD4 and CD8 subsets in single bone marrow chimeras

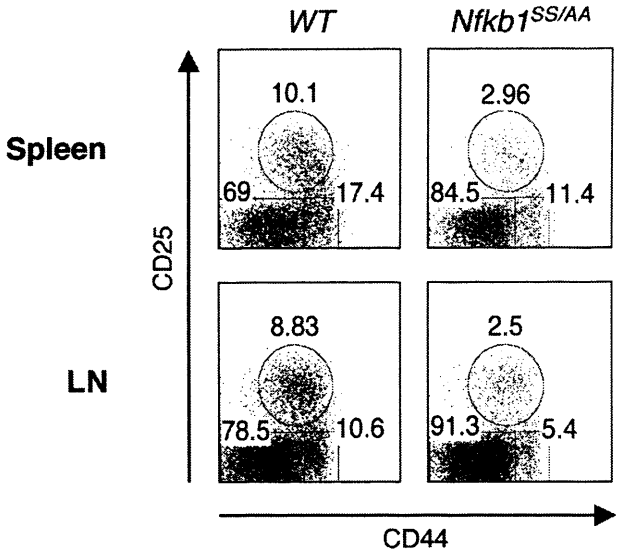


Percentage of peripheral CD4 and CD8 subsets in single bone marrow chimeras

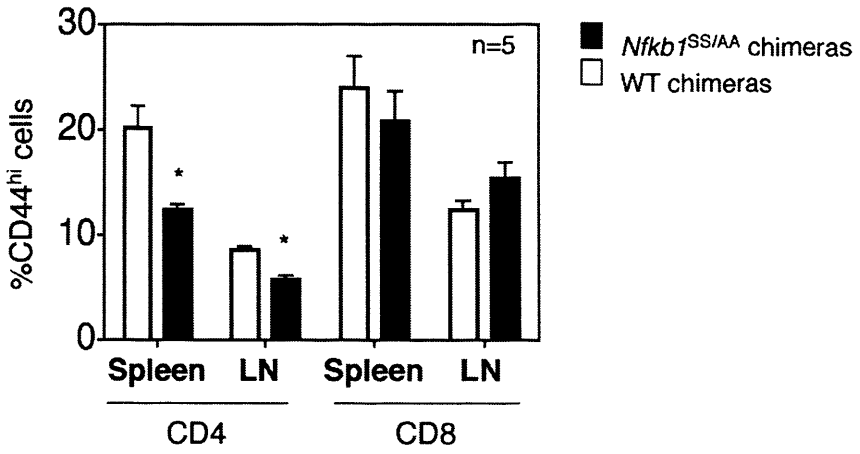


B

Gated:TCRβ+ CD4+



Percentage of CD44hi memory-phenotype cells in single bone marrow chimeras



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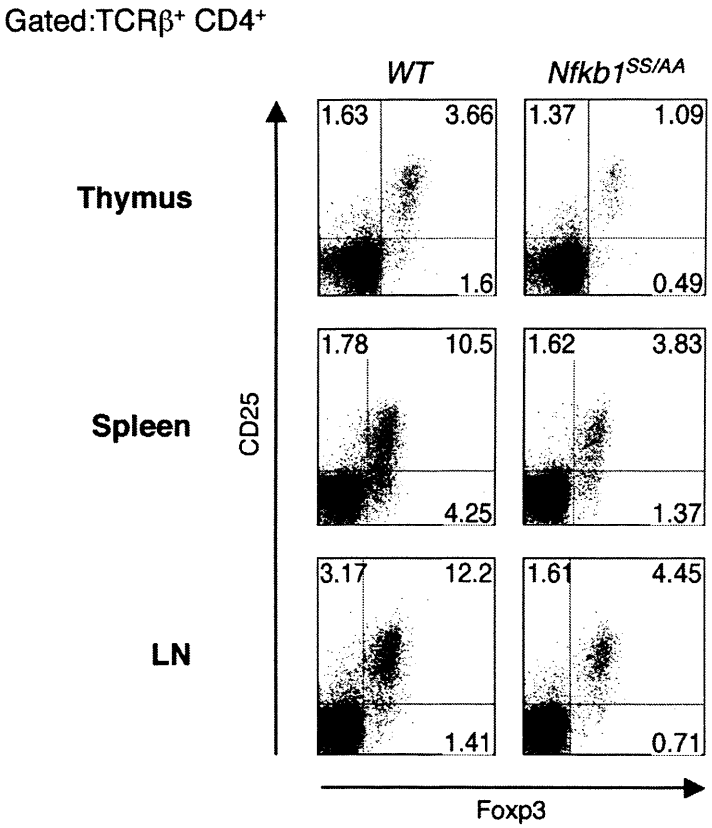
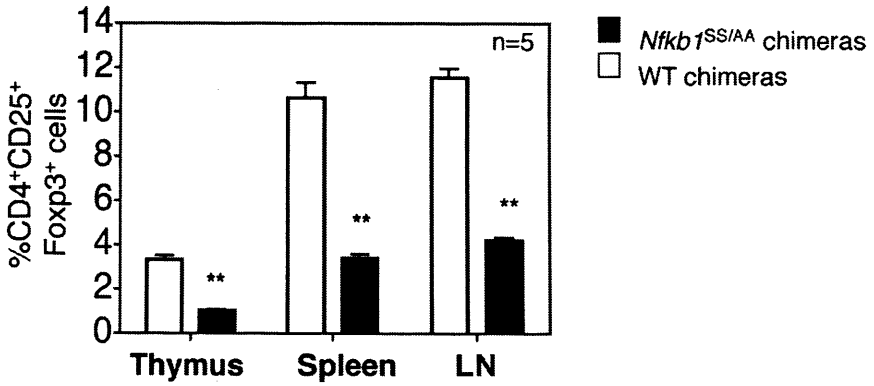


Percentage of CD4+ CD25+Foxp3+ cells in single bone marrow chimeras



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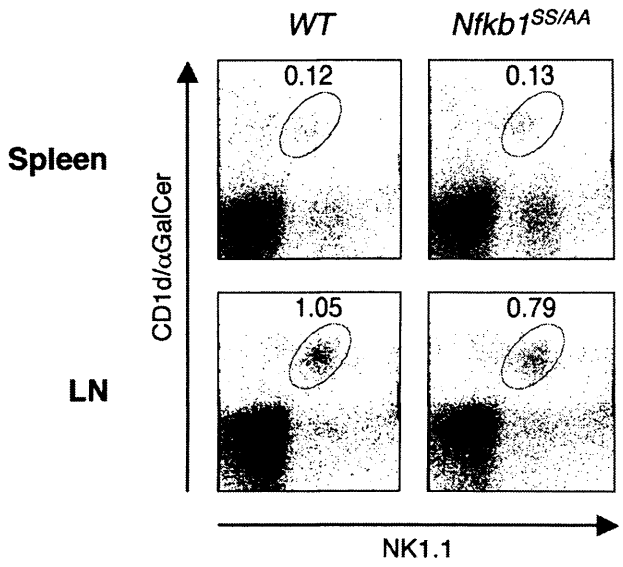


Percentage of NK1.1⁺ Tetramer⁺ NKT cells in single bone marrow chimeras

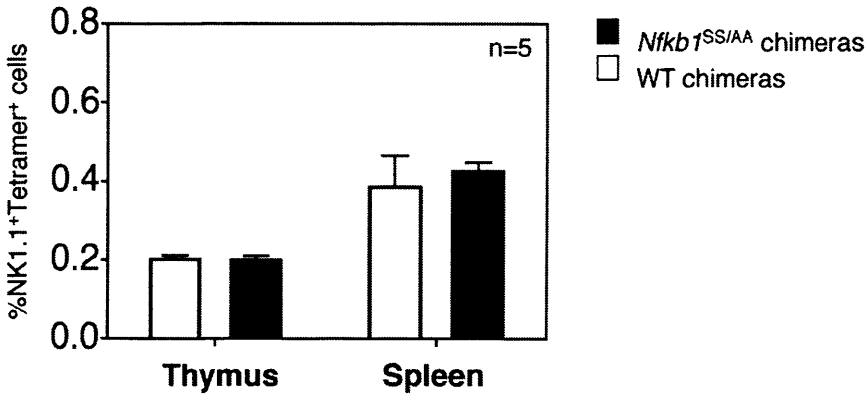


Figure 3.5 Generation of *Nfkb1*$^{SS/AA}$ T cell sub-populations in BM chimeras
T cell-depleted bone marrow cells from *Nfkb1*$^{SS/AA}$ or littermate control WT mice (129S8, C57BL/6 mixed background) were injected into irradiated *Rag1*$^{-/-}$ C57BL/6 recipients to generate single bone marrow chimeras (*Nfkb1*$^{SS/AA}$ and WT chimeras, respectively). After 6-8 weeks, T cells from thymus, spleen and lymph nodes (LN) were analysed by flow cytometry. Data represent results from two experiments with five mice per group. Graphs in this figure show averages ±SEM. *, P≤ 0.05, **, p≤ 0.005; Mann-Whitney test. **(A)** T cell development in chimeras was assessed by surface CD4 and CD8 staining of thymocytes, LN and splenic cells. Graphs show average percentages of live gated CD4 and CD8 subsets in tissues from WT and *Nfkb1*$^{SS/AA}$ chimeras, as indicated. **(B)** CD44 and CD25 expression on LN and splenic cells from WT and *Nfkb1*$^{SS/AA}$ chimeras was determined by flow cytometry. FACS plots show gated TCR$^+$CD4$^+$ live cells from LN and spleen, with numbers indicating fractions of gated cells. Percentages of CD44^{hi} populations from FACS analysis of gated TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ live T cells in chimeras are represented graphically (n=5). **(C)** Intracellular Foxp3 and CD25 surface expression from gated TCRβ$^+$CD4$^+$ live cells of WT and *Nfkb1*$^{SS/AA}$ chimeras are represented as dot plots, with percentages of gated cells indicated. Corresponding graphs show percentages of Foxp3$^+$CD25$^+$ cells from live-gated TCRβ$^+$CD4$^+$ cells of WT and *Nfkb1*$^{SS/AA}$ chimeras (n=5). **(D)** Flow cytometric analysis of NKT cells in thymus and spleen of chimeras are shown, based on staining with α-galactosylceramide (αGal-Cer)-loaded CD1d tetramer and surface expression of NK1.1 on live gated cells. Numbers on plots indicate fraction of cells that were NK1.1$^+$tetramer$^+$, and percentages from five mice are also presented on graphs.

mice. In contrast, the reduction in NKT cells in the thymus of *Nfkb1*$^{SS/AA}$ mice compared to WT mice required p105 mutation in non-haematopoietic cells.

3.6 Impaired production of *Nfkb1*$^{SS/AA}$ T_{reg} and $CD4^+$ T_{mem}– phenotype cells cannot be rescued by WT T cells

Single BM chimera experiments demonstrated that impaired generation of T_{reg} and $CD4^+$ T_{mem} cells resulted from defects in *Nfkb1*$^{SS/AA}$ haematopoietic cells. However, it was still unclear whether these were cell autonomous defects. *Nfkb1*$^{SS/AA}$ mutation may have impaired T-T cell interactions or production of T cell-derived soluble factors critical for the generation of these T cell sub-populations. These possibilities were explored by generating competitive bone marrow chimeras in which *Nfkb1*$^{SS/AA}$ T cells developed in the presence of WT T cells. To do this, BM from $CD45.1^+$ WT C57BL/6 mice were mixed with BM from either $CD45.2^+$ WT (*Nfkb1*$^{+/+}$+ BL6→BL6 chimera) or $CD45.2^+$ *Nfkb1*$^{SS/AA}$ mice (*Nfkb1*$^{SS/AA}$+ BL6→BL6 chimera) and transferred into irradiated $CD45.2^+$*Rag1*$^{-/-}$ C57BL/6 recipients. Antibodies specific for the congenic markers CD45.1 and CD45.2 allowed development of T cells from both sets of transferred BM to be followed in the same recipient. $CD45.1^+$ BM cells were injected at either a 4:1 excess to $CD45.2^+$ BM cells, or at equal ratios. The fraction of T cell populations that developed from $CD45.1^+$ and $CD45.2^+$ cells was then followed by flow cytometry. Those chimeras generated using 4:1 BM cells are presented here, although similar results were obtained from both ratios of BM cells transferred.

3.6.1 Development of *Nfkb1*$^{SS/AA}$ CD4$^+$ and CD8$^+$ T cells is normal in mixed BM chimeras

The ratio of CD45.2:CD45.1 cells found in host tissues from *Nfkb1*$^{SS/AA}$+ BL6→BL6 chimeras were slightly higher than from *Nfkb1*$^{+/+}$+ BL6→BL6 chimeras (Figure 3.6A). However the ratio of CD45.2$^+$ *Nfkb1*$^{SS/AA}$:CD45.1 WT cells initially transferred had also been found to be slightly higher. *Nfkb1*$^{SS/AA}$ cells could therefore reconstitute host tissues as well as *Nfkb1*$^{+/+}$ cells. Consistent with this, development of CD4 and CD8 T cell subsets in the thymus and periphery from CD45.2$^+$ *Nfkb1*$^{SS/AA}$ BM in *Nfkb1*$^{SS/AA}$+ BL6→BL6 chimeras was comparable to that of cells from CD45.2$^+$ *Nfkb1*$^{+/+}$ BM from *Nfkb1*$^{+/+}$+ BL6→BL6 chimeras (Figure 3.6B).

3.6.1 Fractions of *Nfkb1*$^{SS/AA}$ T_{reg} and CD4$^+$ T_{mem}-phenotype cells are reduced in a competitive environment

Using CD44 expression as a marker, the proportion of CD4$^+$ T_{mem} cells that developed from CD45.2$^+$ BM cells was assessed in mixed BM chimeras. *Nfkb1*$^{SS/AA}$ CD45.2$^+$ BM generated 6-fold fewer splenic CD4$^+$ T_{mem}–phenotype cells compared to WT CD45.2$^+$ BM (Figure 3.6C). This reduction was more marked in mixed BM chimeras than in unmanipulated *Nfkb1*$^{SS/AA}$ mice or *Nfkb1*$^{SS/AA}$ single bone-marrow chimeras.

Intracellular Foxp3 staining of thymus, spleen and LN from mixed BM chimeras revealed that the percentage of CD45.2$^+$ T_{reg} cells that arose from *Nfkb1*$^{SS/AA}$ BM

was markedly lower than that of CD45.2$^+$ T_{reg} cells from WT BM (*Nfkb1*$^{+/+}$) (Figure 3.6D). This decrease was more pronounced than that detected in intact *Nfkb1*$^{SS/AA}$ mice. CD45.2$^+$ T_{reg} cells from *Nfkb1* $^{SS/AA}$ BM were seven- to ten-fold fewer than CD45.2$^+$ T_{reg} cells from *Nfkb1*$^{+/+}$ BM. In contrast, intact *Nfkb1*$^{SS/AA}$ mice and *Nfkb1*$^{SS/AA}$ single bone-marrow chimeras only exhibited a three- to four-fold reduction in T_{reg} cells compared with intact WT mice or WT chimeras.

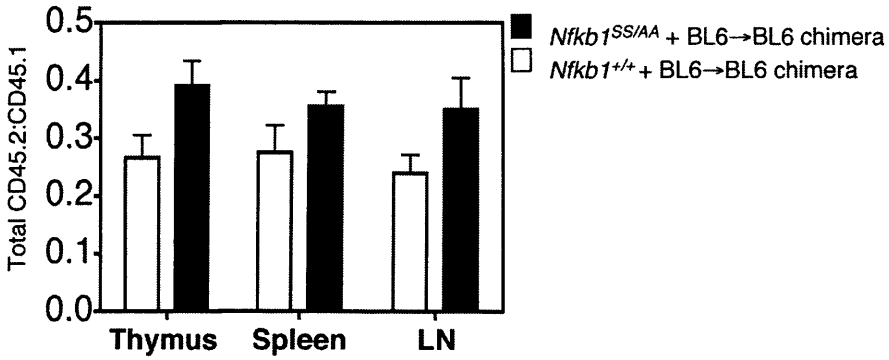
Consistent with these results, the fractions of CD45.1$^+$ T_{reg} cells and CD4$^+$ T_{mem}–phenotype cells generated in *Nfkb1*$^{SS/AA}$+BL6→BL6 chimeras were higher than those CD45.1$^+$ cells from *Nfkb1*$^{+/+}$+BL6→BL6 chimeras (Figure 3.6E). These data therefore suggest that *Nfkb1*$^{SS/AA}$ T_{reg} and CD4$^+$ T_{mem}–phenotype cells were less able to compete with CD45.1 wild-type cells than *Nfkb1*$^{+/+}$ CD45.2 and that the defect in *Nfkb1*$^{SS/AA}$ T_{reg} and CD4$^+$ T_{mem}–phenotype numbers was cell autonomous.

3.7 Turnover of T_{reg} and CD4$^+$ T_{mem}–phenotype cells is unaffected by p105 mutation

The reduced numbers of T_{reg} and CD4$^+$ T_{mem}–phenotype cells in *Nfkb1*$^{SS/AA}$ mice compared to WT mice may have been due to increased turnover of these cells. To investigate this, *in vivo* labelling was carried out using a thymidine analogue, bromo-2'-deoxyuridine (BrdU), which becomes incorporated into newly-synthesised DNA of cells in S-phase of the cell cycle. Mice were labelled continuously for 12 days, after which time LN and spleens were analysed for BrdU content. Those cycling cells that had incorporated BrdU were detected by intracellular staining with

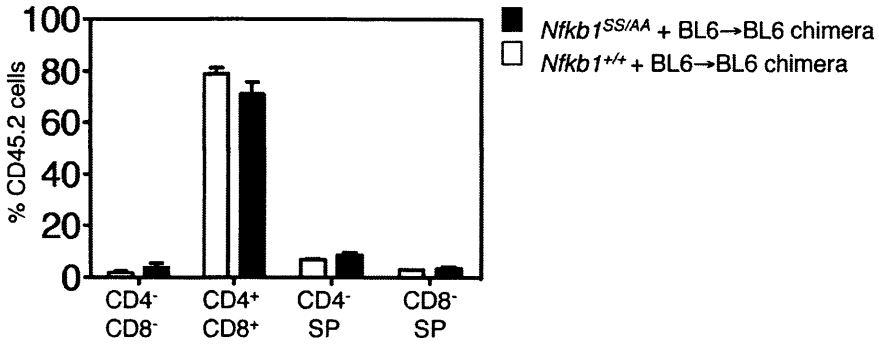
A

Ratio of total CD45.1:CD45.2 cells in mixed bone marrow chimeras

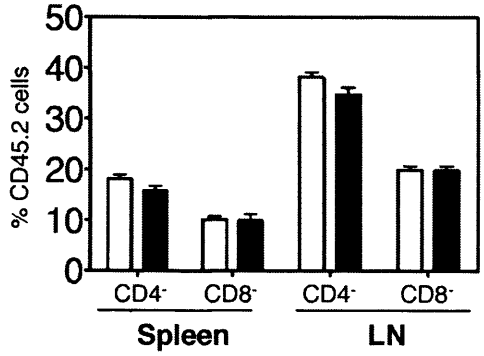


B

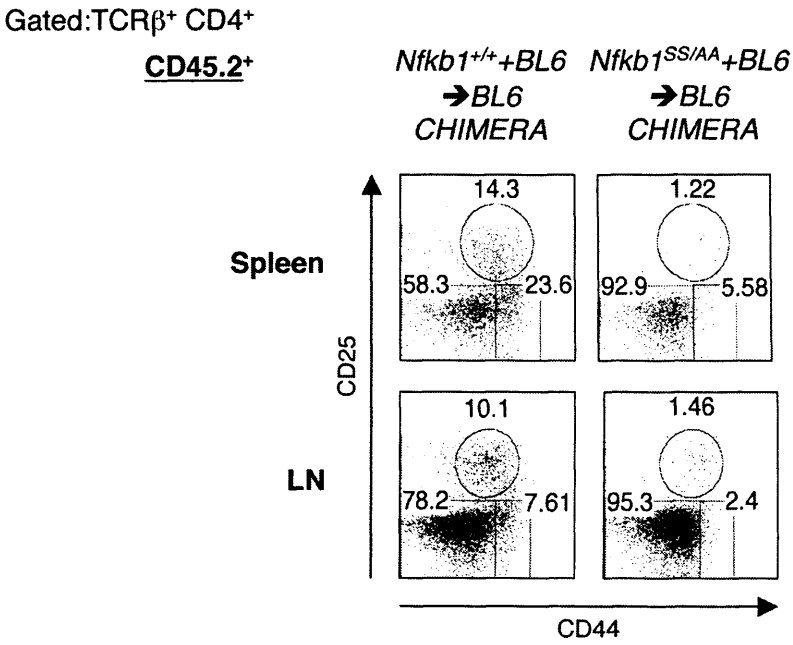
Percentage of thymic CD45.2+ CD4 and CD8 subsets in mixed bone marrow chimeras



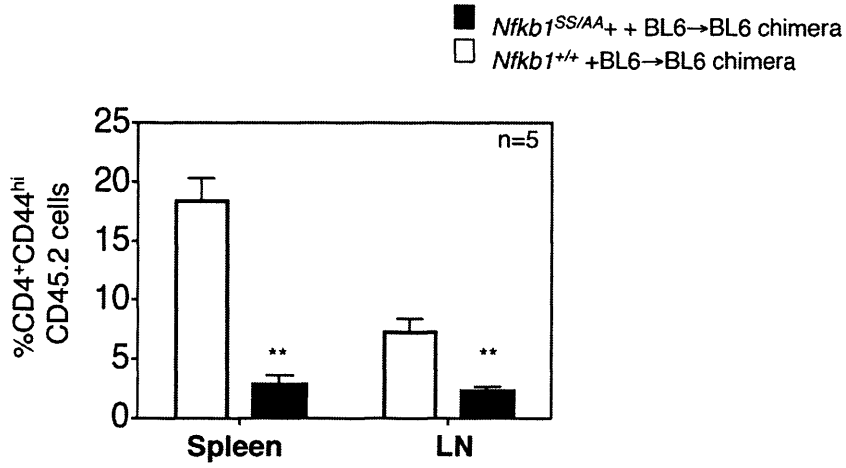
Percentage of peripheral CD45.2+ CD4 and CD8 subsets in mixed bone marrow chimeras



C

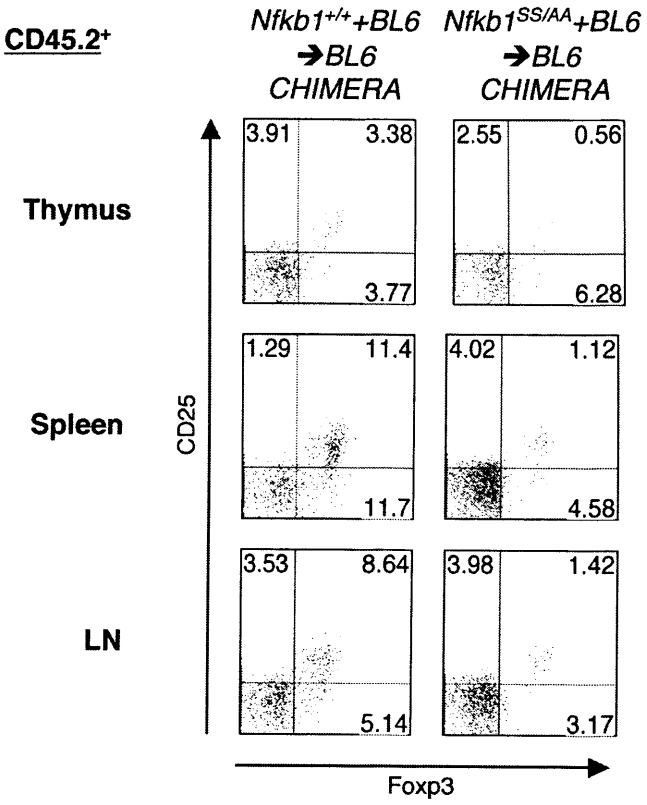


Percentage of CD45.2+ CD44hiCD4+ T cells in mixed bone marrow chimeras

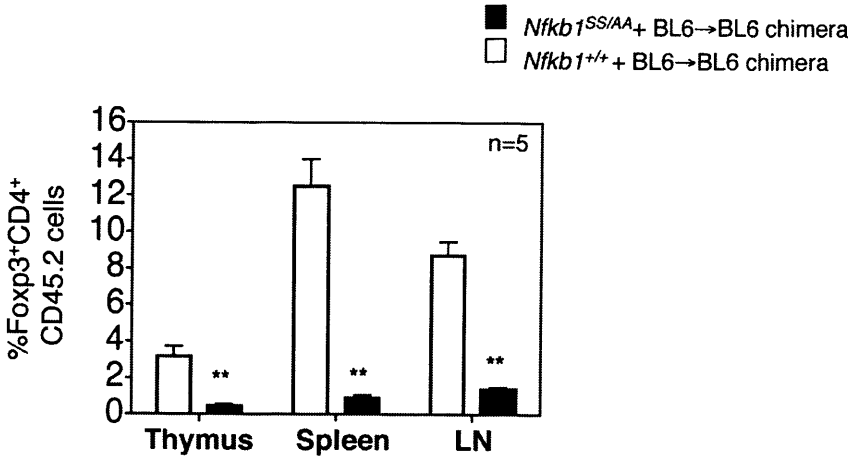


D

Gated:TCRβ⁺ CD4⁺



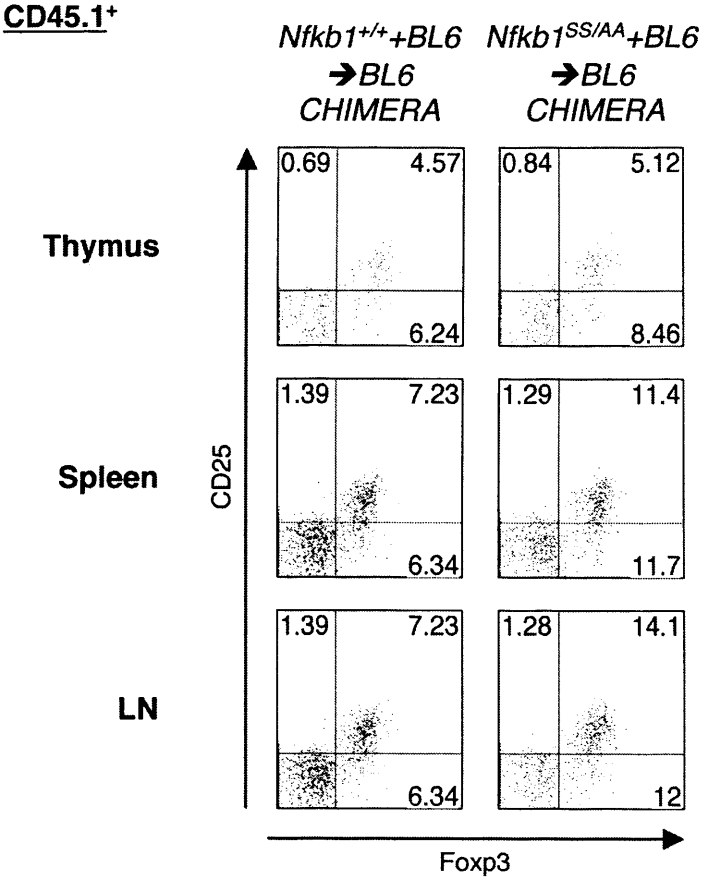
Percentage of CD45.2⁺ Foxp3⁺CD4⁺ T cells in mixed bone marrow chimeras



E

Gated:TCRβ+ CD4+

CD45.1+



Gated:TCRβ+ CD4+

CD45.1+

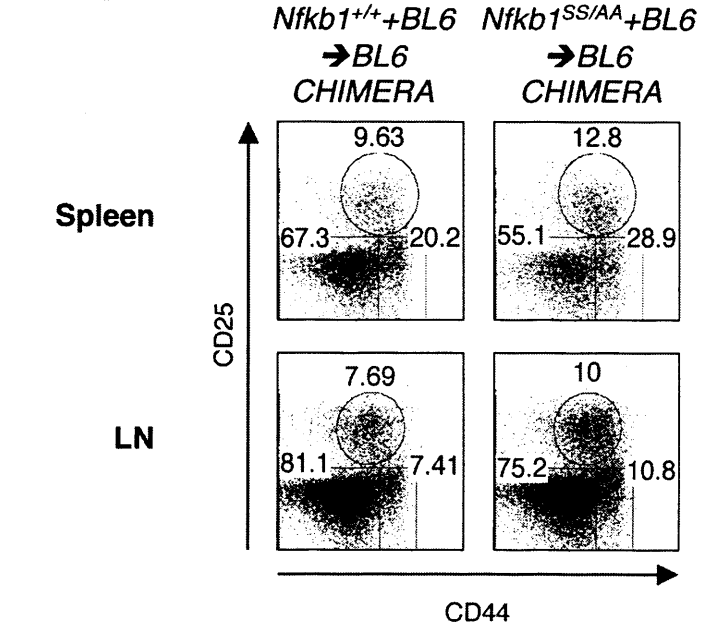
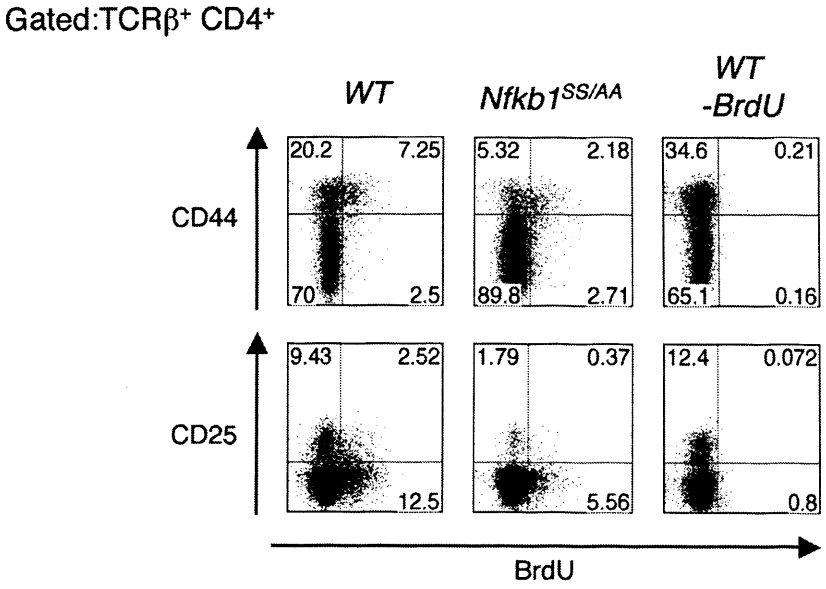


Figure 3.6 Cell autonomous defects contribute to impaired generation of *Nfkb1*$^{SS/AA}$ T_{reg} and CD4$^+$ memory-phenotype sub-populations

Mixed bone marrow (BM) chimeras were generated by injecting irradiated CD45.2$^+$*Rag1*$^{-/-}$ C57BL/6 mice with BM from CD45.1$^+$ WT C57BL/6 mice that was mixed with either BM from CD45.2$^+$ WT (*Nfkb1*$^{+/+}$+ BL6→BL6 chimera) or CD45.2$^+$ *Nfkb1*$^{SS/AA}$ mice (*Nfkb1*$^{SS/AA}$+ BL6→BL6 chimera). After 6-8 weeks, tissues from mixed chimeras were analysed by flow cytometry. Data shown here are from analysis of chimeras generated using a 4:1 excess of CD45.1:CD45.2 BM cells. Results are representative of two independent experiments with five mice in each group. Bar charts in this figure show average ±SEM. *, P≤ 0.05, **, p≤ 0.005; Mann-Whitney test. **(A)** Graphs show the ratio of total CD45.1 and CD45.2 cells present in *Nfkb1*$^{+/+}$+ BL6→BL6 chimeras and *Nfkb1*$^{SS/AA}$+ BL6→BL6 chimeras, as analysed by flow cytometry using antibodies against congenic markers. **(B)** Development of WT (*Nfkb1*$^{+/+}$) and *Nfkb1*$^{SS/AA}$ T cells was assessed based on surface staining for CD4 and CD8 on gated CD45.2$^+$ live cells from *Nfkb1*$^{+/+}$+ BL6→BL6 and *Nfkb1*$^{SS/AA}$+ BL6→BL6 chimeras. Bar charts show percentages of indicated CD4 and CD8 subsets in thymus and periphery. **(C)** Surface staining for CD44 and CD25 on WT (*Nfkb1*$^{+/+}$) and *Nfkb1*$^{SS/AA}$ cells from mixed BM chimeras was carried out. Cells from indicated tissues were gated for expression of TCRβ, CD4 and CD45.2, and data are presented as dot plots. Numbers indicate percentages of cells in the corresponding gate. Percentages are also represented as bar charts of CD45.2$^+$CD4$^+$TCRβ$^+$ cells which had high CD44 expression (CD44^{hi}). **(D)** Flow cytometric analysis of intracellular Foxp3 and surface CD25 expression on gated live TCRβ$^+$CD4$^+$CD45.2$^+$ cells from mixed bone marrow chimeras is shown. Numbers in dot plots represent the percentage of cells in quadrants. The percentage of Foxp3$^+$CD25$^+$ cells from these gated TCRβ$^+$CD4$^+$CD45.2$^+$ cells are also shown graphically. **(E)** Development of WT CD45.1 cells in *Nfkb1*$^{+/+}$+ BL6→BL6 and *Nfkb1*$^{SS/AA}$+ BL6→BL6 chimeric mice was also followed by flow cytometry. Dot plots show Foxp3 and CD25 expression, or CD44 and CD25 expression, on gated TCRβ$^+$CD4$^+$CD45.1$^+$ live cells. Numbers represent the percentage of cells in designated gates.

a BrdU-specific antibody and flow cytometry. Only 2% of naïve $CD4^+$ T cells had incorporated BrdU in both WT and *Nfkb1*$^{SS/AA}$ mice, indicating a low level of turnover in this population that was consistent with previous studies (Tough and Sprent, 1994) (Figure 3.7A and 3.7B). Furthermore, this small fraction of BrdU positive naïve $CD4^+$ T cells also suggested that BrdU labelling occurred mostly in the periphery, and that export of BrdU-labelled thymic precursors was not likely to be the main source of BrdU-positive cells. The fraction of splenic $CD4^+CD25^+$ T_{reg} and $CD4^+CD44^{hi}$ T_{mem}–phenotype cells that had incorporated BrdU was found to be between 20 and 25% in both WT and *Nfkb1*$^{SS/AA}$ mice. There was also no significant difference in the fraction of BrdU-positive $CD4^+CD25^+$ T_{reg} cells from LN. (data not shown) Taken together, these results suggest that *Nfkb1*$^{SS/AA}$ mutation did not alter the turnover of T_{reg} and $CD4^+$ T_{mem}–phenotype cells.

A



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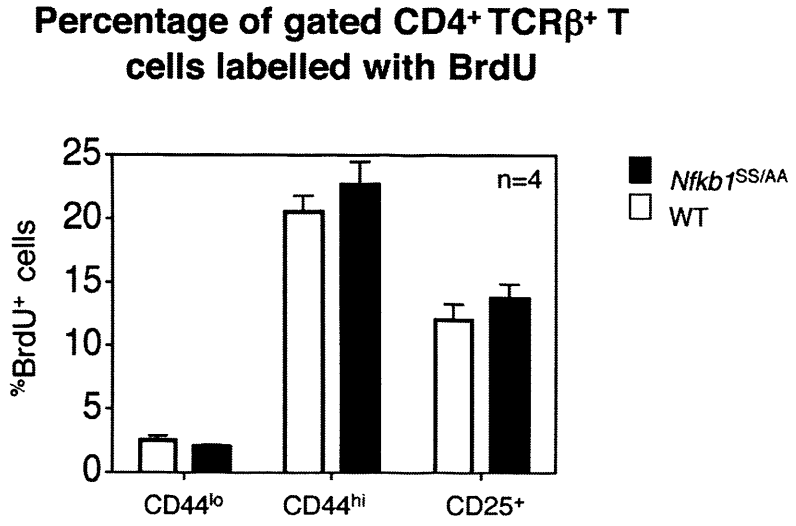


Figure 3.7 Turnover of *Nfkb1*$^{SS/AA}$ T_{reg} and CD4$^+$ T_{mem} cells is normal
BrdU was injected intraperitoneally into WT and *Nfkb1*$^{SS/AA}$ mice, which were supplemented with BrdU in drinking water for 12 d. Control mice were not injected and kept off BrdU. Labelling of gated CD44^{lo}, CD44$^+$ or CD25$^+$, CD4+ T cell sub-populations in the spleen was determined by flow cytometry. **(A)** Dot plots show BrdU incorporation of gated live TCRβ$^+$CD4$^+$ cells stained for CD44 or CD25, as indicated. Numbers indicate the percentages of cells in the designated gate. **(B)** Bar charts show the fraction of BrdU$^+$ cells in the indicated gated live TCRβ$^+$ CD4$^+$ populations (average± SEM) from WT and *Nfkb1*$^{SS/AA}$ mice (n=4). Data represent results from three experiments.

3.8 Chapter 3 summary

Analysis of *Nfkb1*$^{SS/AA}$ mice and BM chimeras presented in this chapter suggest:

- *Nfkb1*$^{SS/AA}$ mutation does not affect conventional TCR αβ T cell development.
- Blocking IKK-induced p105 proteolysis results in a T cell-autonomous defect in generation of T_{reg} cells.
- *Nfkb1*$^{SS/AA}$ mutation does not impair T_{reg} cell suppressor function *in vitro.*
- IKK-induced proteolysis of p105 is required in non-haematopoietic cells for generation of NKT cells.
- A T-cell autonomous defect caused by *Nfkb1*$^{SS/AA}$ mutation contributes to impair the generation and/or maintenance of $CD4^+$ T_{mem}–phenotype cells.
- *Nfkb1*$^{SS/AA}$ mutation impairs $CD4^+$ T_{reg} cells and $CD4^+$ T_{mem}–phenotype cells competitive fitness.
- *Nfkb1*$^{SS/AA}$ mutation in non-haematopoietic cells is required for the reduction in splenic $CD8^+$ T_{mem}–phenotype numbers in *Nfkb1*$^{SS/AA}$ mice.
- Turnover of $CD4^+$ T_{mem}–phenotype and T_{reg} cells in *Nfkb1*$^{SS/AA}$ mice is normal, implying that the reduction in numbers of these sub-populations resulted from their reduced production.

4. Characterisation of *Nfkb1*$^{SS/AA}$ CD4^{+} T cells

4. Characterisation of *Nfkb1*$^{SS/AA}$ CD4^{+} T cells

4.1 IKK-induced p105 proteolysis is required for CD4^{+} T cells proliferation *in vitro* following TCR stimulation

Development of T_{reg} cells requires TCR signalling, with NF-κB activation being of particular importance (Liston and Rudensky, 2007, Schmidt-Supprian et al., 2003). TCR stimulation of naïve cells is also essential for the generation of T_{mem} cells (Seder and Sacks, 2004). Therefore, the cell autonomous reductions in T_{reg} and CD4^{+} T_{mem} cells in *Nfkb1*$^{SS/AA}$ mice may have resulted from defective TCR activation.

To address this possibility, the ability of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells to divide *in vitro* after TCR cross-linking was analysed. CD4^{+}CD25^{-} T cells were enriched by staining with biotinylated antibodies against markers on the cells to be depleted, including B cells and CD8^{+} T cells. Labelled cells were magnetically removed using beads conjugated to streptavidin, which binds to biotin. CD4^{+}CD25^{-} T cells from LN purified in this way were found to give a purity of 90-95 % by flow cytometry (Figure 4.1A). To monitor proliferation, CD4^{+}CD25^{-} T cells were labelled with CFSE. This fluorescent dye stably binds to intracellular amines, such that after labelled cells undergo mitotic division, each daughter cell contains half the starting amount of CFSE, and consequently emits half the fluorescence of the starting labelled cell. Therefore, measurement of CFSE fluorescence intensity permitted analysis of cell division.

4.1.1 Anti-CD3-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells is impaired *in vitro*

LN-derived WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells were triggered to proliferate in response to plate-bound anti-CD3, which crosslinks the TCR. Cells were gated for viability (7AAD^{-}) and TCRβ^{+}CD4^{+} expression. Whilst 35 % of viable WT CD4^{+} T cells had divided after 72h stimulation with 1 μg/ml anti-CD3, only 10 % of viable *Nfkb1*$^{SS/AA}$ CD4^{+} T cells had proliferated (Figure 4.1B). Of the *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells that had divided, the number of divisions undergone in 72 h of culture, known as the proliferation index, was similar to WT cells (Figure 4.1C). Therefore, the *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells that can proliferate undergo normal division. Notably, a proliferation defect of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells was observed over a range of concentrations of anti-CD3 (Figure 4.1D). Costimulation with 1 μg/ml CD28 antibody increased the percentage of dividing cells of both WT and *Nfkb1*$^{SS/AA}$ depleted T cells. However, the fraction of *Nfkb1*$^{SS/AA}$ cells dividing still did not reach levels of WT cells.

Interestingly, *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells purified from spleen, where the purity of the cell population was only 75-85 % (Figure 4.1A), showed no significant defect in proliferation when stimulated with high concentrations of anti-CD3. However, at limiting concentrations of CD3 antibody, fewer *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells were able to divide relative to WT cells (Figure 4.1E). Thus, *Nfkb1*$^{SS/AA}$ mutation resulted in a dose-dependent defect in the percentage of splenic CD4^{+}CD25^{-} T cells triggered to proliferate after TCR stimulation. This discrepancy in proliferation

between splenic and LN $CD4^+CD25^-$ T cells raises the possibility that the 20 % contaminating cells in splenic populations contained cells, such as APCs, which could provide costimulatory signals and/or produce cytokines that enhance the fraction of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells dividing.

In subsequent experiments, $CD4^+CD25^-$ T cells were purified from LN since higher levels of purity were possible and so the proliferative defect was independent of the degree of TCR stimulation.

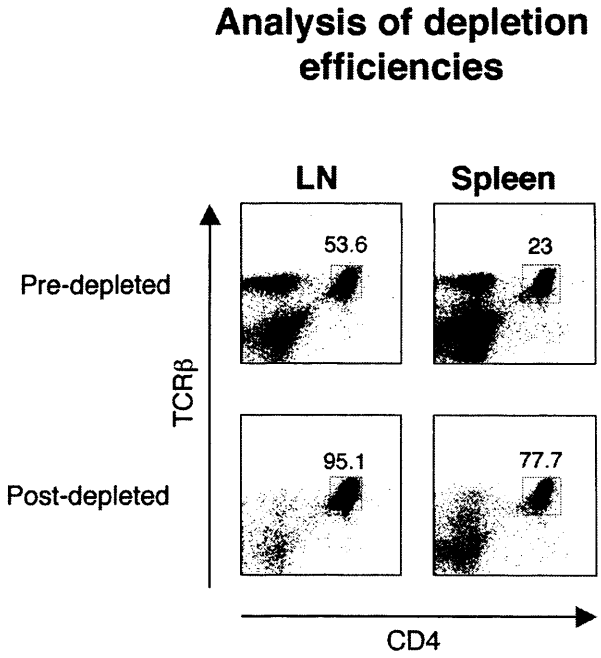
4.1.2 Proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells is normal after PdBU and ionomycin treatment

Stimulation with the pharmacological agents phorbol ester (PdBU) and ionomycin can activate T cells by mimicking TCR stimulation, but independently of the TCR. PdBU can directly activate PKCs, whilst ionomycin binds calcium ions, causing an increase in intracellular calcium levels. It was interesting to determine whether proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ was impaired after PdBU and ionomycin stimulation, which provides a more potent stimulus than anti-CD3. Analysis of CFSE profiles from gated $7AAD^-TCR\beta^+CD4^+$ T cells revealed that PdBU and ionomycin triggered approximately 85 % of cells to divide, with no significant difference observed between WT and *Nfkb1*$^{SS/AA}$ cultures (Figure 4.1F). These data indicate that *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells can divide as normal, if stimulated with a strong pharmacological stimulant. Furthermore, these results suggest that there was no inherent defect in division of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells.

4.1.3 Defects in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cell proliferation are T cell intrinsic

CD4$^+$CD25$^-$ T cells were routinely isolated by negative selection due to the high yields possible using this method. However, a significant fraction of non-T cells were present in proliferation assay starting cultures due to limitations of this method. In addition, it was not possible to selectively deplete CD4$^+$CD44^{hi} T_{mem} cells without also removing CD4$^+$CD44^{lo} T cells. Therefore, depleted CD4$^+$CD25$^-$ T cells used for proliferation assays contained both naïve and memory-phenotype CD4$^+$ T cells, as well as non-T cells. It was possible then that the defect in proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells may have not been intrinsic to the CD4$^+$ T cells or due to defects in *Nfkb1*$^{SS/AA}$ T_{mem} cells. To address these issues, CD4$^+$CD44^{lo}CD25$^-$ naïve T cells were isolated by cell sorting (purity>98 %) and stimulated with soluble anti-CD3 presented on syngeneic WT BMDCs. This stimulus induced efficient activation and proliferation of WT CD4$^+$ T cells, with approximately 64 % of cells triggered to divide. However, the percentage of naïve *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells that had divided was significantly ($p \leq 0.05$) lower than naïve WT CD4$^+$ T cells, amounting to a six-fold reduction (Figure 4.1G). This impaired proliferation of naïve *Nfkb1*$^{SS/AA}$ CD4$^+$ cells was more pronounced than that of depleted *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells. These results indicate that *Nfkb1*$^{SS/AA}$ mutation resulted in a CD4$^+$ T cell intrinsic proliferation defect to anti-CD3 stimulation.

A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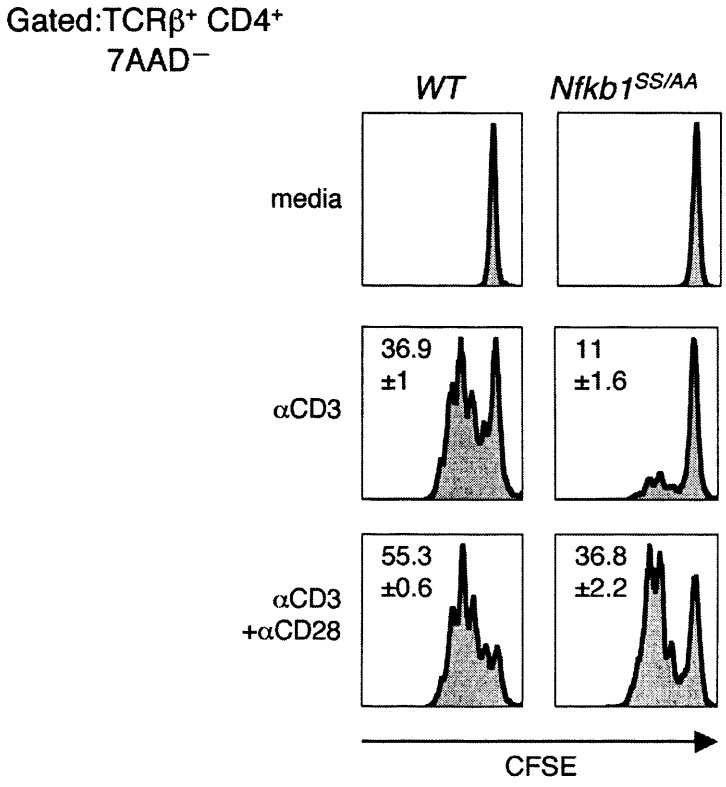
Spleen
Pre-depleted
Post-depleted
TCRβ
53.6
23
95.1
77.7
CD4

B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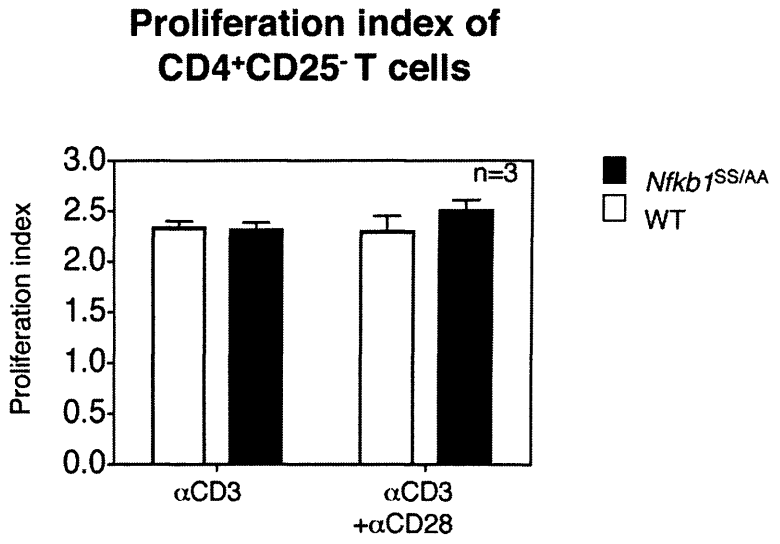
7AAD−
WT
Nfkb1SS/AA
media
αCD3
αCD3
+αCD28
36.9
±1
11
±1.6
55.3
±0.6
36.8
±2.2
CFSE

C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CD4+CD25- T cells
Proliferation index
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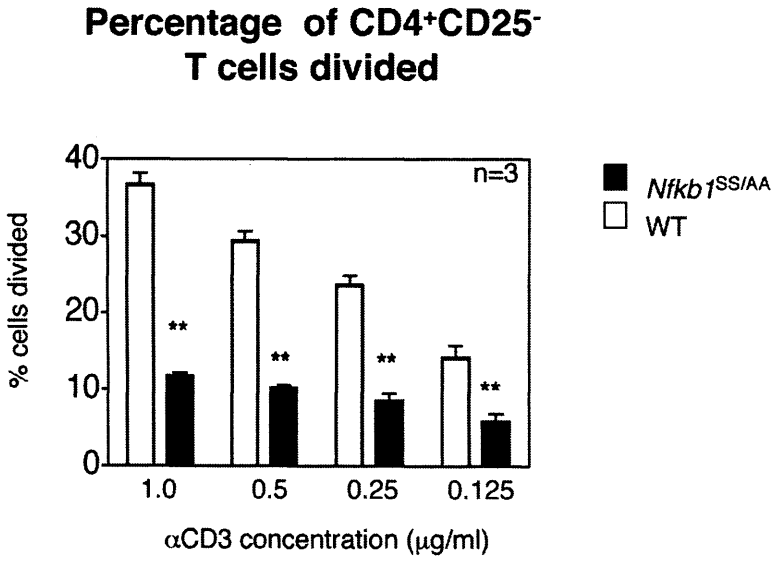
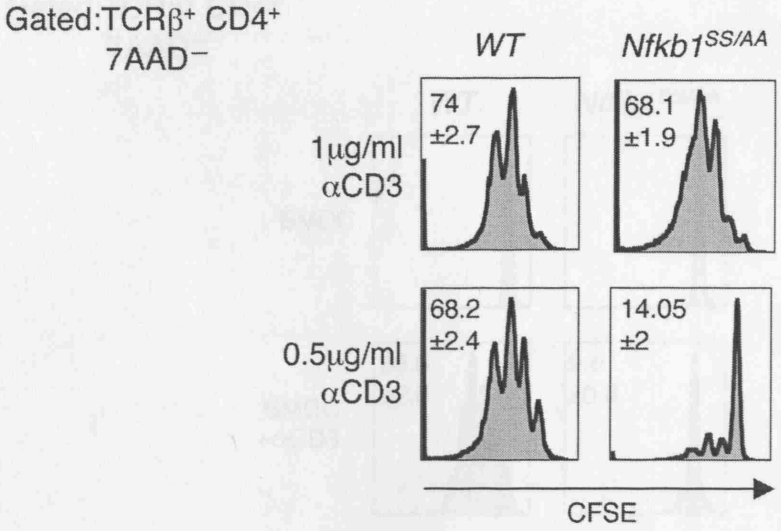
2.5
2.0
1.5
1.0
0.5
0.0
n=3
αCD3
αCD3
+αCD28
Nfkb1SS/AA
WT

D


Percentage of CD4+CD25-
T cells divided
% cells divided
40
30
20
10
0
n=3
**
**
**
**
1.0
0.5
0.25
0.125
αCD3 concentration (μg/ml)
Nfkb1SS/AA
WT

E

SPLEEN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7AAD−
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Nfkb1SS/AA
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αCD3
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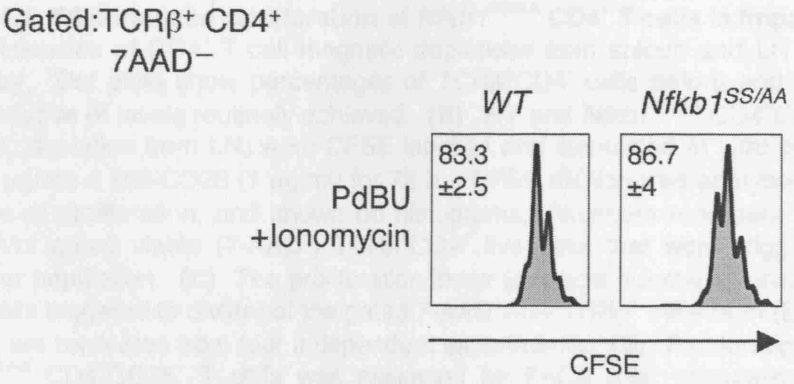
αCD3
68.2
±2.4
14.05
±2
CFSE

F


Gated:TCRβ+ CD4+
7AAD−
WT
Nfkb1SS/AA
PdBU
+Ionomycin
83.3
±2.5
86.7
±4
CFSE

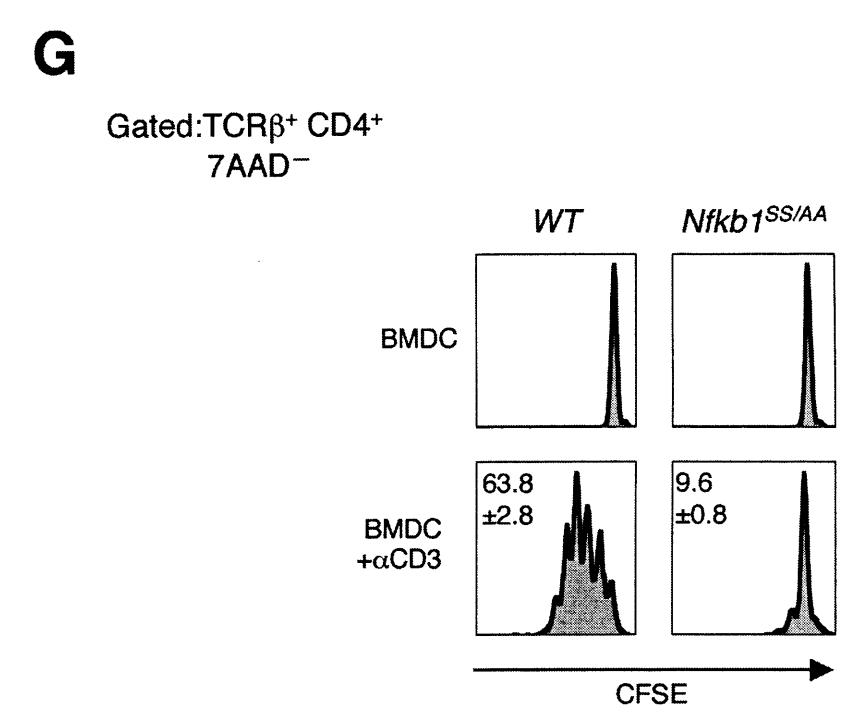


Figure 4.1 TCR-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells is impaired
(A) Efficiencies of CD4$^+$ T cell magnetic depletions from spleen and LN were assessed by flow cytometry. Dot plots show percentages of TCRβ$^+$CD4$^+$ cells before and after depletion, and are representative of levels routinely achieved. **(B)** WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells, isolated by magnetic depletion from LN, were CFSE labelled and stimulated *in vitro* with plate-bound anti (α)-CD3 (1 μg/ml) ± anti-CD28 (1 μg/ml) for 72 h. CFSE dilution was analysed by flow cytometry as a measure of proliferation, and shown on histograms. Numbers represent the average percentage (±SEM) of gated viable (7-AAD$^-$) TCRβ$^+$CD4$^+$ live cells that were triggered to divide from the precursor population. **(C)** The proliferation index (average number of divisions undergone by only those cells triggered to divide) of the gated 7-AAD$^-$CD4 TCRβ$^+$ cells from (B) are shown graphically. Results are replicates from four independent experiments. **(D)** Proliferation of LN-derived WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells was assessed by FACS after stimulation with a range of CD3 antibody concentrations. Bar charts represent percentages (±SEM) of gated 7-AAD$^-$TCRβ$^+$CD4$^+$ live cells that had been triggered to divide after 72 h. Data shown are from triplicate wells and similar to four independent experiments. **, p≤ 0.0.05; Student's t test. **(E)** CD4$^+$CD25$^-$ T cells purified from spleen of WT and *Nfkb1*$^{SS/AA}$ mice, stimulated under conditions indicated, were analysed for CFSE content by flow cytometry. Histograms show gated 7-AAD$^-$TCRβ$^+$CD4$^+$ live cells. Numbers indicate average percentage of cells induced to divide (±SEM). Data are representative of triplicates and four experiments. **(F)** CD4$^+$CD25$^-$ T cells isolated from LN of WT and *Nfkb1*$^{SS/AA}$ mice were stimulated with PdBU (10 ng/ml) and ionomycin (1 μg/ml) for 72 h. Histograms represent CFSE content in 7-AAD$^-$TCRβ$^+$CD4$^+$ gated live cells, with numbers indicating average percentage of starting cells divided (±SEM). Results represent triplicate wells and four independent experiments. **(G)** Sorted naïve CD4$^+$ T cells from WT and *Nfkb1*$^{SS/AA}$ LN were CFSE-labelled and cultured with syngeneic BMDCs ± anti-CD3 (1 μg/ml). Cell proliferation was determined of 7-AAD$^-$TCRβ$^+$CD4$^+$ gated live cells, and shown as CFSE profiles. Numbers indicate percentage of cells triggered to divide (±SEM). Data are representative of three replicate (n=3) experiments.

4.2 Allogeneic proliferation of $CD4^+CD25^-$ T cells is impaired by p105 mutation

4.2.1 *In vitro* allogeneic response is defective in *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells

The previous experiments established a defect in the proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells to CD3 monoclonal antibody, which is a non-physiological stimulus. To examine the response of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells to a physiological stimulus, the MHC-dependent allogeneic response was analysed. BMDC generated from Balb/c mice (H-2^d) were cultured *in vitro* with purified 129S8 (H-2^b) $CD4^+CD25^-$ T cells from WT or *Nfkb1*$^{SS/AA}$ mice. CFSE dilution revealed that the percentage of cells dividing in response to allogeneic Balb/c BMDC stimulation was reduced 3-fold with *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells compared to WT (Figure 4.2A). Furthermore, the mis-matched MHC of Balb/c BMDCs was responsible for the induction of $CD4^+CD25^-$ T cell proliferation, since only a small fraction of WT and *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells divided when cultured with syngeneic 129S8 BMDCs.

4.2.2 *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cell proliferation is impaired *in vivo* following allogeneic stimulation

The *in vivo* T cell response to allogeneic MHC was also assessed. $CD4^+CD25^-$ T cells from *Nfkb1*$^{SS/AA}$ or WT C57BL/6x129S8 mice (N5) were purified and CFSE labelled. These cells were then transferred into H-2^a *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*(C5*$^{-/-}$*)* mice which lack lymphocytes and natural killer cells, and therefore cannot reject

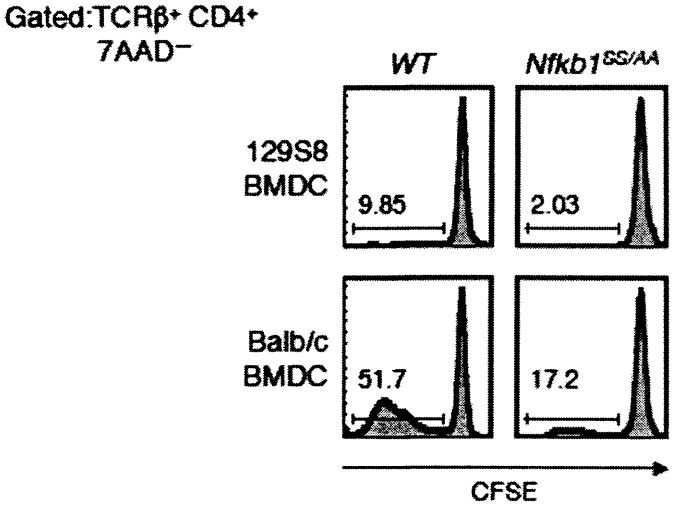
transferred cells (Kassiotis et al., 2003). To verify similar WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cell transfer and migration to the spleen, host mice were analysed one day after transfer. Absolute cell numbers from spleen were counted and the percentage of CD4$^+$ cells analysed by flow cytometry. Equivalent numbers of WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells were found in the spleens of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*(C5*$^{-/-}$*)* mice as expected (Figure 4.2B). Analysis after four and seven days revealed increases in both WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ splenic cell numbers, indicating that transferred cells had proliferated *in vivo*. However, the number of *Nfkb1*$^{SS/AA}$ CD4$^+$CFSE$^+$ T cells in host spleens was significantly lower ($p \leq 0.05$) than in host mice injected with WT CD4$^+$CD25$^-$ T cells. Consistent with this conclusion, analysis of CFSE content revealed a higher percentage of undivided *Nfkb1*$^{SS/AA}$ CD4$^+$ cells compared to WT CD4$^+$ cells (Figure 4.2C). These data suggest that p105 mutation impaired TCR-induced allogeneic proliferation of CD4$^+$CD25$^-$ T cells both *in vitro* and *in vivo*.

4.3 *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells enter the cell cycle but are unable to progress from G1 to S phase after TCR stimulation

4.3.1 TCR-induced growth of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells is normal

Cell growth and division are tightly coupled events, with DNA synthesis and cell division dependent on growth (Conlon and Raff, 1999). Entry of G_0 T cells into the G_1 phase of the cell cycle is accompanied by increase in cell size (blastogenesis). Therefore, growth of cells was measured to investigate whether the block in proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells was due to the inability of cells to move from G_0 into G_1. To this end, forward scatter profiles of CD4$^+$CD25$^-$ T cells

A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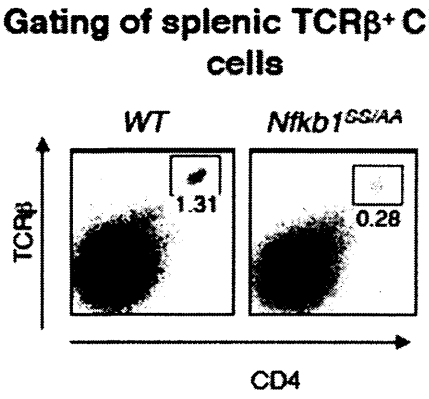
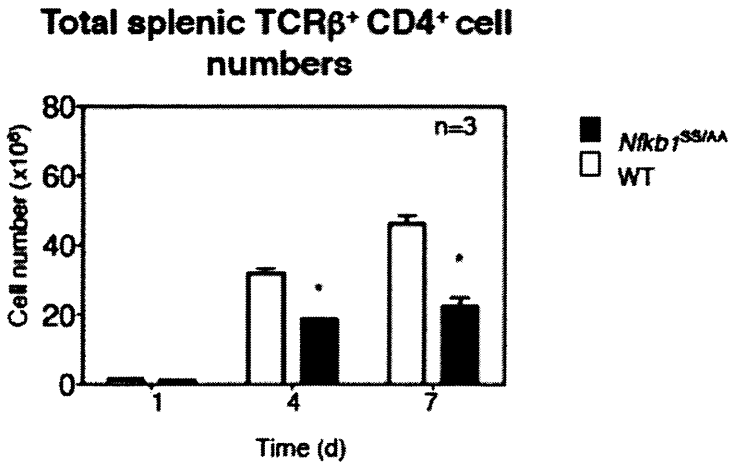
7AAD−
WT
Nfkb1SS/AA
129S8
BMDC
9.85
2.03
Balb/c
BMDC
51.7
17.2
CFSE

B


Gating of splenic TCRβ+ CD4+ cells
WT
Nfkb1SS/AA
TCRβ
1.31
0.28
CD4


Total splenic TCRβ+ CD4+ cell numbers
Cell number (x10⁶)
80
60
40
20
0
n=3
1
4
7
Time (d)
Nfkb1SS/AA
WT

C

Gated:TCRβ$^+$ CD4$^+$

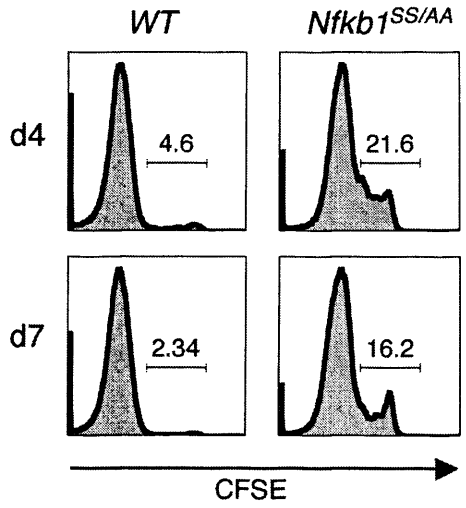


Figure 4.2 Allogeneic proliferation of CD4$^+$CD25$^-$ T cells is impaired by *Nfkb1*$^{SS/AA}$ mutation
(A) CD4$^+$CD25$^-$ T cells purified from LN of WT and *Nfkb1*$^{SS/AA}$ mice (H2^b), were labelled with CFSE and cultured *in vitro* with syngeneic or allogeneic (H2^d) BMDCs, generated from 129S8 and Balb/C mice, respectively. Proliferation of gated 7-AAD$^-$TCRβ$^+$CD4$^+$ live cells was determined by analysis of CFSE dilution after 120h, as shown on CFSE profiles. Numbers indicate percentage of cells that had undergone cell division. Data are representative of three replicate experiments. **(B)** CFSE labelled CD4$^+$CD25$^-$ T cells isolated from spleens of WT and *Nfkb1*$^{SS/AA}$ mice (H2^b) were separately injected into H-2^a *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (*C5*$^{-/-}$) mice. On d1, d4 and d7 post-transfer, numbers of TCRβ $^+$CD4$^+$ splenic cells were determined by flow cytometry and counting of total splenic cellularity. Bar charts represent average number of TCRβ $^+$CD4$^+$ T cells of the indicated genotypes (± SEM). *, $p \leq 0.05$; Student's t test. Flow cytometry was also used to analyse CFSE content in live gated TCRβ $^+$CD4$^+$ splenic T cells, as a measure of proliferation of transferred CD4$^+$ T cells. Numbers indicate the percentage of TCRβ $^+$CD4$^+$ cells positive for CFSE. Data are representative of two experiments.

after 24 h of stimulation with CD3 antibody were analysed. Results show that *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells underwent an increase in size following stimulation, which was similar to WT CD4^{+}CD25^{-} T cells (Figure 4.3A).

An increase in cell size is linked to increased protein synthesis. Cellular levels of particular proteins that function as indicators of cell size must reach a certain threshold in order to trigger entry into S phase. Therefore, protein levels were determined in *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells. Consistent with growth analysis, measurement of protein concentration after 24 h anti-CD3 stimulation revealed no significant difference between *Nfkb1*$^{SS/AA}$ and WT CD4^{+}CD25^{-} T cells (Figure 4.3B). Therefore, anti-CD3 induced growth of CD4^{+}CD25^{-} T cells, and entry into the cell cycle was unaffected by *Nfkb1*$^{SS/AA}$ mutation.

4.3.2 TCR-induced cell cycle S-phase entry is impaired in *Nfkb1*$^{SS/AA}$ CD4^{+} CD25^{-} T cells

The inability of *Nfkb1*$^{SS/AA}$ CD4^{+} CD25^{-} T cells to divide after TCR stimulation could have been due to defective progression into the S-phase cell cycle. To explore this possibility, BrdU labelling was analysed by flow cytometry (Figure 4.3C). CD4^{+} CD25^{-} T cells were stimulated for 24 h with CD3 antibody, the last 16 h of which BrdU was added to cultures (Sato et al., 2005). BrdU can be incorporated into DNA by cells undergoing DNA synthesis, thus those in S-phase of the cell cycle. Measurement of BrdU positive cells therefore allowed analysis of the fraction of cycling cells. In WT cultures, approximately 65% of cells were labelled with BrdU

after 24 h of CD3 antibody stimulation. However, the fraction of BrdU positive cells was approximately three-fold lower in *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cell cultures than WT cultures. Furthermore, co-stimulation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells with anti-CD28 partially rescued this block in S-phase entry. Thus, *Nfkb1*$^{SS/AA}$ mutation did not affect the ability of CD4$^+$CD25$^-$ T cells to move from G_0 to G_1, but did affect their ability to progress into S phase of the cell cycle following anti-CD3 stimulation.

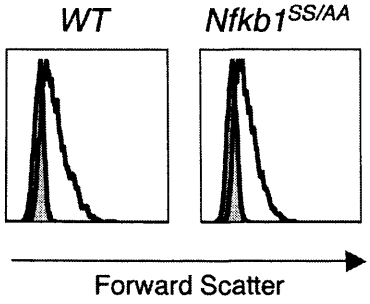
4.4 *Nfkb1*$^{SS/AA}$ mutation does not affect expression of receptors or TCR-induced upregulation of cell surface markers

A possible explanation for the defect in proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells following CD3 stimulation was that TCR expression was impaired by *Nfkb1*$^{SS/AA}$ mutation. Moreover, NF-κB binding sites have been identified in the promoter region of TCRβ (Jamieson et al., 1989). Therefore, surface expression of TCRβ, as well as CD3ε and CD28, was analysed. Expression overlays of these molecules showed similar levels were present on purified WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells (Figure 4.4A). Furthermore, expression of IL-7R (CD127) was also assessed, since IL-7 is important for T cell survival and homeostatic proliferation. This analysis also revealed that levels of IL-7R were similar between WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells.

Activation of CD4$^+$ T cells leads to upregulation of various surface markers. As *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells showed a defect in TCR-induced proliferation, these

A

Gated:TCRβ^{+} CD4^{+}



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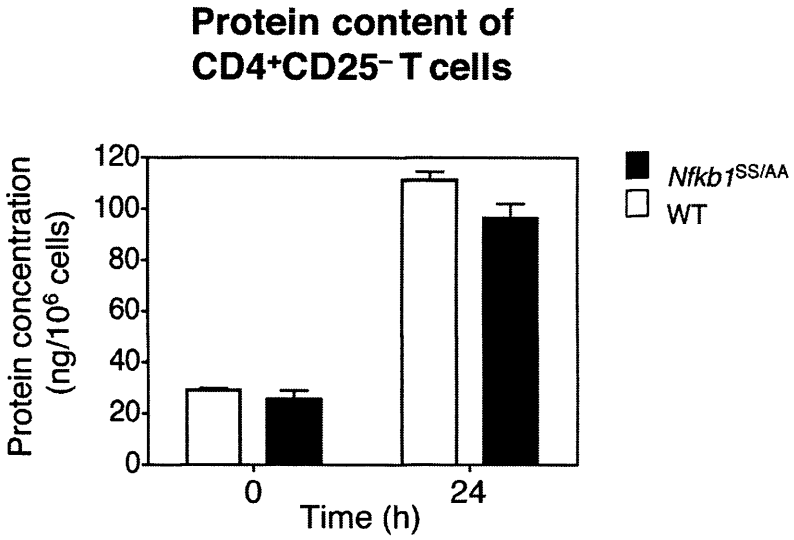


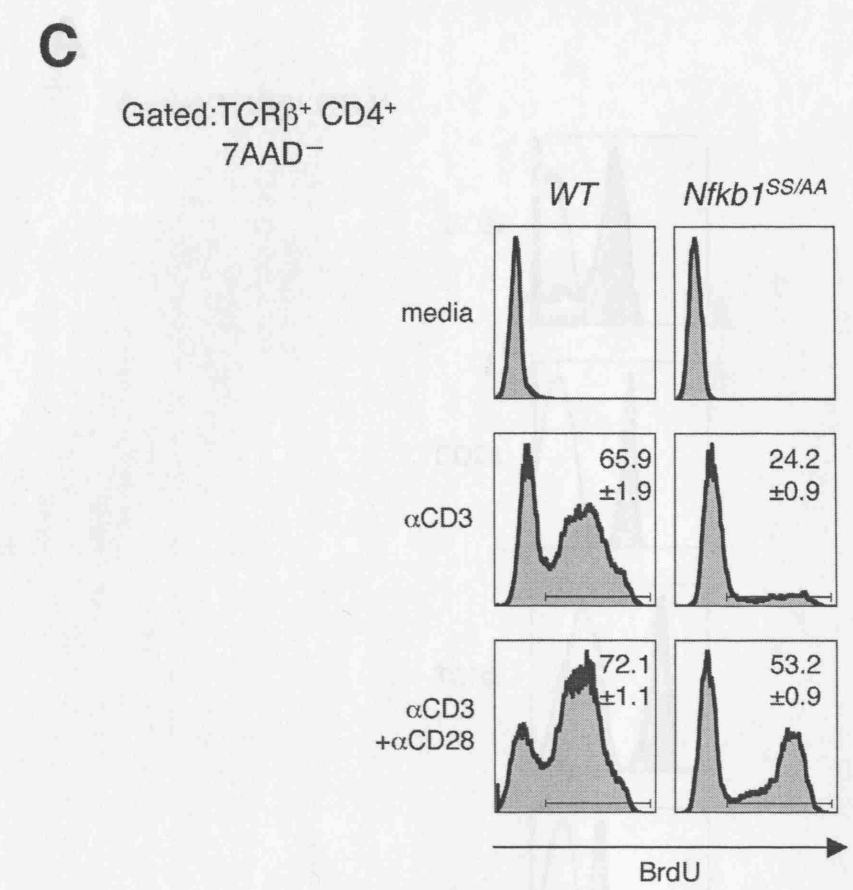
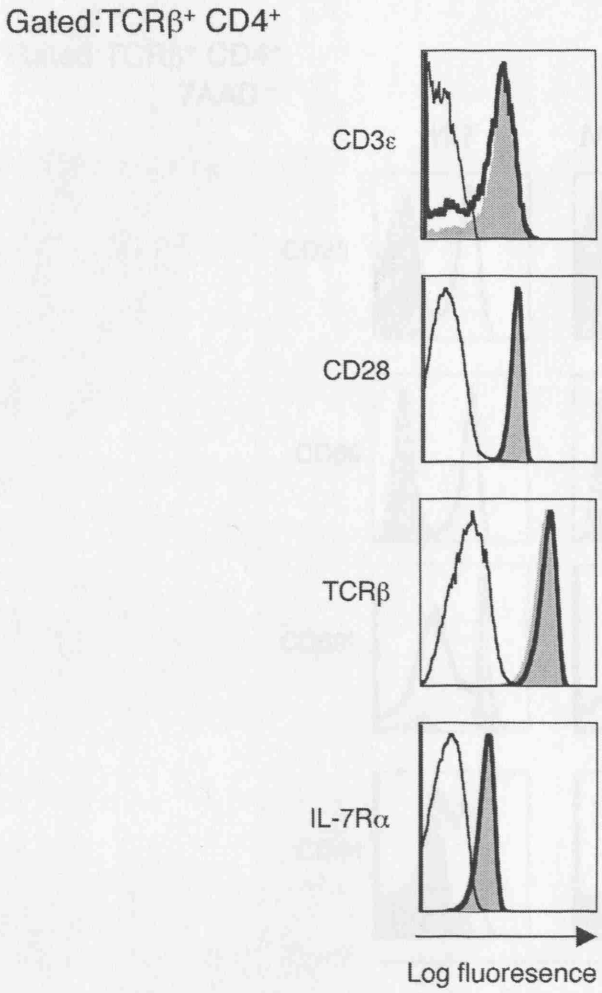


Figure 4.3 G1-S-phase progression is blocked in *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells
(A) WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells were isolated from LN and activated in triplicate wells with plate-bound anti-CD3 (1 μg/ml) for 24 h. Cell size was determined by measuring forward scatter profiles of gated live TCRβ^{+}CD4^{+} cells, as shown. Stimulated cells are represented by thick black lines, and unstimulated cells by shaded histograms. **(B)** Cells were stimulated as in (A), and protein content of lysates determined by Bradford assay. Protein concentrations are shown as bar charts (± SEM). Results are representative of three experiments. **(C)** WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells were isolated from LN and cultured *in vitro* for 24 h with anti-CD3 (1 μg/ml) ± anti-CD28 (1 μg/ml) or medium alone. Cells were pulsed with BrdU for the last 16 h of culture. The percentages of gated live TCRβ $^{+}$CD4^{+} cells labelled with BrdU were determined by flow cytometry and are indicated on histograms. Data are representative of three replicate experiments.

A



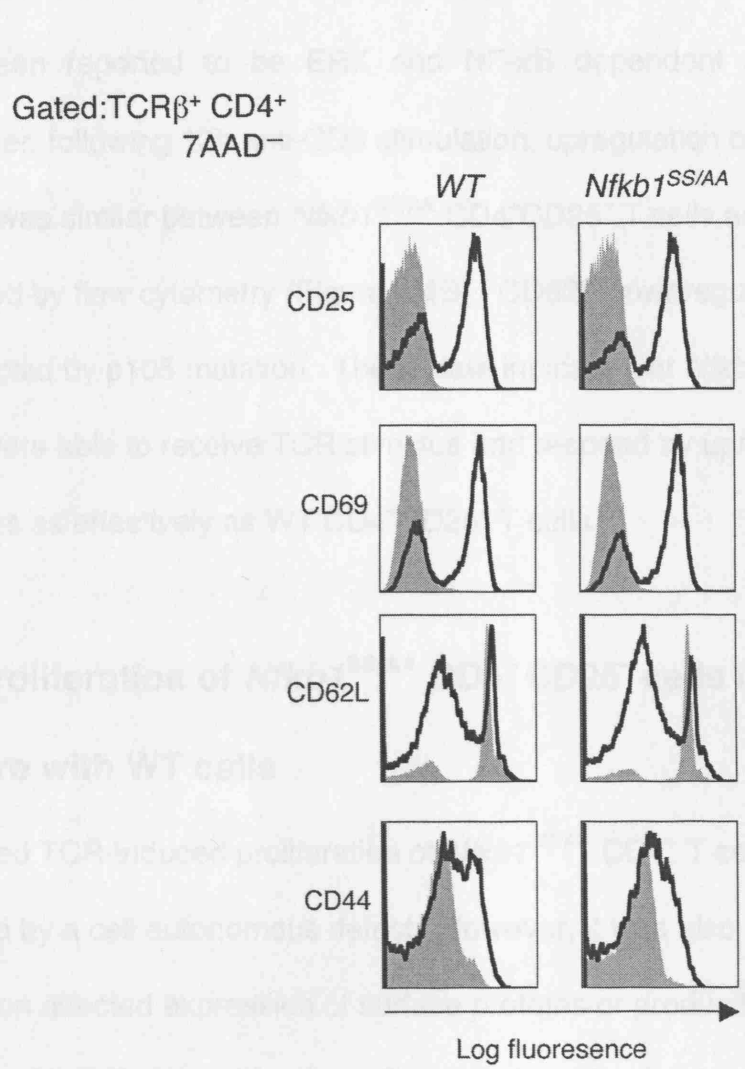


Figure 4.4 Analysis of surface proteins and TCR-induced activation markers on *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells

(A) Surface expression of indicated antigens on CD4$^+$CD25$^-$ T cells purified from LN was analysed by flow cytometry. Histograms show expression overlays of WT (grey filled histograms) and *Nfkb1*$^{SS/AA}$ (thick black lines) from gated TCRβ$^+$CD4$^+$ live cells. Staining for IgG isotype controls (thin black lines) is also shown. Data represent three independent experiments. **(B)** Expression of indicated markers of T cell activation was determined by flow cytometry. CD4$^+$CD25$^-$ T cells purified from WT and *Nfkb1*$^{SS/AA}$ LN were stimulated for 12 h with 1 μg/ml plate bound anti-CD3 (thick black lines), whilst control cells were cultured with medium alone (shaded histograms). Histograms are gated for 7-AAD$^-$TCRβ$^+$CD4$^+$ cells, and represent staining intensity. Data were similar in three separate experiments.

activation events may also have been impaired. Furthermore, expression of CD69 has been reported to be ERK and NF-κB dependent (Villalba et al., 2000). However, following 12h anti-CD3 stimulation, upregulation of CD44, CD25 and CD69 was similar between *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells and WT cells, as followed by flow cytometry (Figure 4.4B). CD62L downregulation was also unaffected by p105 mutation. These data indicate that *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells were able to receive TCR stimulus and respond by upregulating activation markers as effectively as WT CD4$^+$CD25$^-$ T cells.

4.5 Proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ CD25$^-$ cells is enhanced by co-culture with WT cells

Impaired TCR-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells may have been caused by a cell autonomous defect. However, it was also possible that *Nfkb1*$^{SS/AA}$ mutation affected expression of surface proteins or production of soluble factors vital for CD4$^+$ T cell proliferation. This was investigated by addition of CD45.1$^+$ WT C57BL/6 CD4$^+$CD25$^-$ T cells to cultures of CD45.2$^+$ WT and CD45.2$^+$ *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells. Following anti-CD3 stimulation, proliferation of both CD45.1$^+$ and CD4.2$^+$ cells was determined by flow cytometric analysis of CFSE dilution.

The presence of CD45.1$^+$ CD4$^+$ T cells in culture resulted in a small increase in WT CD45.2$^+$ CD4$^+$CD25$^-$ T cell proliferation after anti-CD3 stimulated (Figure 4.5). However, a significant ($p \leq 0.005$) three-fold increase in the fraction of dividing *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells was seen upon CD45.1$^+$ T cell addition. Despite

this increase, CD45.1$^+$ CD4$^+$ T cells could not restore proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells to WT levels. Proliferation of CD45.1$^+$ CD4$^+$CD25$^-$ T cells was slightly reduced in cultures with *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells compared to cultures with WT CD45.2$^+$ CD4$^+$CD25$^-$ T cells, although this was not significant. These results therefore indicate that the impaired proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells after anti-CD3 stimulation resulted, in part, from reduced expression of soluble factors and/or cell surface proteins vital for CD4$^+$ T cell interactions and proliferation. Furthermore, since rescue of proliferation by WT CD4$^+$ T cells was only partial, this experiment suggests that a cell autonomous defect also contributed.

4.6 *Nfkb1*$^{SS/AA}$ mutation impairs IL-2 production by CD4$^+$CD25$^-$ T cells

4.6.1 Upregulation of IL-2 mRNA levels is impaired in *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells

Interleukin-2 (IL-2) is vital for the proliferation of naïve T cells *in vitro* and is produced mainly by T cells and acts both in a paracrine and autocrine manner. Therefore, production of this cytokine by *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells was examined. Upregulation of *Il-2* mRNA in splenic CD4$^+$CD25$^-$ T cells was assessed by quantitative real-time PCR after 4 h stimulation with CD3 antibody. The expression of *Il-2* mRNA was normalised relative to the housekeeping gene *Hprt*. *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells expressed three-fold lower levels of *Il-2* mRNA compared to

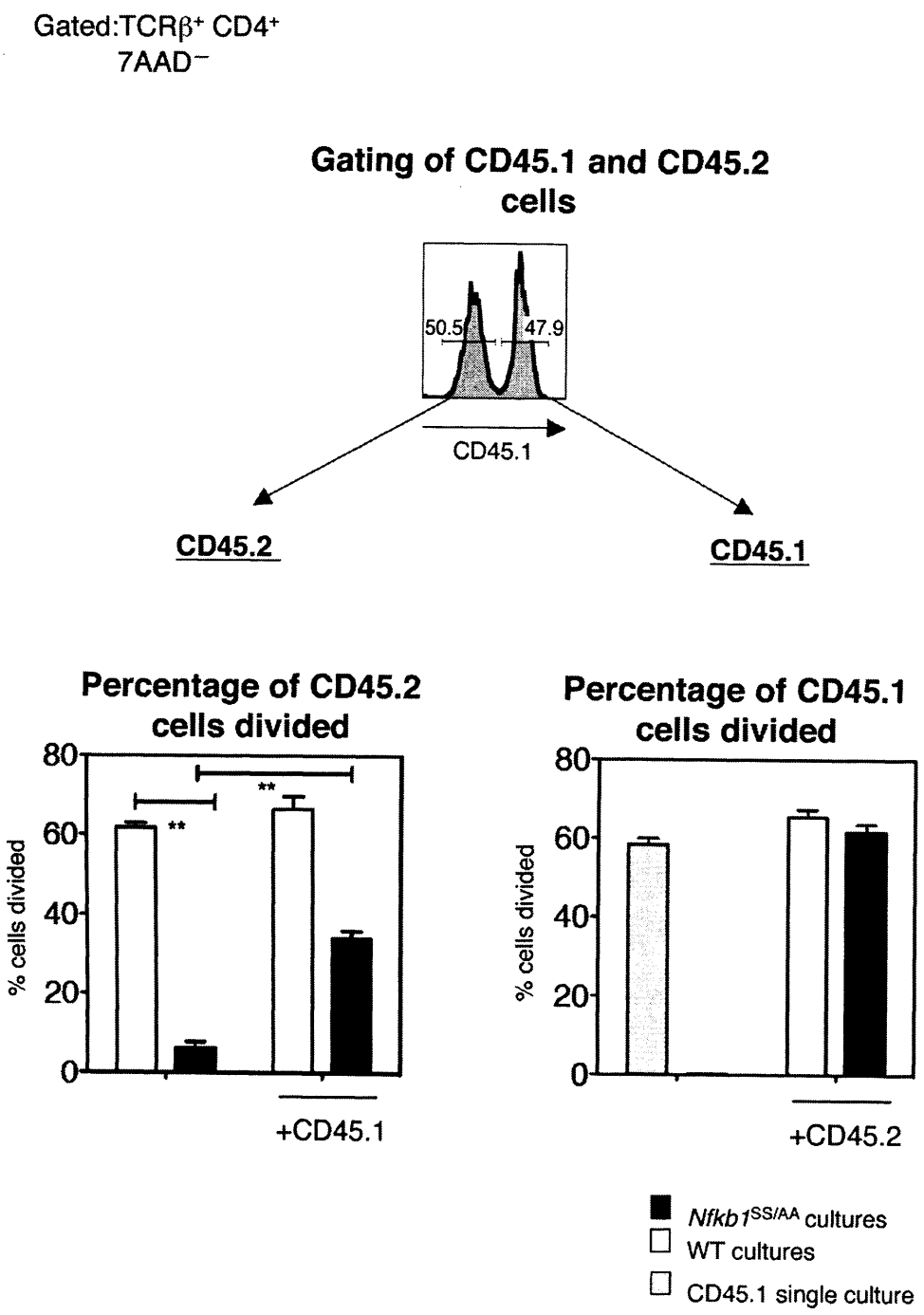


Figure 4.5 Co-culture with WT CD4$^+$ CD25$^-$ cells increases proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ CD25$^-$ cells

CD4$^+$CD25$^-$ T cells isolated from LN of CD45.2$^+$ WT and *Nfkb1*$^{SS/AA}$ mice (129S8, C57BL/6 mixed background) were labelled with CFSE and cultured in the presence or absence of CFSE-labelled WT CD45.1$^+$ CD4$^+$CD25$^-$ T cells, as indicated. Cells were stimulated with plate bound anti-CD3 (1 μg/ml) for 72 h, and CFSE dilution determined by flow cytometry. Bar graphs show percentages of divided cells (average± SEM) from cultures containing CD45.1$^+$ and WT (white) or *Nfkb1*$^{SS/AA}$ (black) CD45.2$^+$ cells, or CD45.1$^+$ cells cultured alone (grey). Analysis was performed on gated 7-AAD$^-$

TCRβ$^+$CD4$^+$ live cells that were either CD45.1$^+$ (right graph) or CD45.2$^+$ (left graph). **, $p \leq 0.005$; Student's t test. Results are representative of triplicates of three separate experiments.

WT cells after CD3 stimulation (Figure 4.6A). Costimulation with anti-CD28 greatly enhanced expression of *Il-2* mRNA in WT CD4$^+$CD25$^-$ T cells, consistent with its established role in IL-2 upregulation (Fraser et al., 1991, Lindstein et al., 1989). Addition of CD28 antibody also markedly enhanced *Il-2* mRNA in *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells, although this still resulted in levels of *Il-2* mRNA that were three-fold lower than those in WT cells.

4.6.2 IL-2 protein production is reduced by *Nfkb1*$^{SS/AA}$ mutation in CD4$^+$CD25$^-$ T cells

The level of IL-2 protein produced by WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cell cultures, was determined by enzyme-linked immunosorbent assays (ELISAs). After 24 h, TCR-induced IL-2 production was three times lower in *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cell supernatants than WT (Figure 4.6B). Addition of CD28 antibody increased levels of IL-2 protein produced by both WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells compared to CD3 antibody activation alone. However, co-stimulation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells still resulted in significantly lower levels of IL-2 relative to WT CD4$^+$CD25$^-$ T cells ($p \leq 0.005$). These results were consistent with *Il-2* mRNA data, although the rescue of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cell IL-2 protein levels provided by CD28 costimulation was greater than that seen with *Il-2* mRNA levels.

These results together demonstrate that p105 mutation can impair the induction of IL-2 in CD4$^+$ T cells after CD3 antibody stimulation.

4.6.3 Impaired IL-2 production is caused by an intrinsic defect in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells

To determine whether the defect in *Nfkb1*$^{SS/AA}$ IL-2 protein levels was cell-intrinsic, naïve CD4$^+$CD44^{lo}CD25$^-$T cells were highly purified by cell sorting. Naïve T cells were then stimulated with CD3 antibody presented on syngeneic BMDCs for 24h. IL-2 production, as detected b ELISA, was significantly reduced from *Nfkb1*$^{SS/AA}$ naïve CD4$^+$ T cells compared to WT CD4$^+$ T cells ($p<0.005$) (Figure 4.6C). A nine-ten-fold reduction in secreted protein was found in *Nfkb1*$^{SS/AA}$ naïve CD4$^+$ T cells compared to WT, which was more pronounced than the defect observed in CD3-stimulated LN *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells purified by negative selection.

4.6.4 Addition of exogenous IL-2 partially restores anti-CD3-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells

Previous IL-2 data raised the possibility that a defect in IL-2 production could be the cause of impaired TCR-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells. Therefore, the effect of adding exogenous recombinant IL-2 to *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cell proliferation was tested. Addition of IL-2 increased the percentage of cells divided in both WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells stimulated with anti-CD3 (Figure 4.6D). This increase was more pronounced for *Nfkb1*$^{SS/AA}$ cells, where the percentage of cells divided was two-fold higher in the presence of IL-2 than compared to *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells stimulated with anti-CD3 alone. However, the rescue with recombinant IL-2 was only partial as

proliferation of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells was still lower than WT CD4^{+}CD25^{-} T cells stimulated with CD3 antibody in the presence of IL-2.

Since proliferation of highly purified naïve *Nfkb1*$^{SS/AA}$ CD4^{+} T cells was severely impaired after stimulation with anti-CD3 presented on BMDCs, the effect of adding recombinant IL-2 to this system was also assessed. Exogenous recombinant IL-2 dramatically increased the fraction of *Nfkb1*$^{SS/AA}$ naïve CD4^{+} T cells that had divided, from 10 % to 62 %, relative to cultures stimulated with anti-CD3 and BMDC alone (Figure 4.6E). This IL-2-induced increase in proliferation of *Nfkb1*$^{SS/AA}$ naïve CD4^{+} T cells was largely comparable to WT CD4^{+} naïve cell proliferation after stimulation with CD3 antibody with or without exogenous IL-2.

4.6.5 TCR-induced cell cycle progression of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells is increased by addition of IL-2

Nfkb1$^{SS/AA}$ mutation impaired the fraction of CD4^{+}CD25^{-} T cells entering S phase of the cell cycle, as shown earlier (Figure 4.1C). Since IL-2 could increase proliferation of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} and sorted T cells, the effect of IL-2 on cell cycle progression was examined. Addition of IL-2 to cultures led to a small increase in BrdU incorporation of WT CD4^{+}CD25^{-} T cells following anti-CD3 stimulation, from 66 % to 69 % of cells (Figure 4.6F). In contrast, the percentage of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells progressing into the cell cycle showed a notable increase upon IL-2 addition, from 24 % to 34 %. The enhanced incorporation of BrdU elicited by IL-2 was still below WT levels, consistent with the effect of IL-2

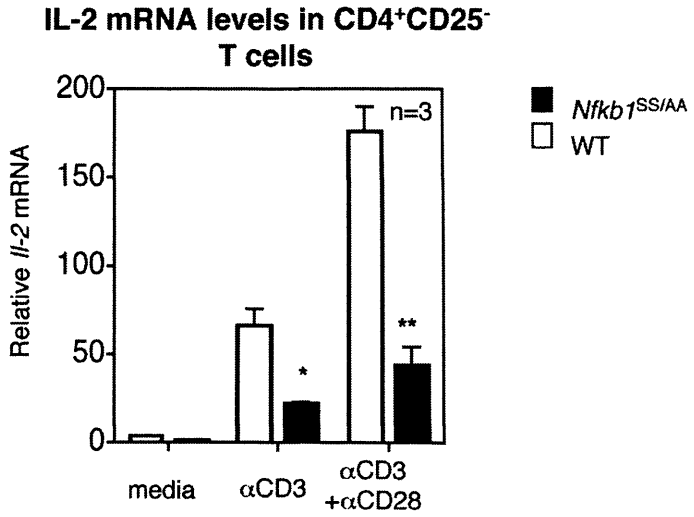
addition on cell division. Therefore, IL-2 could only partially rescue the impaired S-phase entry of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells.

4.6.6 Sustained expression of CD25 is impaired by *Nfkb1*$^{SS/AA}$ mutation in CD4^{+} T cells

Upregulation of CD25 was normal following 12h anti-CD3 stimulation on *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells (Figure 4.4B). However, NF-κB binding sites have been suggested in the promoter of CD122 (IL-2Rβ) (Khoshnan et al., 1999). This prompted analysis of the components of the IL-2R; CD25, CD122 and CD132 (IL-2Rδ). After 12 h of TCR-stimulation, induction of all chains of the IL-2R was comparable between WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells. (Figure 4.6G).

IL-2 itself upregulates expression of CD25 on T cells (Hatakeyama et al., 1989, Plaetinck et al., 1990). Therefore, surface expression of CD25 was examined after longer time in culture with anti-CD3 stimulation. Strikingly, CD25 expression levels were consistently lower on *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells relative to WT cells after 24 h (Figure 14h). These results were consistent with the reduced IL-2 production of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells compared to WT cells. However, levels of surface CD25 remained impaired on *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells in the presence of exogenous IL-2. Data presented here therefore suggest that the maintenance of CD25 expression is impaired by *Nfkb1*$^{SS/AA}$ mutation in a mechanism independent of its affect on IL-2 production.

A


IL-2 mRNA levels in CD4+CD25- T cells
Relative Il-2 mRNA
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150
100
50
0
n=3
*
**
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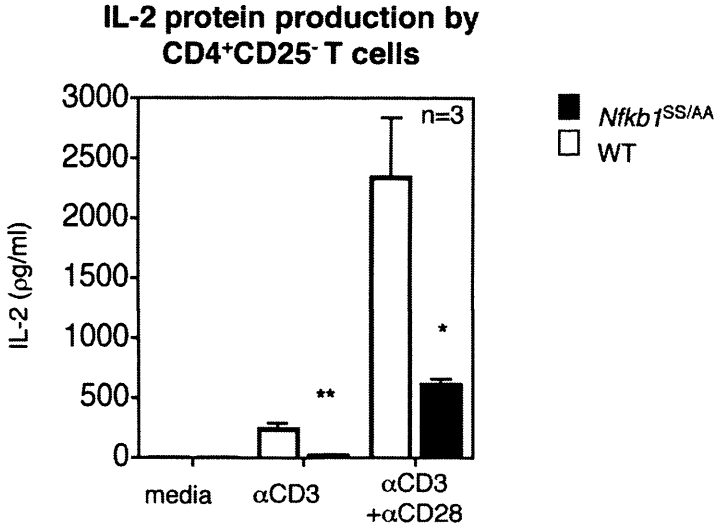
αCD3 +αCD28
Nfkb1SS/AA
WT

B


IL-2 protein production by CD4+CD25- T cells
IL-2 (pg/ml)
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2500
2000
1500
1000
500
0
n=3
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*
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αCD3 +αCD28
Nfkb1SS/AA
WT

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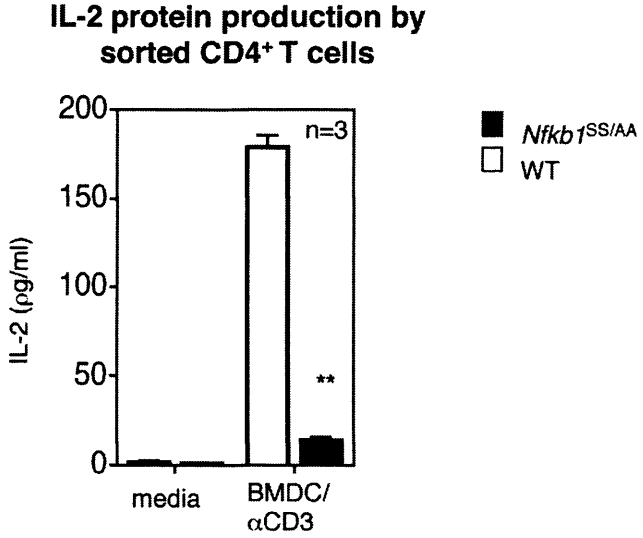
IL-2 protein production by sorted CD4+ T cells
IL-2 (pg/ml)
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WT

D

Gated:TCRβ+ CD4+
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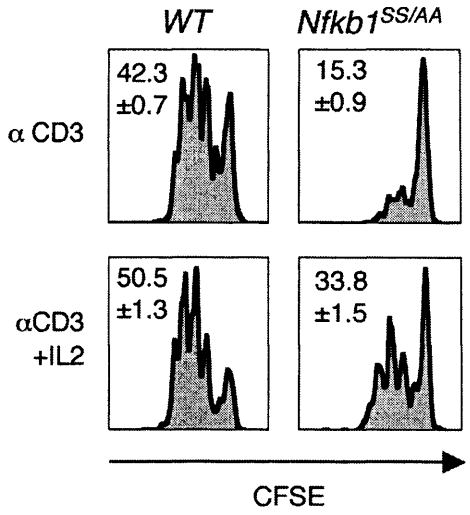

E

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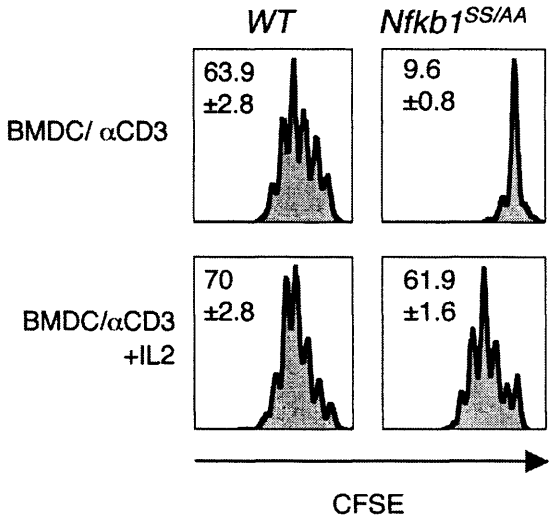
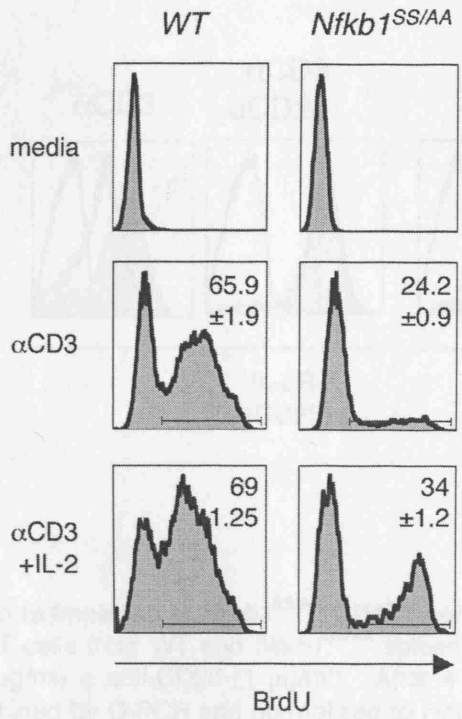


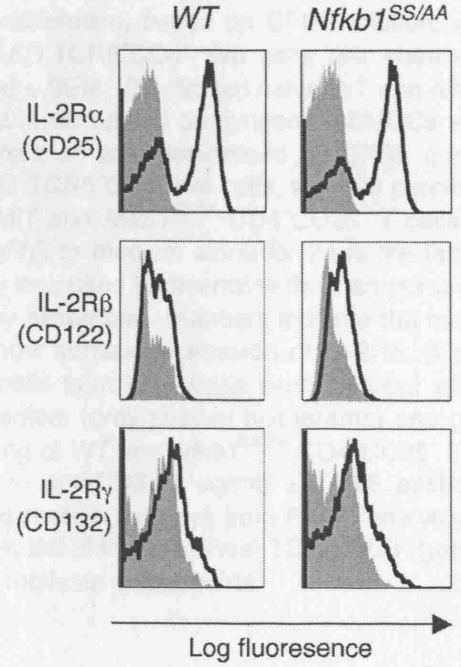
F

Gated:TCRβ+ CD4+
7AAD−



G

Gated:TCRβ+ CD4+
7AAD−



H

Gated:TCRβ$^+$ CD4$^+$

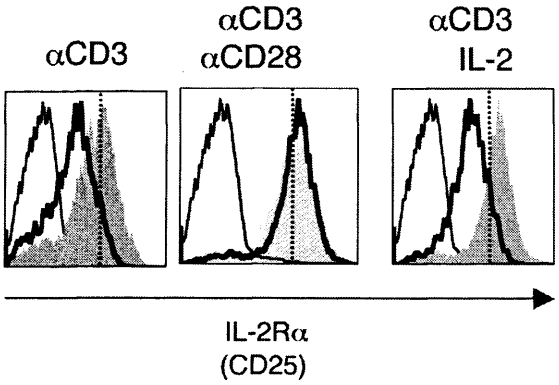


Figure 4.6 IL-2 production is impaired in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells
(A) Purified CD4$^+$CD25$^-$ T cells from WT and *Nfkb1*$^{SS/AA}$ spleens were cultured in triplicates with plate bound anti-CD3 (5 μg/ml) ± anti-CD28 (1 μg/ml). After 4 h, total mRNA was isolated and levels of *Il-2* mRNA determined by Q-PCR and normalized to *Hprt1* mRNA (average± SEM. **, p ≤ 0.0.05; *,p ≤ 0.05; Student's t test). **(B)** CD4$^+$CD25$^-$ T cells isolated from WT and *Nfkb1*$^{SS/AA}$ LN were stimulated with plate bound anti-CD3 (1 μg/ml) ± anti-CD28 (1 μg/ml), or **(C)** sorted WT and *Nfkb1*$^{SS/AA}$ naïve CD4$^+$ T cells, were cultured with syngeneic BMDCs ± anti-CD3 (1 μg/ml). After 24 h, IL-2 present in triplicate culture supernatants was determined by ELISA, as shown by bar charts (± SEM). **, p ≤ 0.0.05; *,p ≤ 0.05; Student's t test. **(D)** WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells purified from LN were labelled with CFSE and cultured for 72 h with plate bound anti-CD3 (1 μg/ml) ± IL-2 (20 ng/ml). Cell proliferation, based on CFSE dilution, was assessed by flow cytometry. Histograms gated on 7-AAD$^-$TCRβ$^+$CD4$^+$ live cells are shown, with numbers indicating mean percentages of cells divided ± SEM. **(E)** Sorted naive WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$CD44^{lo} T cells were labelled with CFSE and stimulated on syngeneic BMDCs with anti-CD3 (1 μg/ml) ± IL-2 (20 ng/ml). CD4$^+$ T cell proliferation was determined by CFSE content analysis after 72 h. CFSE histograms gated on 7-AAD$^-$TCRβ$^+$CD4$^+$ live cells, with the percentages of cells triggered to divide, are shown. **(F)** Purified WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells from LN were cultured with anti-CD3 (1μg/ml) ± IL-2 (20ng/ml) or medium alone for 24 h, the last 16 h of which cells were pulsed with BrdU. Flow cytometry was used to determine the incorporation of BrdU into gated TCRβ$^+$CD4$^+$ live cells, and presented by histogram. Numbers indicate the mean percentages (±SEM) of BrdU$^+$ cells. **(G)** Histograms show surface expression of IL-2Rα, β and γ chains on purified WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells from LN. Cells were cultured with 1 μg/ml anti-CD3 (thick black lines) or kept in control medium (grey shaded histograms) and gated on TCRβ$^+$CD4$^+$ live cells by flow cytometry. **(H)** Staining of WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells isolated from LN, after 24 h stimulation with plate-bound anti-CD3 (1 μg/ml) ± CD28 antibody (1 μg/ml) ± IL-2 (20 ng/ml). Histograms show CD25 expression overlays from FACS analysis of WT (grey shaded), *Nfkb1*$^{SS/AA}$ (thick black lines) and untreated (thin black lines) TCRβ$^+$CD4$^+$ gated live cells. All data in this figure are representative of three replicate experiments.

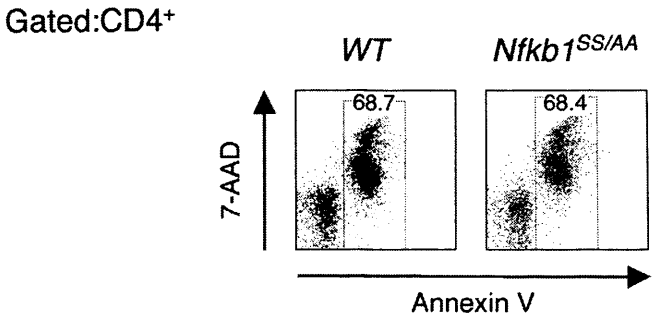
4.7 *Nfkb1*$^{SS/AA}$ mutation does not affect CD4$^+$CD25$^-$ T cell survival

NF-κB regulates the expression of anti-apoptotic genes such as Bcl-2 and Bcl-xL (Khoshnan et al., 2000, Chen et al., 2000) and has been shown to be important for survival of multiple cell types, including CD4$^+$ T cells (Schmidt-Supprian et al., 2003, Zheng et al., 2003, Grumont et al., 2004). Although a defect in TCR-induced proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells has been established, CFSE proliferation analyses were performed on gated live cells, and so gave no information about the effect of *Nfkb1*$^{SS/AA}$ mutation on cell survival. To investigate this, early and late phases of apoptosis were assayed, using annexin V and 7-AAD staining, respectively. The degree of apoptosis, taken as 7-AAD$^+$AnnexinV$^+$ cells, in depleted WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells was found to be similar after culture in media for 0, 24 or 48 h (Figure 4.7A). Furthermore, stimulation with CD3 antibody resulted in an increase in cell death compared to resting cells at equivalent time-points, but was comparable between WT and *Nfkb1*$^{SS/AA}$ CD4$^+$CD25$^-$ T cells.

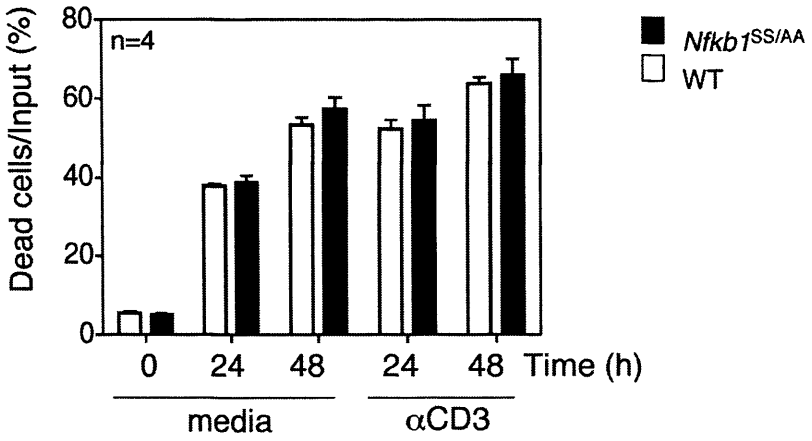
The role of p105 proteolysis in CD4$^+$ T cell survival *in vivo* was also investigated. Purified CFSE-labelled splenic CD4$^+$ T cells from mixed background WT or *Nfkb1*$^{SS/AA}$ C57BL/6x129S8 (N7) were transferred into replete syngeneic CD45.1 C57BL/6 mice. The percentage of transferred cells was followed weekly by analysis of CFSE$^+$CD4$^+$ cells in the total CD4$^+$ T cell population of the blood. The rate of decline of transferred *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells, normalised to levels at d1, appeared similar to WT CD4$^+$ T cells (Figure 4.7B). After 42 d, the percentage of

A

Gating of dead CD4+ cells



Percentage of dead cells in CD4+CD25- culturescells



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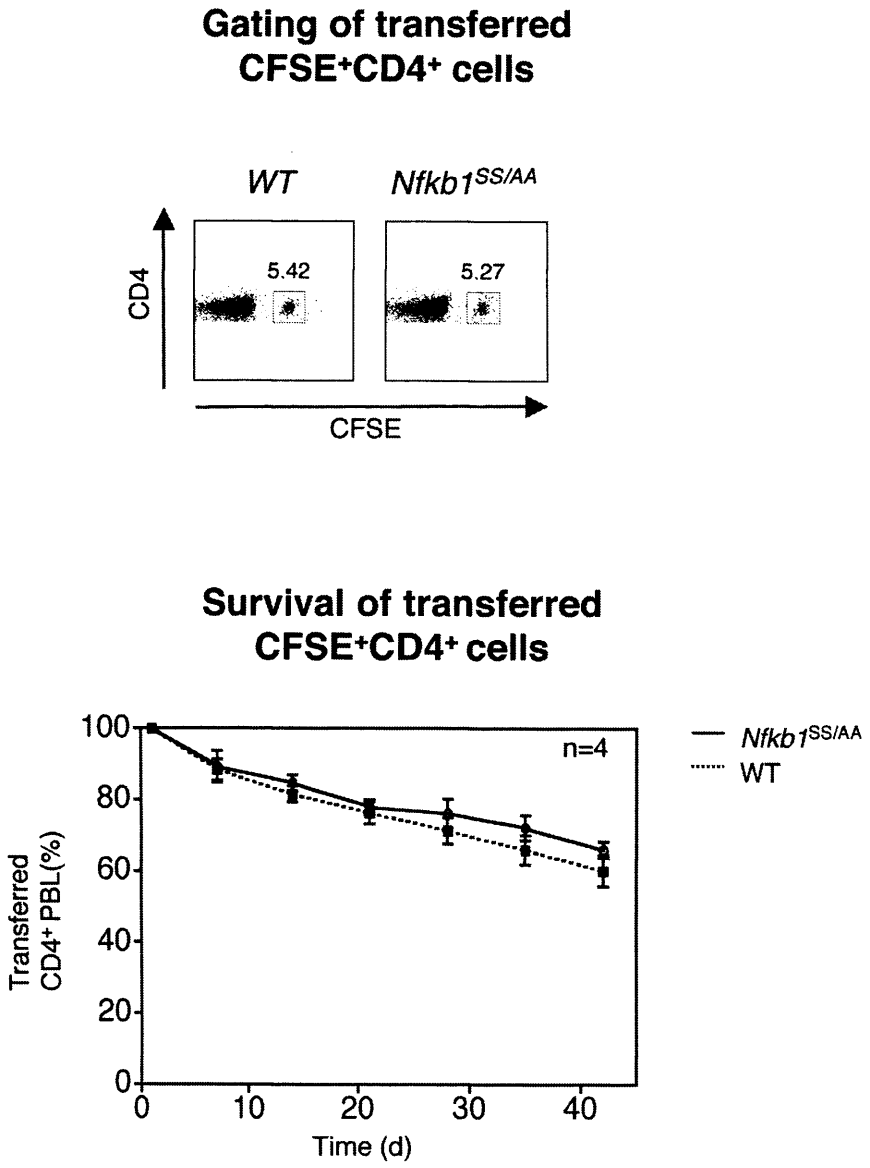


Figure 4.7 Survival of $CD4^+CD25^-$ T cells is unaffected by p105 mutation
(A) $CD4^+CD25^-$ T cells isolated from LN of WT and *Nfkb1*$^{SS/AA}$ mice were stimulated in the absence or the presence of plate bound anti-CD3 (1 μg/ml) for indicated times, and cell death analysed by flow cytometry. $CD4^+$ T cells were electronically gated, and dead cells identified by Annexin V^+7-AAD^+ staining, as shown. Cells were numerated using fluorescent beads. Bar charts represent the fraction of dead cells as a function of culture input (± SEM). Similar results were obtained in three independent experiments. **(B)** Purified $CD4^+CD25^-$ T cells from spleens of WT or *Nfkb1*$^{SS/AA}$ mice (129S8, C57BL/6 mixed background) were labelled with CFSE and injected into replete WT C57BL/6 mice. Blood samples were taken at the indicated times, and fractions of transferred cells determined by flow cytometry. Graphs represent mean values (± SEM) of the percentage of $CD4^+$ cells that were $CFSE^+$, normalized to 100 % at day 1, and are representative of three separate experiments.

CFSE-labelled WT and of *Nfkb1*$^{SS/AA}$ CD4^{+} T cells had decreased to around 60 % of starting levels. No proliferation of transferred cells was evident.

These *in vitro* and *in vivo* data suggest that the survival of CD4^{+} T cells were not affected by *Nfkb1*$^{SS/AA}$ mutation.

4.8 Thymocyte development and mature T cell proliferation are normal in *Map-3k8*$^{-/-}$ mice

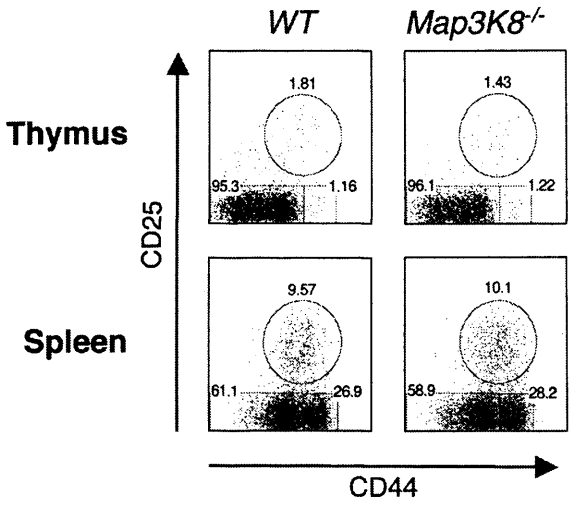
Cellular p105 is not only associated with NF-κB dimers, but also the MAP-3K TPL-2 (Belich et al., 1999). IKK-induced proteolysis of p105 has been shown to regulate the activity of TPL-2 in macrophages (Lang et al., 2004, Belich et al., 1999). Furthermore, TPL-2 MEK kinase activity is blocked in macrophages expressing p105$^{SS/AA}$ through retroviral transduction (Beinke S, 2003). Therefore, to examine whether the phenotype of *Nfkb1*$^{SS/AA}$ T cells could be due to the block in TPL-2 release and its MEK kinase activity, T cells from TPL2-deficient *Map-3k8*$^{-/-}$ mice were examined.

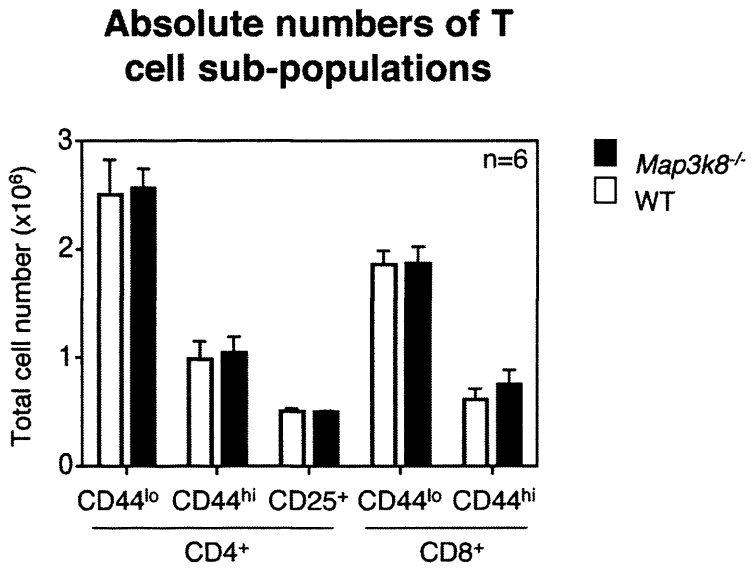
Development of T cell sub-populations in *Map-3k8*$^{-/-}$ mice was followed by flow cytometry. Absolute numbers of splenic CD4 and CD8 T_{mem} cells were comparable between *Map-3k8*$^{-/-}$ and WT mice (Figure 4.8A). Furthermore, generation and peripheral numbers of T_{reg} cells were normal in TPL-2-deficient mice. In contrast to *Nfkb1*$^{SS/AA}$ mice, NK1.1^{+}NKT numbers in the thymus, as well as spleen, were not significantly different between *Map-3k8*$^{-/-}$ and WT mice (Figure 4.8B).

Proliferation of magnetically depleted *Map-3k8*$^{-/-}$CD4^{+}CD25^{-} T cells was assessed by analysis of CFSE dilution, in a similar manner to *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells. Anti-CD3 induced proliferation of *Map-3k8*$^{-/-}$ CD4^{+}CD25^{-} T cells *in vitro* was comparable to WT cells after 72 h(Figure 4.8C). Furthermore, IL-2 production by *Map-3k8*$^{-/-}$ CD4^{+}CD25^{-}T cells was unaffected by TPL-2 deficiency (Figure 4.8D). Analysis of *Map-3k8*$^{-/-}$ mice therefore indicates that impaired generation of T cell sub-populations and TCR-dependent CD4^{+} T cell proliferation in *Nfkb1*$^{SS/AA}$ mice were not primarily due to p105$^{SS/AA}$ inhibition of TPL-2 activity.

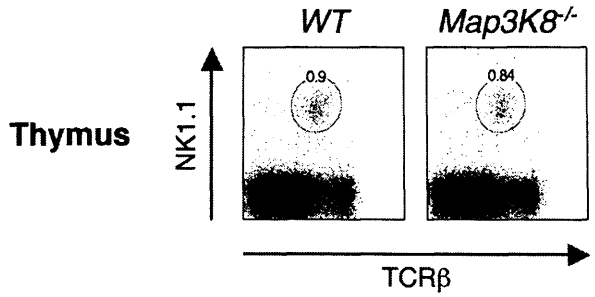
A

Gated:TCRβ+ CD4+

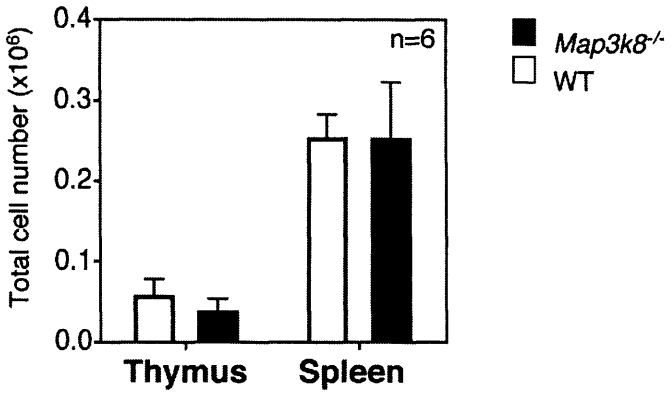




B

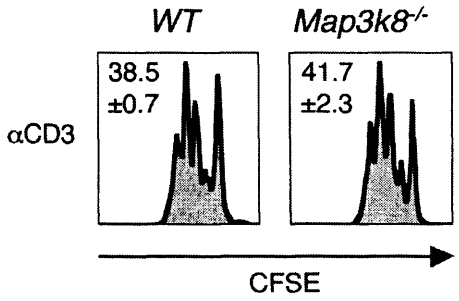


Absolute numbers of TCRβ+ NK1.1+ cells



C

Gated:TCRβ+ CD4+
7AAD−



D

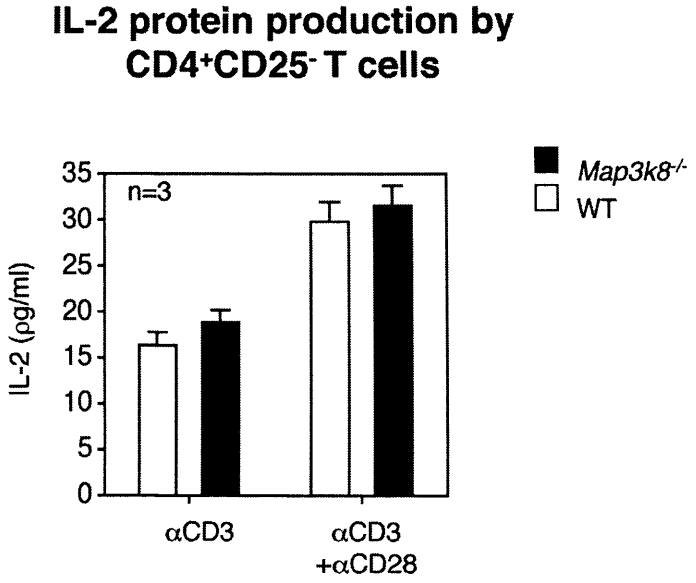


Figure 4.8 TPL-2 deficiency does not affect generation of T cell sub-populations or CD4$^+$ T cell proliferation

(A) Flow cytometric analyses of splenic T cell sub-populations in *Map3k8*$^{-/-}$ mice (n=6) ,compared to WT littermate controls. FACS plots show staining with anti-CD44 and anti-CD25 on gated TCRβ$^+$CD4$^+$ live cells, with numbers indicating percentage of cells in designated gates. Bar graphs show average absolute numbers (± SEM) of the indicated T cell populations. **(B)** Staining of live gated NKT cells, based on NK1.1 and TCRβ expression in thymus and spleen of *Map3k8*$^{-/-}$ and WT littermate controls (n=6). Absolute numbers of TCRβ$^+$NK1.1$^+$ cells are represented graphically (average ± SEM). **(C)** CD4$^+$CD25$^-$ T cells, purified from WT and *Map3k8*$^{-/-}$ LN, were labelled with CFSE and cultured in triplicates with plate bound anti-CD3 (1 μg/ml). Dilution of CFSE was measured by flow cytometry, and percentages of divided live cells, gated on 7AAD-TCRβ$^+$CD4$^+$ staining, are indicated (average ± SEM). **(D)** Purified LN of WT or *Map3k8*$^{-/-}$ CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3 (1 μg/ml) ± anti-CD28 (1 μg/ml) for 24 h, and culture supernatants analysed for IL-2 by ELISA. Average concentrations (± SEM) are shown. Data in **C** and **D** are representative of replicates (n=3) of three separate experiments.

4.9 Chapter 4 summary

The *in vitro* characterisation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells presented in this chapter demonstrate:

- A requirement for IKK-induced p105 proteolysis in CD4$^+$ T cell cycle progression and proliferation after TCR stimulation, but dispensable for CD4$^+$ T cell survival.
- CD28 costimulation can largely overcome the defect in G1-S phase progression and proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells.
- *Nfkb1*$^{SS/AA}$ mutation in CD4$^+$ T cells impairs TCR-induced IL-2 production compared to WT cells. Since addition of exogenous IL-2 can provide some rescue of proliferation, an IL-2 deficiency is implicated as one cause of the reduced proliferative capacity of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells.
- Prolonged surface expression of CD25, but not initial upregulation, is impaired in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells following TCR stimulation. This defect occurs independently of the reduced IL-2 production.
- *Nfkb1*$^{SS/AA}$ mutation decreases proliferation of CD4$^+$ T cells in response to allogeneic stimuli *in vivo.*

5. Discussion

5. Discussion

5.1 The significance of *Nfkb1*$^{SS/AA}$ mutation

5.1.1 Development of T cell sub-populations

This study provides evidence that IKK-induced proteolysis of p105 is required for the generation of T_{reg}, NKT and CD4$^+$ T_{mem}–phenotype cells. Impaired generation and maintenance of these T cell sub-populations has been described in a number of mice deficient of NF-κB activity, supporting a role for p105 as an IκB regulating NF-κB activity. For example, *Nfkb1*$^{-/-}$*crel*$^{-/-}$ mice show a reduction in thymic and peripheral CD4$^+$ T_{mem}–phenotype and T_{reg} cell populations, despite normal generation and survival of CD4$^+$ and CD8$^+$ T cells (Zheng et al., 2003). Moreover, activation of NF-κB specifically *via* the classical pathway appears to be important, as *CD4-Cre/Ikk2*$^{FL/D}$ mice have reduced T_{reg}, CD4$^+$ T_{mem}–phenotype and Vα14i NKT cell numbers, although development of CD4$^+$ and CD8$^+$ SP T cells is slightly impaired in this case (Zheng et al., 2003, Schmidt-Supprian et al., 2004a). Therefore, a T-cell intrinsic requirement for NF-κB activity in the generation of these T cell sub-populations is well established. However, the exact role NF-κB plays is unclear. One suggestion has been that NF-κB is required for optimal signalling downstream of the TCR to trigger proliferation of T cells, since generation of T_{mem}–phenotype cells requires TCR/MHC interactions and expansion of naïve T cells to effector cells (Moulton and Farber, 2006, Harrington et al., 2008), and NKT cells express markers of memory cells, (Schmidt-Supprian et al., 2003, Sivakumar et al., 2003). Furthermore, the generation of NKT and T_{reg} cells

has been proposed to require expression of TCR with increased avidity for spMHC than conventional T cells (Jordan et al., 2001, Apostolou et al., 2002, Kronenberg and Gapin, 2002), raising the possibility that inhibiting NF-κB may reduce the selecting MHC signal, blocking T_{reg} and NKT generation. These ideas will be considered later in this chapter.

5.1.2 *Nfkb1$^{SS/AA}$* CD4$^+$ T cell function *in vitro*

Mice expressing T cells in which total NF–κB activity is blocked or reduced, or which lack particular NF–κB subunits, have revealed a role for NF–κB in CD4$^+$ T cell proliferation, growth and survival (summarised in table 1.1 (Gerondakis et al., 2006)). Furthermore, a requirement for NF–κB in IL-2 transcription has been established (Garrity et al., 1994, Kontgen et al., 1995). Therefore, results presented in this study which demonstrate a requirement for IKK-induced p105 proteolysis in IL-2 production, cell cycle progression and subsequent proliferation of CD4$^+$ T cells are consistent with a role for p105 as an IκB protein, and suggest that TCR-induced proteolysis of p105 can regulate NF–κB activity.

5.2 NF-κB activation by IKK-induced p105 proteolysis

Does Nfkb1$^{SS/AA}$ mutation impair NF-κB activity?

As described above, this study suggests a role for p105 as an IκB in retaining NF-κB subunits. This conclusion was confirmed by parallel EMSA and NF-κB ELISA analysis in the Ley lab, which reveal that p105 mutation markedly reduces total NF-κB activity, blocking release of all major Rel subunits in T cells after anti-CD3/CD28

stimulation (M.Belich, S. Papoutsopoulos; unpublished data). These data are consistent with earlier interaction studies in cell lines, which show that p105 can bind c-Rel, RelA and p50 subunits (Rice et al., 1992, Mercurio et al., 1992, Mercurio et al., 1993, Hatada et al., 1992).

Why is NF-κB activity impaired in Nfkb1$^{SS/AA}$ CD4^{+} T cells?

Although the IκB function of p105 may alone explain the reduction in NF-κB activation in *Nfkb1$^{SS/AA}$* CD4^{+} T cells, analysis of protein levels by immunoblotting revealed that basal levels of p105 were higher in *Nfkb1$^{SS/AA}$* CD4^{+} T cells compared to WT cells (M.Belich, unpublished data). This suggests that p105 in *Nfkb1$^{SS/AA}$* CD4^{+} T cells can bind and inhibit the activity of more NF-κB subunits than p105 in WT cells. The result is a marked reduction in both basal NF-κB activity, and NF-κB activity after TCR-stimulation, in *Nfkb1$^{SS/AA}$* CD4^{+} T since IKK-induced degradation of p105$^{SS/AA}$ is blocked. These findings have significant implications to the role of IKK-induced proteolysis of p105. Firstly, they suggest that levels of p105 are regulated by IKK-induced proteolysis *in vivo*, thus when blocked by *Nfkb1$^{SS/AA}$* mutation, p105 accumulates. Furthermore, these results indicate that a tonic signal can activate IKK-induced p105 proteolysis *in vivo*, since basal levels of p105 are increased in *Nfkb1$^{SS/AA}$* CD4^{+} T cells. This mechanism is similar to the proposed role for BAFF in stimulating the basal IKK-induced p100 processing observed in B cells *in vivo* (Kayagaki et al., 2002). Moreover, analysis of *Nik$^{-/-}$* T cells suggests that a tonic signal can determine basal levels of p100 in T cells, although in contrast to IKK-induced p105 proteolysis, this is believed to occur

via transcriptional regulation of p100 by p52/RelB dimers (Ishimaru et al., 2006). However, the identity of this tonic signal in T cells is unknown. One possibility may be signalling downstream of the TCR, since naïve T cells constantly sample spMHC *via* their TCR, in an interaction necessary for survival (Takeda et al., 1996b, Seddon et al., 2000, Kirberg et al., 1997, Tanchot et al., 1997). Alternatively, a cytokine such as IL-7, which is also required for naïve T cell homeostasis (Tan et al., 2001), may be involved. Examination of this signal requirement will be an interesting future direction or research.

Why does impaired TPL-2 activity not affect Nfkb1$^{SS/AA}$ CD4^{+} T cells?

Analysis of mice lacking TPL-2 in this study demonstrates a block in TPL-2 kinase activity is not primarily responsible for the impaired differentiation of T cell sub-populations in *Nfkb1$^{SS/AA}$* mice. Furthermore, TPL-2-deficient CD4^{+} T cells proliferate and produce IL-2 as normal *in vitro*, suggesting these defects in *Nfkb1$^{SS/AA}$* CD4^{+} T cells are not due to impaired TPL-2 activation. Accordingly, activation of ERK after TCR stimulation is unaffected by a lack of TPL-2 ((Dumitru et al., 2000); M. Belich, unpublished results). Therefore, TPL-2 appears to play a redundant role in CD4^{+} T cells, and function of other MAP-3Ks, such as Raf-B, may be sufficient to induce ERK activity in these cells.

5.3 Thresholds of activation for different NF-κB target genes

Why are Nfkb1$^{SS/AA}$ mice viable?

Impairing NF-κB activity in mouse strains can be lethal, due to the anti-apoptotic role and ubiquitous expression of NF-κB transcription factors. For example, mice lacking RelA or IKK2 die *in utero* due to massive TNFα-induced hepatic apoptosis (Beg et al., 1995b, Li et al., 1999b). Therefore it is interesting that the reduction in NF-κB activity observed in *Nfkb1*$^{SS/AA}$ CD4^{+} T cells, including impaired RelA activity (M.Belich, S. Papoutsopoulos; unpublished data), is not reflected in the viability of *Nfkb1*$^{SS/AA}$ mice and susceptibility of *Nfkb1*$^{SS/AA}$ hepatocytes to TNFα-induced cell death. This raises the possibility that IKK-induced proteolysis of p105 may not occur to an appreciable extent in hepatocytes. However, IKK-mediated proteolysis of p105 has been observed after TNFα-stimulation in macrophages and fibroblasts (Salmeron et al., 2001, Beinke S, 2003), suggesting this may not be a cell-type specific event. Alternatively, the partial activity of NF-κB subunits may be sufficient for protection against TNFα-induced cell death. This latter idea is supported by evidence from transgenic mice expressing a T cell-specific IκBα super-repressor (Boothby et al., 1997), as discussed below.

Why is conventional T cell development unperturbed by p105 mutation?

NF-κB subunits show a certain degree of functional redundancy. Therefore, T cell development is unaffected in mice deficient in single NF-κB subunits (Sha et al., 1995, Kontgen et al., 1995, Beg et al., 1995b), whereas an inhibition of multiple NF-κB transcription factors, such as in T cells of mice expressing IκBα super-repressor, reveal a role for NF-κB in thymocyte selection and survival (Boothby et al., 1997, Mora et al., 2001b). Since IκBα super-repressor and p105$^{SS/AA}$ both

inhibit p50, RelA and c-Rel subunits in $CD4^+$ T cells (M.Belich, S. Papoutsopoulos; unpublished data), it may be surprising that mice expressing these proteins do not show similar defects in T cell differentiation. However, NF-κB activity after TCR ligation is almost completely abolished in $CD4^+$ T cells expressing the IκBα super-repressor (Boothby et al., 1997), whilst NF-κB activity is only partially blocked in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells, probably due to the higher affinity IκBα possesses for RelA heterodimers compared to p105. Therefore, the remaining NF-κB activity in *Nfkb1*$^{SS/AA}$ T cells appears to be sufficient for normal αβ T cell development, unlike in mice expressing T cells with IκBα super-repressor (Boothby et al., 1997). Consistent with this idea, a complete absence of NF-κB activity in T cells, via expression of a kinase dead form of IKK2 or deletion of NEMO using Cre expressed under the control of the CD4cre transgene, results in the total lack of SP thymocytes and peripheral T cells (Schmidt-Supprian et al., 2003).

Why is there a selective defect in NF-κB target genes?

Several $CD4^+$ T cell functions have been described to be regulated by NF-κB (Gerondakis et al., 1999). However, defects in only some of these were seen in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells, including impaired TCR-induced proliferation and generation of T cell sub-populations, whilst conventional T cell development, growth and survival are unaffected by p105 mutation. Similarly, $CD4^+$ T cells from *CD4-Cre/Ikk2*$^{FL/D}$ mice, which also only have a partial block in NF-κB activity after TCR stimulation, show defects in TCR-induced proliferation but not survival (Schmidt-Supprian et al., 2003). These results may be reconciled by the idea that

different NF-κB target genes have different thresholds of activation. Thus a partial block in NF-κB activity may affect transcription of only certain genes such as IL-2, which could require higher levels of NF-κB for their transcription. Other genes, including those required for survival, may have a lower requirement for NF-κB, and are thus unaffected by the reduced levels of NF-κB activity in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells after TCR stimulation. Therefore, the level of NF-κB activity in a cell may be critical for expression of target genes.

5.4 IL-2 signalling in proliferation of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells and generation of *Nfkb1*$^{SS/AA}$ T_{reg} cells

How does p105 mutation affect IL-2 transcription?

The defects in IL-2 production and proliferation observed in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells are not due to defective activation of the TPL-2-MEK1/2-ERK pathway, as evident by analysis of *Map3k8*$^{-/-}$ CD4$^+$ T cells, but are likely to be due to impaired NF-κB activation after TCR activation. Moreover, the rescue of TCR-induced *Nfkb1*$^{SS/AA}$ CD4$^+$ T cell proliferation by CD28 costimulation, and impaired production of IL-2 after TCR stimulation is very similar to the phenotype of *crel*$^{-/-}$ CD4$^+$ T cells (Liou et al., 1999, Kontgen et al., 1995). A role for c-Rel in regulating IL-2 production has been well established, with c-Rel able to bind κB elements of the IL-2 promoter after TCR activation, which induces chromatin remodelling of the IL-2 promoter (Rao et al., 2003, Algarte et al., 1995). Therefore, the reduction in IL-2 production by both *crel*$^{-/-}$ and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells suggest that signal-induced p105 proteolysis may be important for the regulation of c-Rel activation in T cells.

Analyses of nuclear levels of c-Rel are consistent with p105 able to inhibit c-Rel in the cytoplasm of $CD4^+$ T cells (M.Belich; unpublished data). TCR activation results also increases NF-κB-dependent transcription of c-Rel (Grumont and Gerondakis, 1990, Venkataraman et al., 1995), thus p105 may inhibit NF-κB dimers containing c-Rel in the cytoplasm and also regulate the transcription of c-Rel. These results therefore suggest that IL-2 production is impaired in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells due to reduced c-Rel activation,

Is impaired IL-2 production the cause of the proliferation defect in Nfkb1$^{SS/AA}$ $CD4^+$ T cells?

Transcription and secretion of IL-2 is impaired by *Nfkb1*$^{SS/AA}$ mutation in $CD4^+$ T cells. However, evidence presented here suggests that production of IL-2 is not the sole cause of the impaired proliferative capacity of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells. Firstly, addition of exogenous IL-2 only provides a partial rescue in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cell proliferation after anti-CD3 stimulation. Furthermore, although TCR-induced upregulation of CD25 (IL-2Rα), and assembly of the high-affinity IL-2R is initially unaffected by *Nfkb1*$^{SS/AA}$ mutation in $CD4^+$ T cells, the ability to sustain this expression is impaired, even in the presence of exogenous IL-2. Since CD25 expression is dependent both on signals downstream of the TCR and IL-2R, this raises the possibility that blocking p105 proteolysis impairs signalling downstream of the IL-2R (Hatakeyama et al., 1989, Plaetinck et al., 1990). The tyrosine kinases Janus-associated kinase (JAK)-1 and JAK3 have been suggested to selectively associate with the IL-2Rβ and γ-chains respectively (Miyazaki et al., 1994).

Binding of IL-2 to its receptor brings these kinases together, promoting their transphosphorylation and phosphorylation of IL-2Rβ (Sharon et al., 1989). Phosphorylated tyrosine resides on IL-2Rβ act as docking sites for STAT5a and STAT5b transcription factors (Sharon et al., 1989, Mui et al., 1995), which upon binding themselves undergo JAK-mediated phosphorylation. This activation triggers STAT5 dimerisation, nuclear translocation and binding of target genes containing γ-interferon activated sequences (GAS) (Moriggl et al., 1999b, Hennighausen and Robinson, 2008). Interestingly, NF-κB deficiency has previously been shown to impair STAT5 activation after IL-2 stimulation (Mora et al., 2001a). Consistent with this, work in the Ley lab has revealed that, after initial stimulation with anti-CD3 to upregulate IL-2R, IL-2 induced STAT5 phosphorylation occurs, but is markedly impaired in *Nfkb1*$^{SS/AA}$ CD4^{+} T cells (S. Papoutsopoulos; unpublished data). Levels of STAT5 protein were unaffected by p105 mutation in these cells. Thus, as well as impairing production of IL-2, these findings support the idea that signalling downstream of the IL-2R is impaired by blocking IKK-induced p105 proteolysis in CD4^{+} T cells.

How does CD28 costimulation rescue the proliferation defect caused by Nfkb1$^{SS/AA}$ mutation?

Work in this study has shown that CD28 costimulation can largely overcome the proliferative defect seen in *Nfkb1*$^{SS/AA}$ CD4^{+} T cells stimulated with CD3 antibody alone, although the reduced levels of IL-2 mRNA in *Nfkb1*$^{SS/AA}$ CD4^{+} T cells compared to WT cells are only partially rescued by CD28 ligation. In contrast,

transcription of IL-2 in WT $CD4^+$ T cells is markedly enhanced upon CD28 costimulation, presumably due to the presence of a CD28RE in the promoter of IL-2 (Fraser et al., 1991), and stabilisation of IL-2 mRNA (Lindstein et al., 1989). This suggests that IL-2 production is unlikely to be the main mechanism by which CD28 costimulation rescues proliferation, particularly since addition of recombinant IL-2 did not increase proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+CD25^-$ T cells to the same extent as CD28 ligation. One possible means of rescue comes from results presented here that suggest CD28 costimulation can increase levels of CD25 on *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells, consistent with reports that CD28 promotes sustained expression of CD25 (Kahn-Perles et al., 1997). Thus, CD28 costimulation may upregulate expression of CD25, and hence the high affinity IL-2R, and increase levels of signalling sufficiently to rescue proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells, despite the impaired IL-2 responsiveness proposed here. Although CD28 ligation has been suggested to augment the TCR signal rather than deliver a unique signal (Schmitz and Krappmann, 2006), an alternative explanation is that the defect in IL-2R signalling may be circumvented by costimulation, in a pathway independent of IKK-induced p105 proteolysis. This may conceivably occur through CD28 costimulation augmenting transcription of proteins that positively regulate IL-2 signalling, or by activating such proteins post-translationally. Consistent with the rescue of proliferation, IL-2-induced STAT5 phosphorylation after CD28 costimulation is comparable between WT and *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells (S. Papoutsopoulos; unpublished data). Strikingly, addition of IL-2 can largely restore proliferation of sorted naïve *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells when stimulated with CD3 antibody on BMDCs. It seems likely in this case that BMDCs can provide costimulation, in a

similar manner to CD28 antibody, to overcome proliferation defects in *Nfkb1*$^{SS/AA}$ when exogenous IL-2 is added.

Does the IL-2 - IL-2R signalling pathway affect T_{reg} cell generation?

The development of T_{reg} cells requires NF-κB activation, but is specifically dependent on signals downstream of the TCR, as evident by a lack of T_{reg} cells in mice lacking PKCθ or BCL10, two signalling molecules essential for TCR-induced NF-κB activation (Schmidt-Supprian et al., 2004a). Therefore, the T cell intrinsic defect in *Nfkb1*$^{SS/AA}$ T_{reg} cell generation could be a result of a block in TCR-induced activation of NF-κB. Furthermore, results presented here demonstrate that TCR-induced production of IL-2, a cytokine that is important for maintenance of T_{reg} cells (D'Cruz and Klein, 2005, Fontenot et al., 2005a), is impaired in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells *in vitro*. However, IL-2 produced by Rag-2-deficient hosts is sufficient to generate T_{reg} cells from *Il2*$^{-/-}$ BM cells (Almeida et al., 2002), but cannot rescue numbers of T_{reg} cells in chimeras generated using *Nfkb1*$^{SS/AA}$ BM in this study. Therefore, although the defect in IL-2 production may contribute to a reduction in maintenance of T_{reg} cells in *Nfkb1*$^{SS/AA}$ mice, deficient IL-2 cannot explain their impaired differentiation. This conclusion is supported by analysis of mixed BM chimeras, in which *Nfkb1*$^{SS/AA}$ T_{reg} cell numbers are still reduced in the presence of WT haematopoietic cells, which can produce IL-2.

The finding that IL-2R signalling is impaired in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells (S. Papoutsopoulos; unpublished data) provides an alternative explanation for the

defect in T_{reg} cell generation in *Nfkb1*$^{SS/AA}$ mice, since several reports have indicated that STAT5 activation is required for T_{reg} cell differentiation (Yao et al., 2007, Burchill et al., 2007). Burchill and colleagues suggest that TCR and CD28 signals activate T cells and induce their responsiveness to cytokines, and that activation of STAT-5 is then required to complete T_{reg} cell differentiation. Accordingly, the impaired IL-2 signalling proposed in this study may explain the competitive disadvantage of *Nfkb1*$^{SS/AA}$ T_{reg} cells in mixed BM chimeras, as T_{reg} cells generated from *Il2ra*$^{-/-}$ BM could not repopulate host mice as effectively as WT T_{reg} cells in mixed BM chimeras (Fontenot et al., 2005a). To explore these possibilities, the effect of transgenic expression of constitutively active STAT5 in *Nfkb1*$^{SS/AA}$ cells could be tested. Furthermore, we propose that the requirement previously suggested for NF-κB activity in spMHC:TCR-mediated selection of T_{reg} cells (Liston and Rudensky, 2007, Schmidt-Supprian et al., 2003, Siebenlist et al., 2005), may be to promote cytokine-driven STAT5 activation and thus completion of T_{reg} cell differentiation.

How does NF-κB regulate IL-2R signalling?

Impaired IL-2 induced phosphorylation of STAT5 has been previously attributed to reduced levels of NF-κB activity (Mora et al., 2001a). Therefore, it seems likely that the block in TCR-induced NF-κB activity (M. Belich, unpublished data) also results in the impaired IL-2R signalling in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells. However, little is known about the manner in which NF-κB regulates IL-2 signalling or activation of STAT5. One possibility is that NF-κB is involved in the transcriptional regulation of

proteins that modulate IL-2R signals. For example, the suppressors of cytokine signaling (SOCS) are thought to play a key role in modulating cytokine responses, and are upregulated by and inhibit the JAK/STAT pathway in a classical negative-feedback mechanism (Yoshimura et al., 2007). SOCS proteins can function in different manners, including by inhibiting JAK kinase activity, or as E3 ubiquitin ligases that target components of cytokine signalling such as JAKs for degradation (Zhang et al., 1999, Kamura et al., 1998). However, recent work has reported that SOCS2 can enhance IL-2 signalling by promoting the degradation of the inhibitory SOCS3 protein (Tannahill et al., 2005). Therefore, one possibility is that TCR-induced NF-κB activation is necessary for transcriptional regulation of SOCS2. It is notable that mice lacking SOCS2 show enhanced growth hormone-receptor signalling and are larger than WT mice, a defect not observed in *Nfkb1*$^{SS/AA}$ mice (Greenhalgh et al., 2005). Therefore, if such regulation of SOCS2 by NF-κB exists, it may occur in a cell-type specific manner.

Another mechanism by which NF-κB influences IL-2 signalling could be *via* transcriptional regulation of JAK1 and/or JAK3. Thus, inhibiting NF-κB activity would result in reduced activation of STAT5 as a result of reduced JAK1/JAK3. However, preliminary data examining IL-4R signalling in *Nfkb1*$^{SS/AA}$ CD4^{+} T cells, which also activate STAT5 (Lischke et al., 1998, Friedrich et al., 1999), suggests that this may not be the case. Engagement of the IL-4R activates JAK1 and JAK3 through association with the IL-4Rα and common γ-chains, respectively (Miyazaki et al., 1994, Russell et al., 1994). Analysis of anti-CD3 induced proliferation in the

presence of IL-4 revealed a significant enhancement in the percentage of dividing cells in both WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells compared to in the absence of IL-4 (data not shown), consistent with published work on its mitogenic role in T cells (Mitchell et al., 1989). Interestingly proliferation was largely restored to comparable levels between the two genotypes (unpublished data). These data suggest that IL-4R signalling is intact in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells and indicates that the defect in IL-2-induced STAT5 activation is not downstream of all γ-chain cytokines, and may not be due to impaired expression of JAK3 or JAK1. Furthermore, activation of STAT6 has been shown downstream of IL-4Rα, and contributes to proliferation of T cells (Takeda et al., 1996a, Kaplan et al., 1998). Thus these data suggest that *Nfkb1*$^{SS/AA}$ mutation does not impair the activation of all STAT proteins. Consistent with these results, IL-6R ligation, which activates STAT3 and to a lesser extent STAT1 in a JAK1-dependent pathway downstream of the gp130 chain (Heinrich et al., 1998), can induce proliferation in WT and *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells to similar extents (data not shown). However, there may be some functional redundancy between the JAK and STAT families, and therefore direct analysis at the protein level should be carried out. Additionally, since IL-2 and IL-15 share the IL-2Rβ chain (Giri et al., 1994), it will be interesting to examine the responsiveness of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells to this cytokine. These data may help provide a more targeted search of candidate proteins that are involved in NF-κB regulation of STAT5 activation by IL-2.

Given that STAT5 is the predominant isoform activated by the γ-chain cytokines

(Johnston et al., 1995), it is perhaps surprising that the effect of *Nfkb1*$^{SS/AA}$ mutation in the lymphoid compartment is not more severe (Leonard et al., 1994, Nakajima et al., 1997). Indeed, in complete absence of STAT5, mice failed to develop T, B, and natural killer (NK) cells (Hoelbl et al., 2006, Yao et al., 2006). However, mice that express N-terminally truncated STAT5 displayed less severe immunological defects, since this STAT5 protein was found to still retain some functional activity (Teglund et al., 1998, Moriggl et al., 1999a, Sexl et al., 2000). Therefore, the partial activity of STAT5 in *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells may be sufficient for normal lymphoid development in *Nfkb1*$^{SS/AA}$ mice, but not for T_{reg} cell generation or *in vitro* responses to IL-2 in $CD4^+$ T cells.

5.5 Colitis and *Nfkb1*$^{SS/AA}$ mice

Why does the reduction in T_{reg} cells not lead to colitis in Nfkb1$^{SS/AA}$ mice?

T_{reg} cells are able to dampen immune responses and prevent autoimmune diseases, including the colitis that arises after transfer of naïve $CD4^+$ T cells into immuno-deficient mice (Powrie et al., 1993, Sakaguchi et al., 1995). Furthermore, mice with reduced T_{reg} cell numbers, such as those lacking IL-2 or IL-2 signalling, present with signs of autoimmunity (Papiernik et al., 1998, Malek et al., 2002, Almeida et al., 2002). Despite the significant reduction in T_{reg} cells, *Nfkb1*$^{SS/AA}$ mice showed no signs of spontaneous colitis, or development of colitis with age.

This may be due to a number of reasons. Naïve $CD4^+$ T cells transferred into *Rag*$^{-/-}$ or SCID mice can induce colitis through their aberrant inflammatory responses towards the resident bacterial flora of the gut. Activation and differentiation into

Th1 cells, and subsequent production of inflammatory cytokines such as TNFα and IFNγ drive the pathology of colitis in this model (Coombes et al., 2005). Since *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells display a block in TCR-induced proliferation, a possible explanation for the lack of colitis in *Nfkb1*$^{SS/AA}$ mice is that expansion of naive T cells is impaired. Indeed, CD4$^+$CD25$^-$CD45Rb^{hi} T cells from *CD4-Cre/Ikk2*$^{FL/D}$ mice, which have a partial block in NF-κB activity and impaired proliferation following plate-bound anti-CD3 stimulation, cannot induce colitis when transferred into *Rag1*$^{-/-}$ mice (Schmidt-Supprian et al., 2004b). A similar approach using the T cell-transfer model of colitis may be utilised to investigate this possibility with *Nfkb1*$^{SS/AA}$ naïve CD4$^+$ T cells. Furthermore, histopathological signs of colitis and proliferation of colitis-inducing CD4$^+$ T cells are prevented by co-transfer of CD4$^+$CD25$^+$ T_{reg} cells (Annacker et al., 2001, Sakaguchi et al., 1995). Thus, the data presented here that *Nfkb1*$^{SS/AA}$ T_{reg} cells are functional *in vitro*, can be assessed *in vivo*.

Nfkb1$^{SS/AA}$ mutation may affect function of other cell types that are important in maintaining intestinal homeostasis and which are implicated in colitis ((Coombes et al., 2005, Xavier and Podolsky, 2007). For example, a model of colitis induced by the naturally occurring bacteria *Helicobacter hepaticus* in susceptible immune-deficient mouse strains, demonstrates the contribution of innate immune cells and their production of pro-inflammatory cytokines in this disorder (Leung et al., 2004). Interestingly, blocking NF-κB activity using antisense or decoy oligonucleotides has been described to reduce the severity of colitis in this model, consistent with the

pro-inflammatory functions of NF-κB (Fichtner-Feigl et al., 2005, Neurath et al., 1996). However, there is also evidence to the contrary from mice lacking the NF-κB subunits p50 or heterozygous for RelA (Erdman et al., 2001, Tomczak et al., 2006). These mice are more susceptible to *H. hepaticus*-induced colitis, apparently resulting from a requirement for NF-κB activation downstream of the IL-10R in innate immune cells, vital for suppression of inflammatory cytokine expression. Therefore, analysis of *Nfkb1*$^{SS/AA}$ innate immune cell-induced colitis may reveal functions of IKK-induced p105 proteolysis in these cell types.

5.6 NKT cells

What causes the reduction in thymic Vα14i NKT cells in Nfkb1$^{SS/AA}$ mice?

Generation of Vα14i NKT cells is impaired in *Nfkb1*$^{SS/AA}$ mice, whilst peripheral Vα14i NKT cell populations are normal. Similarly, mice with T cell-specific expression of an IκBα super-repressor have reduced frequencies of thymic NKT cells, but only a modest reduction of this population in the periphery, compared to WT mice (Sivakumar et al., 2003). However, in contrast to the T-cell intrinsic defect of the IκBα super-repressor mice, defects in radiation-resistant non-haematopoietic cells contributed to reduced generation of *Nfkb1*$^{SS/AA}$ Vα14i NKT cells. This is consistent with previous data from *Relb*$^{-/-}$ and *aly/aly* mice, which demonstrate that early NKT cell development requires NF-κB activity in stromal cells (Elewaut et al., 2003, Sivakumar et al., 2003). Furthermore, transcription of IL-15, a cytokine that is necessary for NKT generation and whose promoter contains a κB site (Ohteki et al., 1997, Ranson et al., 2003, Azimi et al., 1998), is

impaired in *Relb*$^{-/-}$ thymocytes (Sivakumar et al., 2003). Therefore, the introduction of *Nfkb1*$^{SS/AA}$ mutation may impair activation of RelB in stromal cells. This is unlikely to be through p105$^{SS/AA}$ protein blocking release of RelB-containing NF-κB dimers, since RelB does not bind to p105 (Dobrzanski et al., 1995, Solan et al., 2002). Instead, the block in signal-induced proteolysis of p105 in *Nfkb1*$^{SS/AA}$ stromal cells may inhibit transcription of RelB, as RelB is an NF-κB target gene (Bours et al., 1994). This potential requirement for signal-induced p105 proteolysis in stromal cells and NKT cell generation can be investigated by transferring WT BM into irradiated *Nfkb1*$^{SS/AA}$ mice. Furthermore, measurement of IL-15 mRNA levels in *Nfkb1*$^{SS/AA}$ thymic cells may provide an insight into the mechanism by which NKT cell development is impaired.

Why is NKT cell frequency only reduced in the thymus of Nfkb1$^{SS/AA}$ mice?

CD4-Cre/Ikk2$^{FL/D}$ mice lack Vα14i NKT cells in both the thymus and periphery (Schmidt-Supprian et al., 2004a). Therefore, It is surprising that Vα14i NKT cells are reduced only in the thymus, and that peripheral NKT numbers are normal in *Nfkb1*$^{SS/AA}$ mice. However, a similar phenotype is seen in transgenic mice expressing IκBα super-repressor in their T cells (Sivakumar et al., 2003). This discrepancy may be explained by the complete absence of Vα14i NKT cells in *CD4-Cre/Ikk2*$^{FL/D}$ thymii, whereas only a two-fold reduction of these cells was seen in the thymus of*Nfkb1*$^{SS/AA}$ mice. Therefore, the *Nfkb1*$^{SS/AA}$ Vα14i NKT cells that are generated in the thymus may be able to proliferate in the periphery and fill their niche. The role of NF-κB in NKT cell homeostasis is unknown, but there is

evidence that TCR-induced NF-κB activation is not required, since NKT cell numbers are normal in *Carma1*$^{-/-}$ and *Malt1*$^{-/-}$ mice (Hara et al., 2003, Ruland et al., 2003). However, BCL10-deficient mice have a defect in maintenance of peripheral NKT cells, although thymic numbers are normal, which suggests a TCR-independent pathway requiring BCL10 is necessary for NKT cell homeostasis (Schmidt-Supprian et al., 2004a).

5.7 T_{mem}-phenotype cells

Why are CD4$^+$ T_{mem}–phenotype cells particularly reduced in Nfkb1$^{SS/AA}$ mice?

It has previously been proposed from studies of *CD4-Cre/Ikk2*$^{FL/D}$ mice, that the CD4$^+$ T_{mem}–phenotype pool is more sensitive to a reduction in NF-κB activity than the CD8$^+$ T_{mem}–phenotype population (Schmidt-Supprian et al., 2003). Moreover, CD8$^+$ T_{mem}–phenotype cell numbers are normal when TCR-induced activation of NF-κB is blocked in mice lacking BCL10 or PKCθ, despite a significant reduction in CD4$^+$ T_{mem}–phenotype cells (Schmidt-Supprian et al., 2004a). Consistent with these findings, we show here an impaired generation and/or survival of the *Nfkb1*$^{SS/AA}$ splenic CD4$^+$ T_{mem}–phenotype population which is more severe than for that of the splenic CD8$^+$ T_{mem}–phenotype population. Furthermore, haematopoietic cell-defects in *Nfkb1*$^{SS/AA}$ mice lead to a reduction in CD4$^+$ T_{mem}–phenotype cell numbers, whilst the reductions in the CD8$^+$ T_{mem}–phenotype pool required *Nfkb1*$^{SS/AA}$ mutation in non-haematopoietic cells. Thus, these results suggest that generation and/or survival of CD8$^+$ T_{mem}–phenotype cells require distinct signalling pathways compared to CD4$^+$ T_{mem}–phenotype cells, with CD4$^+$ T cells particularly

requiring TCR-induced NF-κB activity. This is in accordance with the disparate cytokine requirements between $CD4^+$ and $CD8^+$ T_{mem}–phenotype cells. For example, IL-15 has been suggested to be dispensable for the maintenance of the $CD4^+$ pool but necessary for the $CD8^+$ pool of T_{mem}–phenotype cells (Lantz et al., 2000, Judge et al., 2002, Tan et al., 2002, Zhang et al., 1998c).

How does NF-κB affect generation and/or survival of memory cells?

T_{mem}–phenotype cells are believed to be generated in response to antigens from resident commensal bacteria and self-peptides, which induce a weak but chronic activation of T cells (Surh et al., 2006, Moulton and Farber, 2006). Therefore, the defect in TCR-induced proliferation of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells described in this study may indicate that effector cell expansion is also impaired *in vivo*. Consistent with this idea, *CD4-Cre/Ikk2*$^{FL/D}$ $CD4^+$ T cells, which have a partial reduction in TCR-induced NF-κB activity, show reduced recall responses compared to WT cells, which appeared to result from defective expansion of effector cells (Schmidt-Supprian et al., 2004b). Furthermore, the reduced allogeneic response of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells compared to WT cells reported here is consistent with a defect in TCR-dependent proliferation *in vivo*. However, the IL-2 deficiency and proposed defect in IL-2 signalling presented in this study are unlikely to be the cause of the impaired expansion of *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells *in vivo*, since proliferation of T cells *in vivo* is thought to occur largely independently of IL-2 signalling (Kneitz et al., 1995), and the main role for IL-2 *in vivo* has been suggested to be in maintaining the T_{reg} cell population (Kundig et al., 1993, Schorle

et al., 1991, Sadlack et al., 1993, Willerford et al., 1995, Leung et al., 2000). Therefore, it is likely that *Nfkb1*$^{SS/AA}$ mutation affects CD4$^+$ T cell proliferation *in vivo* in an as-yet-unidentified manner. This may involve impairing production of cytokines that support effector T cell proliferation and/or generation of memory cells from effector cells. Recently, IL-2 signals have been shown to upregulate IL-7Rα surface expression on CD4$^+$ T cells during an antigen response, which is suggested to be necessary for the long-term survival and generation of memory cells once antigen has been cleared (Dooms et al., 2007). Therefore, the reduced production of IL-2 and defective signalling downstream of the IL-2R in *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells may impair the generation of the CD4$^+$ T_{mem}-phenotype pool by reducing signals from the IL-7R. Analysis of IL-7Rα expression on CD4$^+$ T_{mem}–phenotype cells may clarify this.

5.8 Future directions

In vivo functions The *in vitro* proliferative defects of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells, described in this study, raises the question as to how this translates to the generation of an effective immune response *in vivo*. Numerous approaches can be taken to address this, since CD4$^+$ T cells provide helper function for the activation of various immune cells. For example, production of antibodies by B cells in response to T-dependent antigens requires CD4$^+$ T cell activation. Therefore, assays to measure production of antigen-specific antibodies can demonstrate helper activity of *Nfkb1*$^{SS/AA}$ CD4$^+$ T cells. The physiological role of signal-induced p105 proteolysis can further be examined through generation of TCR transgenic

Nfkb1$^{SS/AA}$ mice, such as those recognizing ovalbumin proteins (OT-II) (Barnden et al., 1998). By transferring OT-II *Nfkb1*$^{SS/AA}$ $CD4^+$ T cells into T-cell deficient mice and immunization with ovalbumin, antigen-induced expansion can be studied, since antigenic challenge results in the activation of the entire T cell population. The memory response generated can also be assessed by *in vitro* rechallenge.

Effector differentiation NF-κB has been implicated in differentiation of T-helper effector cells by regulating expression of GATA-3, expansion of Th1 cells and EAE-pathology (Das et al., 2001, Artis et al., 2002, Hilliard et al., 2002, Hilliard et al., 1999). Therefore, secretion of cytokines and differentiation into Th1 or Th2 polarized upon polyclonal activation *in vitro* would be interesting to analyse. This can also be examined *in vivo* by infection with pathogens that require Th1 or Th2-driven responses for their clearance, or assessing differentiation into Th17 cells through induction of EAE.

Clinical relevance NF-κB transcription factors mediate the rapid expression of pro-inflammatory cytokine genes in response to activation under many pathogenic conditions. Thus, NF-κB is implicated in chronic inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease (reviewed in (Yamamoto and Gaynor, 2004) with an emerging role in cancers (Karin et al., 2002). Furthermore, commonly used anti-inflammatory agents, such as salicylates, appear to reduce inflammation by blocking NF-κB activity. Therefore, targeting the NF-κB pathway of activation for inhibition has gained

interest for drug development. However, NF-κB activity is vital for survival and function of many cell types, limiting its use in such applications. Since T cells play an important role in autoimmunity and inflammatory responses, this study demonstrates a specific pathway leading to NF-κB activation that affects $CD4^+$ T cell activation, without affecting survival or naïve T cell generation, which may provide a novel therapeutic target.

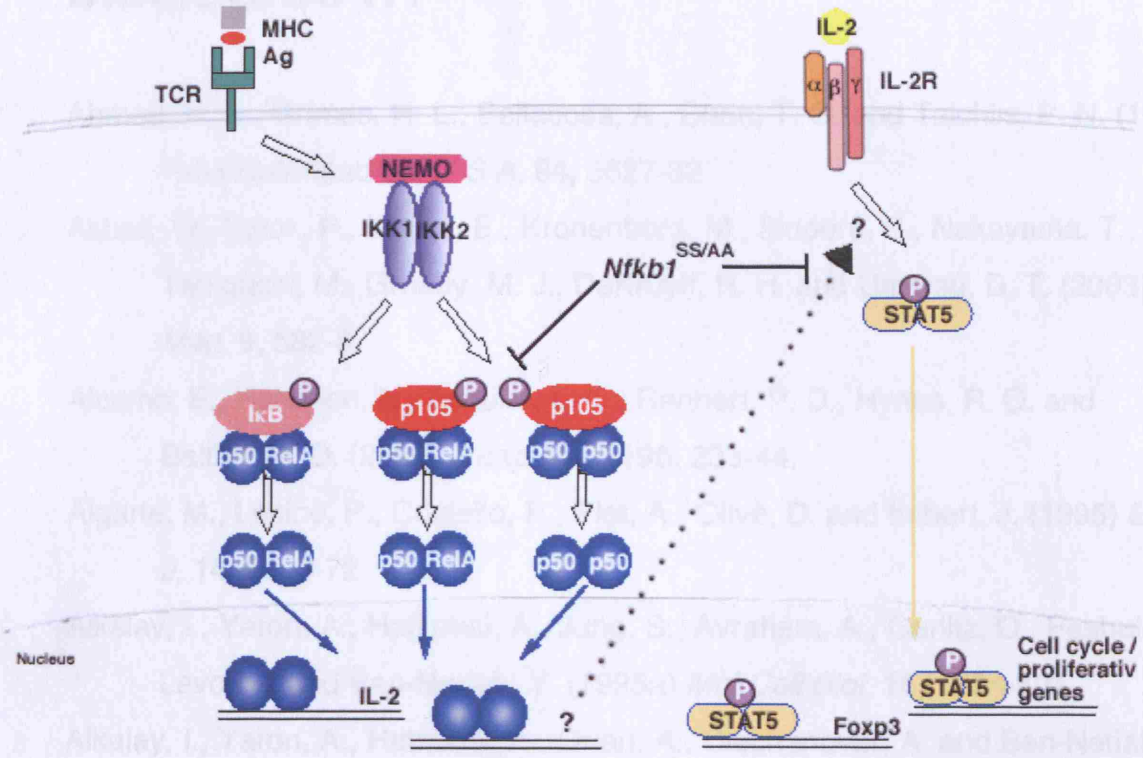


Figure 5. IKK-induced proteolysis is required to regulate basal levels of NF-κB1 p105 in T cells and determines NF-κB activation.
Introduction of *Nfkb1*$^{SS/AA}$ mutation causes an increase in p105 levels in resting T cells, implying that IKK-induced phosphorylation determines the cellular level of p105 protein through a constitutive signal such as TCR:spMHC. Higher levels of non-degradadable p105$^{SS/AA}$ results in reduced NF-κB activation through cytoplasmic retention of both p50 homodimers and "classical" NF-κB heterodimers. This impaired activation of NF-κB results in reduced transcription of the NF-κB target gene IL-2, which contributes to a reduction in *Nfkb1*$^{SS/AA}$ CD4^{+} T cell proliferation. *Nfkb1*$^{SS/AA}$ mutation also compromises responses to IL-2 in an undefined manner, possibly by dysregulating transcription of an NF-κB-target involved in the IL-2R signalling pathway. This impaired transduction of IL-2R signals leads to a decrease in STAT5 activation, a transcriptional regulator of the Foxp3 gene, thereby reducing numbers of T_{reg} cells in *Nfkb1*$^{SS/AA}$ mice.
Abbreviations: Ag, antigen; IKK, inhibitor of NF-κB kinase; IL-2, interleukin 2; MHC, major histocompatibilty complex; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κ B cells; STAT, signal transducer and activators of transcription; TCR, T cell receptor; T_{reg}, natural regulatory T cells.

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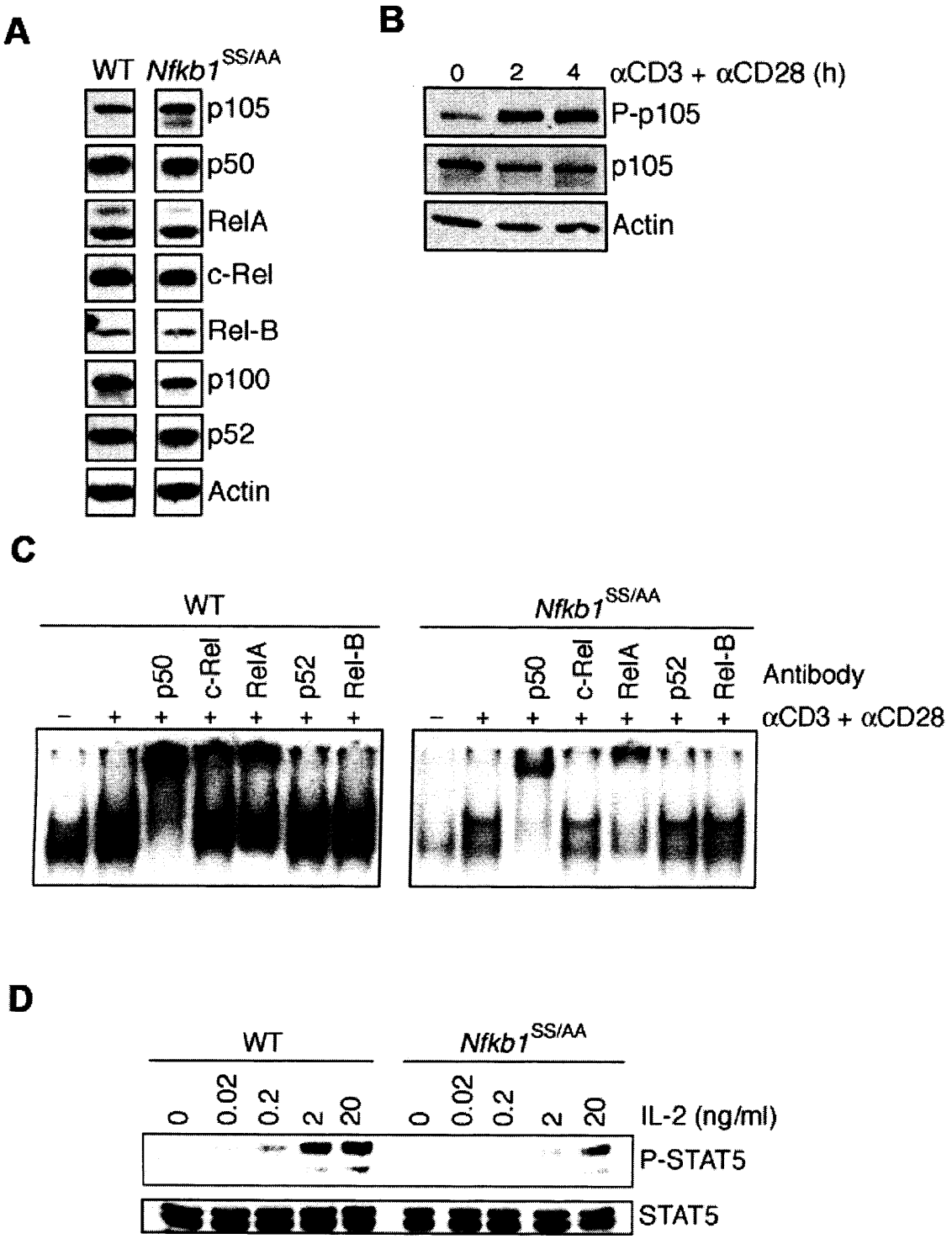
Appendix


A
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p50
RelA
c-Rel
Rel-B
p100
p52
Actin
B
0
2
4
αCD3 + αCD28 (h)
P-p105
p105
Actin
C
WT
Nfkb1SS/AA
p50
c-Rel
RelA
p52
Rel-B
Antibody
αCD3 + αCD28
D
WT
Nfkb1SS/AA
0
0.02
0.2
2
20
IL-2 (ng/ml)
P-STAT5
STAT5

Figure 6.1 Protein analysis of *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells (A-C carried out by M. Belich; D by S. Papoutsopoulos)

(A) Expression of NF-κB subunits was analysed by immunoblotting total lysates of purified WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells. **(B)** TCR-induced phosphorylation of p105 at serine 927 and serine 932 was confirmed in WT CD4^{+}CD25^{-} T cells by immunoblot analysis. **(C)** NF-κB complexes in nuclear extracts from WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} T cells were detected using a radiolabelled NF-κB probe by electrophoretic mobility shift assay (EMSA) with (+) or without (–) CD3 and CD28 antibody stimulation for 4 h. Composition of probe-bound dimers was assessed by supershift analysis using antibodies indicated. **(D)** Purified WT and *Nfkb1*$^{SS/AA}$ CD4^{+}CD25^{-} cells were stimulated for 12 h with CD3 and CD28 antibodies to induce expression of the high affinity IL-2R. After washing and resting for 2 h, cells were restimulated with indicated concentrations of IL-2 for 15 mins. STAT5 phosphorylation was assessed by immuoblotting total cell lysates.

Acknowledgements

Firstly I would like to thank my supervisor Steve Ley for his guidance, support and time over the course of my PhD and during writing of this thesis. I am also indebted to my second supervisor, Ben Seddon, who has provided invaluable advice and help with experiments.

I am grateful to the other members of the Division of Immunology at the NIMR for their suggestions, particularly George Kassiotis and Brigitta Stockinger. In addition, I thank Georgina Cornish for critical reading of this thesis.

The Cell Sorting Lab (Aaron and Graham), animal staff and Histology department (Elena) at the NIMR have provided much help with this work and I would also like to acknowledge.

Many thanks to all the members of the Ley lab, past and present for their support. In particular, I would like to acknowledge Monica Belich, Julia Janzen, Tharsana Tharmalingam and Stamatia Papoutsopoulos for their contributions and advice, both scientifically and personally.

I dedicate this thesis to my family, whose support has been endless.