The Role of IKK Signalling in T cells

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Declaration

I, Scott James Layzell 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.

Scott James Layzell
Abstract

The Inhibitor of Kappa B Kinase (IKK) complex is a key component of canonical NF-κB signalling. More recently, the IKK complex has emerged as a regulator of extrinsic cell death pathways by directly limiting the activity of RIPK1. Normal thymocyte development is solely reliant on repression of TNF triggered cell death by the IKK complex, independently of NF-κB activation. However, the role of IKK signalling in T cells after activation remains unclear. To address this, we analysed activation of IKK2 deficient TCR transgenic T cells. While early activation events were normal, proliferation of T cell blasts was impaired. Cell cycle progression in IKK2 deficient T cells was also unperturbed. Instead, dividing cells were more sensitive to extrinsic apoptosis since inhibition of RIPK1 kinase activity almost completely rescued cell survival. Consequently, anti-viral IKK2 deficient T cell responses were profoundly diminished in vivo.

Transcriptomic analysis of activated IKK2 deficient T cells revealed defective expression of Tnfaip3, that encodes A20, a negative regulator of NF-κB. We examined whether A20 induction was required to protect IKK2 deficient T cells from death. Inhibition of IKK activity in A20 deficient T cells in vitro uncovered a role for A20 in regulating cell death. Mice with combined A20 and IKK2 deficiency exhibited defective CD8+ SP thymocyte development and substantial peripheral T cell lymphopenia. However, this phenotype was almost completely reversed by inhibition of RIPK1 kinase activity and partially rescued upon deletion of TNFR1 in vivo. Since receptors additional to TNFR1 appeared to be triggering naïve T cell death, we subsequently identified roles for IKK and A20 in regulation of the Fas apoptotic pathway.

Together, our data reveals that IKK signalling protects T cells from RIPK1 dependent death in both an NF-κB dependent and independent manner, and downstream of multiple receptors.
Impact Statement

The canonical NF-κB signalling pathway links multiple cell surface receptors to the activation of NF-κB, which drives the transcription of genes important for cell survival, differentiation, and function. Paradoxically, receptors which promote cell survival, by inducing NF-κB activation, can also trigger cell death. Regulating this cell fate decision are the kinases and adaptors which constitute the canonical NF-κB signalling pathway, including the IKK complex. Traditionally, it was thought that NF-κB activation protects cells from cell death induced by TNF solely through induction of pro-survival proteins. But recently, IKK has been shown to directly phosphorylate and inhibit the kinase RIPK1 to block TNF induced cell death (Dondelinger et al., 2015, 2019). As such, the IKK complex is a crucial regulator of cell survival.

Understanding how the canonical NF-κB signalling pathway is regulated is of important scientific interest and it’s becoming clear that control of NF-κB activation and cell death differs between cell types. Dysregulation of NF-κB has been implicated in human diseases such as cancers, autoimmunity and during infection, in which T cells have critical roles. Patients with mutations in canonical NF-κB signalling components present with primary immunodeficiencies including T cell defects. Thus, knowledge of how T cell survival, homeostasis and function is controlled by components of the canonical NF-κB signalling pathway, such as IKK, is important to determine if and how this pathway can be targeted therapeutically in human disease.

This thesis has expanded our understanding of how IKK functions in different T cell populations. While it had been shown recently that IKK is required for thymocyte development through direct repression of RIPK1 dependent cell death, independently of NF-κB activation, it was not known if this regulatory circuit was active in peripheral T cells (Webb et al., 2019). We show that IKK inhibition of RIPK1 is important for survival of mature and activated T cells and that this mechanism appears to be functioning downstream of multiple death receptors including TNFR1 and Fas. We further identify A20 as
another regulator of RIPK1 in T cells, acting to fine tune control of RIPK1 by IKK. The role of A20 appears to be particularly prominent during T cell activation where its expression is regulated not only by NF-κB but also TCR signalling (Düwel et al., 2009). In contrast, normal development and homeostasis of regulatory and memory T cells appears to require IKK predominantly for activation of NF-κB rather than repression of extrinsic cell death pathways.

Collectively, the role of IKK in T cells is complex. While IKK activation of NF-κB appears to be important for T cell survival and homeostasis, as has been traditionally thought, so too is IKK repression of RIPK1 dependent and independent cell death pathways downstream of multiple death receptors. In light of these findings, careful consideration needs to be made before targeting canonical NF-κB signalling for human disease as to the impact on the regulation of extrinsic cell death versus NF-κB activation in different T cell populations and whether or not this would be beneficial in the disease context.
Acknowledgments

Firstly, I want to thank my supervisor Prof. Benedict Seddon for the opportunity to study and complete my PhD in his lab. I’m extremely grateful for all your help and support throughout this time. I’ve really enjoyed our scientific discussions, which would always leave me feeling inspired.

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To my Mum, Dad and sister, I can’t thank you enough for everything you have done for me to get to this point. I’m very lucky to have a family as supportive as you are.

Finally, to everyone at the IIT, thanks for making it a really enjoyable experience to work here.
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List of Abbreviations

Aire  Autoimmune regulator
ACD  Accidental cell death
aFasL Aggregated FasL
APAF1 Apoptotic protease-activating factor-1
APC  Antigen presenting cell
BAD  BCL-2 associated agonist ff cell death
BAFF-R B cell activating factor receptor
BCL-2 B cell lymphoma 2
BCL10 B cell lymphoma/leukemia 10
BCR  B cell receptor
Bfl-1 Bcl-2 related gene expressed in fetal liver 1
BH   BCL-2 homology
BID  BH3-interacting domain death agonist
BIK  Bcl-2 interacting killer
Blimp-1 B lymphocyte-induced maturation protein-1
BMF  BCL-2 modifying factor
BSA  Bovine serum albumin
CAD  Caspase activated DNase
CARD11 Caspase Recruitment Domain Family Member 11
CARMA1 CARD-containing MAGUK protein 1
CASP8 Caspase-8
CBM  CARD11-BCL10-MALT1
CD   Cluster of differentiation
CDK  Cyclin dependent kinase
CFA  Complete freund's adjuvant
c-FLIP Cellular FADD-like IL-1β-converting enzyme
c-FLIP_L Cellular FADD-like IL-1β-converting enzyme long isoform
c-FLIP_R Cellular FADD-like IL-1β-converting enzyme Raji isoform
c-FLIP_S Cellular FADD-like IL-1β-converting enzyme short isoform
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>cIAP</td>
<td>Cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>Cpdm</td>
<td>Chronic proliferative dermatitis</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per million mapped reads</td>
</tr>
<tr>
<td>cTEC</td>
<td>Cortical thymic epithelial cell</td>
</tr>
<tr>
<td>CTV</td>
<td>CellTrace Violet</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
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<td>DED</td>
<td>Death effector domain</td>
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<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP-Binding protein with Low PI</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EOMES</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FC</td>
<td>Fold change</td>
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<td>FN14</td>
<td>Fibroblast growth factor-inducible 14</td>
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<td>G1</td>
<td>Gap/growth 1</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNFR-related protein</td>
</tr>
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<td>GLD</td>
<td>Generalized lymphoproliferative disease</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
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<td>HOIL-1</td>
<td>Heme-oxidized IRP2 ubiquitin ligase 1</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>HOIP</td>
<td>Heme-oxidized IRP2 ubiquitin ligase 1 interacting protein</td>
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<tr>
<td>HRK</td>
<td>Harakiri</td>
</tr>
<tr>
<td>huCD2</td>
<td>Human CD2</td>
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<tr>
<td>ICAD</td>
<td>Inhibitor of caspase activated DNase</td>
</tr>
<tr>
<td>IEC</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of nuclear factor kappa B</td>
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<td>IKK</td>
<td>Inhibitor of nuclear factor kappa B kinase</td>
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<td>IKK2i</td>
<td>IKK2 inhibitor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
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<td>I.P</td>
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<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<td>Janus kinase</td>
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<td>KLF2</td>
<td>Kruppel like factor 2</td>
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<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
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<td>KLRG1</td>
<td>Killer Cell Lectin Like Receptor G1</td>
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<td>LAT</td>
<td>Linker for activation of T cells</td>
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<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<tr>
<td>LogFC</td>
<td>Log$_2$ fold change</td>
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<td>LPR</td>
<td>Lymphoproliferation</td>
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<td>LTβR</td>
<td>Lymphotoxin beta receptor</td>
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<tr>
<td>LUBAC</td>
<td>Linear ubiquitin chain assembly complex</td>
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<td>M1</td>
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<td>MALT1</td>
<td>Mucosa-associated lymphoid tissue lymphoma translocation 1</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<tr>
<td>mFasL</td>
<td>Membrane-bound Fas ligand</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIB2</td>
<td>Mind bomb E3 ubiquitin protein ligase 2</td>
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<tr>
<td>MK2</td>
<td>MAPK-activated kinase 2</td>
</tr>
<tr>
<td>MLKL</td>
<td>Mixed lineage kinase domain-like</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>MP</td>
<td>Memory phenotype</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory precursor effector cell</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
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<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
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<tr>
<td>NAP1</td>
<td>Nucleosome assembly protein 1</td>
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<td>NBD</td>
<td>Nuclear factor kappa B essential modulator binding domain</td>
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<td>Nec-1</td>
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<td>NEMO</td>
<td>Nuclear factor kappa B essential modulator</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor binding near the κ light-chain gene in B cells</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
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<td>nFPKM</td>
<td>Normalised fragments per kilobase per million mapped fragments</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
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<tr>
<td>PAMP</td>
<td>Pattern associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PKCθ</td>
<td>Protein kinase C theta</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>pTreg</td>
<td>Peripherally induced regulatory T cell</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RCD</td>
<td>Regulated cell death</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RHIM</td>
<td>RIP homotypic interaction motif</td>
</tr>
<tr>
<td>RIPK</td>
<td>Receptor-interacting serine/threonine-protein kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>S1P1</td>
<td>Sphingosine 1-phosphate receptor</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp, Cullin, F-box containing complex</td>
</tr>
<tr>
<td>SDD</td>
<td>Scaffolding and dimerization domain</td>
</tr>
<tr>
<td>sFasL</td>
<td>Soluble Fas ligand</td>
</tr>
<tr>
<td>Sharpin</td>
<td>SHANK Associated RH Domain Interactor</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology region 2 domain-containing phosphatase 1</td>
</tr>
<tr>
<td>SLEC</td>
<td>Short lived effector cell</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SPATA2</td>
<td>Spermatogenesis Associated 2</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK1 binding protein</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β activated kinase 1</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NF-kappa B activator</td>
</tr>
<tr>
<td>tBID</td>
<td>Truncated BH3-interacting domain death agonist</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK binding kinase 1</td>
</tr>
<tr>
<td>T&lt;sub&gt;cm&lt;/sub&gt;</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;em&lt;/sub&gt;</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>TNF Alpha Induced Protein 3</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TNFRSF</td>
<td>Tumour necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumour necrosis factor receptor type 1-associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor–associated factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRAIL-R</td>
<td>TNF-related apoptosis-inducing ligand receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;rm&lt;/sub&gt;</td>
<td>Resident memory T cell</td>
</tr>
<tr>
<td>tTreg</td>
<td>Thymic derived regulatory T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;vm&lt;/sub&gt;</td>
<td>Virtual memory T cell</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Zeta-chain-associated protein kinase 70</td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction

1.1 Adaptive immunity

Eukaryotic organisms are under continual threat from pathogenic microorganisms which exploit the host and subsequently cause disease. To combat this, multicellular organisms have developed an intricate system involving an array of different organs and cell types: the immune system. As such, immune pressure has spurred an evolutionary arms race between host and pathogen. The first line of defence is the innate immune response, comprised of cell types including macrophages and DCs. Underpinning innate immunity is the principle of pattern recognition. Germline encoded receptors termed pattern recognition receptors (PRRs), such as TLRs, recognise conserved repeated structures called pathogen associated molecular patterns (PAMPs) (Akira et al., 2006). Although the innate immune response is rapidly activated following infection, it has limited specificity and, as such, is escapable. This necessitates a second line of defence termed the adaptive immune response.

Adaptive immunity is slower to respond to infection and requires innate immunity for activation, but is highly specific. It primarily consists of T and B lymphocytes expressing randomly generated receptors with an extraordinarily large number of potential antigen specificities. T and B cell receptors are formed by rearrangement of the Tcr and Ig gene loci during which there is random addition and deletion of nucleotides (Flajnik and Kasahara, 2010). Due to the large range of receptor specificities which can be generated, T and B lymphocytes need to undergo a process of selection to delete cells with receptors reacting to self-antigens and avoid autoimmunity. In order to maintain an extensive diversity of antigen receptors, the body maintains a huge number of lymphocytes with only a very few expressing the same receptor. B cell responses constitute the humoral arm of the adaptive response, secreting antibodies which act to neutralise and opsonise toxins and foreign antigens (Cyster and Allen, 2019).
T cells are considered the cell mediated arm of the adaptive response, acting to target infected cells. Adaptive immune responses need to be tightly controlled and resolved after infection to prevent the potentially damaging consequences of excessive inflammation. Nonetheless, after a pathogen has been cleared, some lymphocytes remain, allowing more rapid adaptive responses if infected a second time and forming the basis of immunological memory.

1.2 T cells – a natural history

T lymphocytes are characterised as cells possessing a T cell receptor (TCR). However, they begin life without expressing a TCR in the bone marrow. From there, they migrate to the thymus where they develop and mature to express functional TCRs. T cells can differentiate into different lineages and this decision begins in the thymus. CD4+ T cells, termed helper T cells, recognise antigens presented by antigen presenting cells (APCs) on MHC Class II. CD8+ T cells, termed cytotoxic T cells, recognise antigens presented on any cell (apart from red blood cells) on MHC class I (Rossjohn et al., 2015). Following exit from the thymus, these cells are maintained in a quiescent state and recirculate around the body. T cells congregate in secondary lymphoid organs such as lymph nodes and the spleen, scanning APCs for cognate antigen. During an infection, recognition of foreign antigen leads to T cell activation. This is characterised by rapid clonal expansion, migration to the site of infection and acquisition of effector functions. Depending on the cytokine milieu, CD4+ T cells can differentiate into further subsets, each characterised by expression of different master transcription factors and production of different cytokines. In this way, different CD4+ T cell subsets are specialised to combat different types of pathogens. CD8+ T cells are particularly important for killing cells infected with viruses or intracellular bacteria, utilising enzymes to puncture holes in the cell membrane. Once the infection has been resolved, the majority of the responding T cells die due to a lack of survival factors. However, some T cells persist and differentiate further into memory T cells. Memory T cells can leave non-lymphoid tissues
and recirculate or can remain in tissues, such as the skin, gut and lungs, to become resident memory T cells (T<sub>r</sub>m) (Schenkel and Masopust, 2014). To maintain normal T cell homeostasis and prevent responses to harmless or self-antigens, regulatory T cells (Tregs), which are generated in both the thymus and periphery, act to shut down T cell responses both through direct interaction and by production of anti-inflammatory cytokines (Vignali et al., 2008). Ultimately, the development, maintenance and activation of T cells are complex processes mediated by carefully orchestrated cell programmes controlling survival and differentiation.

1.2.1 T cell development in the thymus

The site of T cell development and maturation is the thymus. Within the thymus, there are four main areas: the corticomedullary junction, cortex, subcapsular region and medulla. As thymocytes migrate through the different areas of the thymus, they receive different signals and make different interactions with different cell types. This acts to guide their development to ultimately generate mature thymocytes which have functional but not self-reactive TCRs.

Thymic progenitors produced in the bone marrow enter the thymus via the corticomedullary junction. Following thymic entry, these cells do not express CD4 or CD8 and are termed double negative (DN) thymocytes. DN thymocytes undergo a process of maturation which can be divided into 4 stages characterised by differing expression of CD25, CD44 and Kit (CD117) (Ceredig and Rolink, 2002). Notch signalling is crucial throughout DN thymocyte development, until β selection, to drive commitment to the T lineage (Radtke et al., 1999; Sambandam et al., 2005; Tan et al., 2005; Yui and Rothenberg, 2014). DN1 thymocytes consist of the early thymic progenitors that seed the thymus. They are defined as expressing high levels of Kit and CD44, but absent CD25 expression (CD44<sup>+</sup>CD25<sup>-</sup>) (Ceredig and Rolink, 2002). At this stage, they rapidly proliferate and still possess some potential to differentiate into other cell types such as DCs (Yui and Rothenberg, 2014). DN1 thymocytes then migrate into the cortex, via CXCR4.
and CCR7 signals, where they upregulate CD25 to become DN2 thymocytes (CD44+CD25+) (Ceredig and Rolink, 2002; Takahama, 2006). IL-7R induction at this stage promotes survival. The DN2 stage can be further split into DN2a and DN2b based on the expression of Kit (Masuda et al., 2007). DN2a thymocytes have high expression of Kit and still have potential to differentiate into DCs but following downregulation of Kit at the DN2b stage, they lose the potential to differentiate into other cell types (Rothenberg et al., 2008; Yui and Rothenberg, 2014). At the DN2b stage, commitment to the T cell lineage is complete.

Transition from the DN2b to the DN3a stage is characterised by downregulation of CD44 (CD44−CD25+) and migration to the subcapsular region of the thymus via CCR9 (Ceredig and Rolink, 2002; Takahama, 2006). Proliferation almost ceases and thymocytes express Rag1 and Rag2, allowing rearrangement of Tcrb, Tcrg and Tcrd loci. Other genes required for pre-TCR formation such as Cd3e and Ptcra are also induced. Upon successful completion of Tcrg and Tcrd gene rearrangement, those thymocytes expressing the γδ TCR continue development via a separate pathway to the αβ TCR lineage (Yui and Rothenberg, 2014). Once the Tcrb locus has been successfully rearranged, the TCRβ chain forms the pre-TCR together with the invariant pre-Tα chain and CD3. DN3a thymocytes then undergo the process of β selection whereby thymocytes with a functional pre-TCR are rescued from apoptosis and transition to the DN3b stage (von Boehmer, 2005). At this point thymocytes no longer rely on Notch signals, downregulate IL-7R and rapidly proliferate. Thymocytes subsequently transition to the DN4 stage which is associated with downregulation of CD25 (CD44−CD25−) and enter the thymus cortex (Ceredig and Rolink, 2002).

Pre-TCR signals induce the differentiation of DN4 thymocytes to double positive (DP) thymocytes, which are characterised by upregulation of both CD4 and CD8. Rag proteins are re-expressed in DP thymocytes to allow rearrangement of the Tcra locus. Following successful rearrangement, the TCRα chain pairs with TCRβ chain, forming the TCRαβ on the cell surface. DP thymocytes then undergo positive and negative selection to select TCRs
of the appropriate affinity but which do not react to self, ultimately leading to
differentiation to single-positive (SP) thymocytes (Takaba and Takayanagi,
2017). Within the cortex, cortical thymic epithelial cells (cTECs) present
peptides on MHC class I and II (Klein et al., 2014). Thymocytes with TCRs
which fail to interact with MHC undergo apoptosis in a process termed “death
by neglect” (Klein et al., 2014). Those thymocytes with TCRs that can
interact with MHC are rescued from cell death. DP thymocytes subsequently
commit to the CD8 or CD4 lineage depending on which MHC class the TCR
interacts with. Thus, those which interacted with class I express only CD8,
while those interacting with class II express only CD4. Following positive
selection, SP thymocytes upregulate CCR7 and migrate to the medulla.
Here, medullary thymic epithelial cells (mTECs) and DCs present tissue
restricted antigens on MHC (Takaba and Takayanagi, 2017). Expression of
autoimmune regulator (Aire) within mTECs permits the generation of
peripheral self-antigens of other cell types (Anderson et al., 2002). During
negative selection, thymocytes with TCRs which interact with the self-
peptide-MHC complexes, induce the expression of Bcl-2 family member Bim
and undergo Bim mediated apoptosis (Bouillet et al., 2002).

Once SP thymocytes have completed selection, they undergo further
maturation events in the medulla to prime their survival and function in the
periphery (Hogquist et al., 2015). Initially SP thymocytes express high levels
of CD24 and CD69, but low levels of MHC class I and are considered
“immature” (Kishimoto and Sprent, 1997; Xing et al., 2016). These SP
thymocytes are not competent to proliferate after TCR stimulation and
instead undergo apoptosis. As they mature, SP thymocytes receive NF-κB
and interferon signals, the latter of which is required for induction of Qa2
(Xing et al., 2016). SP thymocytes are termed “mature” following
downregulation of CD24 and CD69 and upregulation of MHC class I
(Kishimoto and Sprent, 1997; Xing et al., 2016). Mature SP thymocytes are
competent to proliferate following TCR stimulation (Xing et al., 2016). For
mature SP thymocytes to egress the thymus, the transcription factor Kruppel-
like factor 2 (KLF2) drives the upregulation of CD62L and S1P1 (Carlson et
al., 2006; Weinreich et al., 2011).
1.2.2 Naïve T cell homeostasis

Once T cells exit from the thymus and before they encounter antigen, they are termed “naïve”. Naïve T cells are characterised as expressing low levels of CD44 but high levels of CD62L, allowing them to circulate through the lymphatics. To ensure maintenance of the extraordinarily diverse TCR repertoire, naïve T cells are primarily maintained by two crucial signals: TCR and IL-7R.

Naïve T cells have TCRs with high affinity for foreign antigens but also have low affinity for self-antigens. The interaction of TCRs with self-peptides loaded onto MHC class I or class II is crucial for maintenance of CD8+ and CD4+ naïve T cells in the periphery (Kawabe et al., 2021). In mice, deficient in either MHC, TCR or intracellular TCR signalling components, survival of naïve T cells is reduced and there is a decline in T cell numbers (Labrecque et al., 2001; Martin et al., 2003; Polic et al., 2001; Seddon and Zamoyska, 2002; Takeda et al., 1996; Tanchot et al., 1997). These peptide-MHC-TCR interactions do not result in T cell activation. Those TCRs with relatively high self-reactivity are tuned following positive selection to keep these TCR signals below the threshold required for T cell activation. This involves the upregulation of negative regulators of TCR signalling such as CD5, CD45 and SHP-1 (Cho et al., 2016; Stephen et al., 2009; Tarakhovsky et al., 1995).

Alongside TCR signals, naïve T cells also require IL-7 signals for peripheral survival. The IL-7R, comprised of IL-7Rα and the common γ chain, is expressed on naïve T cells (Kondo et al., 1994). This allows them to sense IL-7, the majority of which is thought to be produced by fibroblastic reticular cells and lymphatic endothelial cells within lymph nodes (Hara et al., 2012; Link et al., 2007; Miller et al., 2013; Onder et al., 2012). The binding of IL-7 to IL-7R promotes naïve T cell survival by driving Jak/Stat signalling and inducing the upregulation of Bcl-2, which blocks Bim induced apoptosis (Akashi et al., 1997). IL-7 deficient mice have reduced numbers of peripheral naïve T cells, however forced expression of Bcl-2 can rescue naïve T cells
(Akashi et al., 1997; Schluns et al., 2000; Tan et al., 2001). Similarly, naïve T cells numbers are reduced in Bcl-2 deficient mice, but can be rescued upon additional ablation of Bim (Wojciechowski et al., 2007). IL-7 mediated survival of naïve T cells can be mediated at the level of IL-7R expression. The Foxo1 transcription factor is crucial for IL-7R upregulation and maintenance (Kerdiles et al., 2009; Ouyang et al., 2009). IL-7 itself can negatively regulate the surface expression of IL-7R on naïve T cells (Park et al., 2004). Nonetheless, IL-7 is usually limiting and thus survival of naïve T cells depends on competition between themselves and other cell types such as innate lymphoid cells (Martin et al., 2017).

1.2.3 T cell activation

At the steady state, naïve T cells exist in a quiescent state and circulate between the blood and secondary lymphoid organs. But upon encountering their cognate antigen, T cells undergo a substantial transformation into activated, highly proliferative T cells which acquire a range of effector functions to combat infection. The binding of the TCR to peptide-MHC on APCs leads to formation of the immunological synapse (Dustin, 2014). Within the synapse, further co-stimulatory ligand-receptor interactions take place. In particular, the binding of CD28 on T cells with CD80 and CD86 on APCs, inducing activation of PI3K and NF-κB, is crucial for T cell activation (Chen and Flies, 2013). Further co-stimulatory receptors on T cells include members of the TNFRSF including 4-1BB, OX40, CD27 and CD30 which activate NF-κB (Chen and Flies, 2013). CD8 and CD4 co-receptors also bind to MHC class I or II. Proximal TCR signalling events involve multiple tyrosine kinases (Gaud et al., 2018). LCK bound to either CD4 or CD8 is brought into close proximity with the TCR and phosphorylates the ITAMs of the CD3. ZAP70 binds to the phosphorylated CD3 chains and subsequently phosphorylates LAT. Consequently, LAT can bind to other proteins to form the LAT signalosome. This drives the activation of distal TCR signalling pathways including ERK1/2, Ca²⁺, NF-κB and p38/JNK MAPK signalling (Chen and Flies, 2013). Ultimately, these signals drive T cell priming, proliferation and effector functions.
Within the first 24 hrs after receiving a TCR stimulus, T cells rewire their metabolic pathways to prepare for rapid cell division. This process is characterised by a shift in metabolism from fatty acid β oxidation to glycolytic and pentose phosphate pathways (Shyer et al., 2020). Furthermore, there is a dramatic increase in the synthesis of proteins, lipids and nucleic acids. Facilitating this is an increase in the expression of glucose transporters and amino acid transporters. As a result, the T cells increase in size in a process termed T cell growth or blasting. Underpinning these changes is the transcription factor myc (Marchingo et al., 2020; Wang et al., 2011). TCR and CD28 signals induce the expression of myc through Akt, ERK and mTOR signalling pathways (Wang et al., 2011). Inducible deletion of myc in T cells leads to a block in T cell growth and proliferation in vitro and in vivo (Wang et al., 2011).

T cell activation drives entry into cell cycle phase G1 by inducing the expression of cell cycle proteins including cyclins, CDKs and E2F proteins (Appleman et al., 2000; Lewis and Ly, 2021). The expression of some of these proteins is regulated by myc including Cyclin A, Cdk2 and Cdk4 (Wang et al., 2011). High levels of the cell cycle inhibitor p27 are expressed in naïve T cells, which binds to and inhibits the activity of CDK1/2 (Wells and Morawski, 2014). TCR/CD28 stimulation induces the activation of the kinases Lyn and AKT which phosphorylates p27. Cyclin E is also upregulated which further leads to p27 phosphorylation by activating CDK2 (Appleman et al., 2000; Boussiotis et al., 2000). This results in the degradation of p27 which is necessary for progression to S phase. Indeed, forced expression of p27 leads to cell cycle arrest and failure of T cells to proliferate and produce IL-2 (Boussiotis et al., 2000). On the other hand, T cells lacking p27 proliferate more in absence of co-stimulation or following co-stimulation blockade (Rowell et al., 2005, 2006). Mice deficient in p27 exhibit increased CD4+ T cell expansion during LCMV infection in vivo (Jatzek et al., 2012).
For sustained proliferation, activated T cells also require cytokine signals, especially IL-2. Before TCR activation, T cells do not express the high affinity IL-2R. NFAT, NF-κB and AP-1 signalling downstream of TCR and CD28 drive induction of CD25, allowing surface expression of the high affinity IL-2R (Liao et al., 2013a). Activated T cells also secrete high amounts of IL-2 which acts in both an autocrine and paracrine manner (Boyman and Sprent, 2012). When activated in the absence of IL-2, T cells exhibit reduced survival and downregulate the expression of cyclins and CDKS while upregulating the expression of p27, thereby impairing cell cycle progression (Rollings et al., 2018). But although IL-2 was identified as important for T cell proliferation in vitro, adoptive transfer and bone marrow chimera experiments revealed that IL-2R signalling had a minimal impact on T cell expansion during primary responses (Williams et al., 2006; Yu et al., 2003). Nonetheless, some studies have suggested IL-2 does sustain late CD8⁺ T cell proliferation (D’Souza and Lefrançois, 2003; Obar et al., 2010). Instead, it’s thought that IL-2R signalling guides CD4⁺ and CD8⁺ T cell differentiation by regulating the expression of transcription factors such as Eomes, T-bet, and Blimp-1, cytokine receptors such as IL-12R and cytokines such as IFN-γ (Ross and Cantrell, 2018).

1.2.4 Memory T cell generation and maintenance

After recognising antigen, T cells can persist and differentiate into memory cells. In mice, memory T cells express high levels of CD44 and can be subdivided based on the expression of CD62L. Central memory T cells (Tcem) have high CD62L expression and circulate via lymphoid tissues. Effector memory T cells (Tem) have low CD62L expression and circulate via non-lymphoid tissues.

CD8⁺ memory T cells derive from memory precursor effector cells (MPECs) generated during the T cell response, which have low expression of KLRG1 but high expression of IL-7R (Kaech et al., 2003). The differentiation of MPECs and memory T cells over short lived effector cells (SLECs), which do not become memory cells, is decided by TCR, costimulatory and cytokine signals (Kawabe et al., 2021). The effector T cells which receive short TCR
signals, which occurs at the later stages of infection, preferentially differentiate into MPECs whereas those with prolonged stimulation do not (Sarkar et al., 2008). Co-stimulation by receptors such as OX40, CD27 and 4-1BB is also thought to be important for generation of memory T cells. CD27, OX40L and 4-1BB knockout mice exhibit impaired CD8\(^+\) memory generation in response to influenza (Hendriks et al., 2000, 2005). Furthermore, OX40 appeared to be particularly important for the generation of MPECs in response to listeria infection (Mousavi et al., 2008). Both IL-2 and IL-12 signalling are thought to have a negative impact on memory generation. Those effector T cells with relatively low CD25 expression, exhibit less sustained IL-2R signalling and have greater propensity to differentiate into MPECs (Kalia et al., 2010; Pipkin et al., 2010). High IL-12 concentrations during T cell priming promotes SLEC differentiation by inducing the expression of T-bet, whereas low IL-12 concentrations promote MPEC differentiation resulting from less T-bet expression (Joshi et al., 2007).

The signals which drive the development of CD4\(^+\) memory T cells are less defined but are thought to be similar to those required for CD8\(^+\) memory cells (Kawabe et al., 2021). While CD27 and OX40 signals are important for CD4\(^+\) memory generation, strong TCR and sustained IL-2R signalling are detrimental to this process (Hendriks et al., 2000; Snook et al., 2018; Soroosh et al., 2007).

For maintenance of CD4\(^+\) and CD8\(^+\) memory T cell populations, IL-7 and IL-15 signalling is crucial, without a need for peptide-MHC (Kawabe et al., 2021). In vivo blockade of IL-7R signalling leads to impaired survival and homeostatic proliferation of CD8\(^+\) and CD4\(^+\) memory T cells due to defective Bcl-2 expression (Goldrath et al., 2002; Kondrack et al., 2003; Lenz et al., 2004; Schluns et al., 2000). IL-15 also plays a role for maintenance of memory T cell populations, particularly for CD8\(^+\) memory T cells since they have high expression the IL-15R. IL-15 knockout mice exhibit impaired homeostatic proliferation of CD8\(^+\) memory T cells generated after LCMV infection (Becker et al., 2002).
1.2.5 Memory phenotype T cells

Memory T cells also exist in unchallenged mice which have not been exposed to foreign antigen. These populations are termed “memory phenotype” (MP) cells and are thought to derive after recognising self-antigens since they are present in germ and antigen free mice (Kim et al., 2016). CD4+ MP cells are generated from peripheral naïve T cells in response to self-peptide-MHC and costimulation (Kawabe et al., 2017). In lymphopenic conditions, such as in neonates, this leads to rapid proliferation (Kawabe et al., 2021). More recently, it’s been shown that CD4+ MP cells are also generated throughout the life course (Kawabe et al., 2017). For CD8+ MP cells, the vast majority express CD122 and are termed “virtual” or “innate” memory T cells (T\(\text{vm}\)). Unlike CD4+ MP cells, CD8+ T\(\text{vm}\) are thought to have a thymic origin. They are generated from mature CD8+ SP thymocytes and upregulate Eomes in response to self-peptide-MHC (Daniels and Teixeiro, 2020; Miller et al., 2020). Once they exit the thymus into the periphery, other currently unknown signals are thought to be required for full differentiation into T\(\text{vm}\) cells.

Cytokine signalling is thought to be important for the maintenance of both CD4+ and CD8+ MP cells (Kawabe et al., 2021). CD4+ MP cells continue to proliferate and turnover at a rate dependent on the affinity of the TCR for self-peptide-MHC (Kawabe et al., 2017). This proliferation is not thought to be dependent on TCR signals but is dependent on CD28 and IL-7 signals (Kawabe et al., 2017; Younes et al., 2011). Since CD8+ T\(\text{vm}\) express high levels of IL-15R, its thought that IL-15 signalling is important for maintenance of this population, alongside IL-7R signalling (Tan et al., 2002; Zhang et al., 1998).

1.2.6 Regulatory T cell generation and homeostasis

Regulatory T cells (Tregs) are crucial for suppression of immune responses to ensure peripheral tolerance and prevention of autoimmunity. Tregs can develop either in the thymus (tTregs) or the periphery (pTregs) and are
characterised by the expression of the transcription factor FoxP3. Scurfy mice with a mutation in Foxp3, resulting in a lack of expression, develop a lethal lymphoproliferative syndrome, due to an absence of FoxP3+ Tregs (Brunkow et al., 2001).

During selection in the thymus, some CD4+ SP thymocytes possessing TCRs with high affinity for self-peptide-MHC manage to avoid deletion. A two-step model has been established to describe Treg development. Firstly, TCR and CD28 signals drive differentiation of Treg precursors, of which there are two distinct populations (Lio et al., 2010; Moran et al., 2011; Tai et al., 2005; Vang et al., 2010). Stronger TCR signals induce the expression of CD25 leading to generation of CD25+FoxP3- precursors whereas weaker TCR signals lead to formation of a CD25-FoxP3lo precursor population (Lio and Hsieh, 2008; Marshall et al., 2014; Owen et al., 2019a, 2019b). In the second step, IL-2 and IL-15 induce STAT5 signalling and promote the induction of FoxP3 expression to generate mature Tregs (CD25+FoxP3+) (Burchill et al., 2007; Lio and Hsieh, 2008; Marshall et al., 2014). It’s thought that FoxP3 expression can also lead to death of developing Tregs unless it is countered by IL-2 signals (Tai et al., 2013). TCR/CD28 signalling also lead to upregulation of TNFRSF members such as GITR, OX40 and TNFR2, with the level of expression corresponding to the strength of TCR signalling (Mahmud et al., 2014). The ligands of these receptors are produced by APCs and augment the IL-2 dependent induction of FoxP3 (Mahmud et al., 2014).

Following the export of Tregs into the periphery, IL-2 signalling plays an important role for Treg homeostasis. CCR7 expression directs Tregs to the T-cell zone of secondary lymphoid organs where IL-2 is produced by T cells (Smigiel et al., 2013). Tregs continue to express the high affinity IL-2R comprised of CD25, CD122 and the common γ chain. In thymectomised mice, treatment with anti-IL-2 leads to a reduction in peripheral Tregs resulting in autoimmunity (Setoguchi et al., 2005). Specific ablation of CD122 or CD25 in mature Tregs leads to a lethal lymphoproliferative syndrome, but milder compared to Scurfy mice (Chinen et al., 2016; Fan et al., 2018; Toomer et al., 2019). This was characterised by a reduction in Treg numbers
and function. It’s thought that IL-2 signalling is required for continued FoxP3 and CD25 expression by Tregs, to maintain Treg stability and suppressive function (Fontenot et al., 2005). IL-2 also promotes the survival of peripheral Tregs, which is thought to occur by regulation of the Bcl-2 family member Mcl-1 (D’Cruz and Klein, 2005; Pierson et al., 2013).

In a similar fashion to naïve T cells, Tregs also receive TCR/CD28 signals which contribute to peripheral Treg homeostasis (Moran et al., 2011). Ablation of the TCR in Tregs showed that although TCR signalling is not required for maintenance of FoxP3 expression, it is required for peripheral homeostatic expansion (Vahl et al., 2014). In support, DC numbers correlate with Treg numbers and have been shown to drive the homeostatic proliferation of Tregs in a manner dependent on the expression of MHC class II (Darrasse-Jèze et al., 2009).

1.3 NF-κB transcription factors

Nuclear factor binding near the κ light-chain gene in B cells (NF-κB) is a family of transcription factors first identified in the lab of David Baltimore 36 years ago (Sen and Baltimore, 1986). As the name suggests, NF-κB was originally found to regulate expression of the κ light-chain in activated B cells, however it is now known to be expressed in a wide variety of cell types and has been highly conserved throughout vertebrate evolution. This is primarily because NF-κB allows cells to sense changes to the local microenvironment, such as inflammation, and rapidly respond by inducing the transcription of genes involved in cellular survival, differentiation, and function. Linking NF-κB to an array of receptors on the cell surface are a network of signalling pathways involving many protein complexes. Such is the sophistication of these pathways, there is a need for them to be tightly regulated; and dysregulation of NF-κB signalling has been implicated in a variety of diseases including arthritis, cancer and inflammatory bowel disease.
1.3.1 The NF-κB family

Five protein subunits constitute the NF-κB family: RelA (p65), RelB, c-Rel, p50 and p52. Common between the 5 subunits is the presence of a Rel homology domain (RHD) and a nuclear localisation sequence (NLS) at the N-terminus (Zhang et al., 2017). The RHD enables the subunits to form hetero- and homo-dimers with each other, with up to 15 different combinations thought possible, although the most common are RelA:p50, c-Rel:p50 and RelB:p52 (Zhang et al., 2017). The RHD is also the region which binds to the NF-κB DNA consensus sequence located in gene promoters, otherwise known as the κB site: 5’-GGGRNWYYCC-3’ (Zhang et al., 2017). NF-κB subunits can be further divided into 2 groups based on their structures. RelA, RelB and c-Rel all possess a transactivation domain (TAD) at their C-terminus, which is required to drive gene transcription (Zhang et al., 2017). Instead, p50 and p52 lack this domain and therefore homodimers of these can serve as transcriptional repressors. Unlike the Rel proteins, p50 and p52 are not directly formed by protein synthesis but are generated upon proteasomal processing of NF-κB1 (p105) and NF-κB2 (p100) respectively (Zhang et al., 2017).

At the steady state, NF-κB dimers are sequestered in the cytoplasm by the Inhibitor of κB (IkB) proteins: IkBα, IkBβ, IkBε, IkBz, IkBNS, p100, p105 and BCL-3. These are characterised by the presence of ankyrin repeats at their C-terminus which act to mask the NLS of the Rel proteins, thereby blocking translocation of NF-κB dimers to the nucleus (Zhang et al., 2017). Following induction of NF-κB signalling, the IkB proteins are phosphorylated and subsequently K48-linked ubiquitinated by SCF E3 ligases, targeting them for proteasomal degradation (Zhang et al., 2017). NF-κB dimers can then translocate into the nucleus and initiate gene transcription. The elegance of this system, and perhaps an important reason for its conservation across species, is that the NF-κB dimers are pre-formed and can induce gene transcription within 30 mins of activation, allowing a rapid cellular response to external stimuli.
Nuclear translocation of distinct NF-κB dimers can be induced by different cell surface receptors via different signalling pathways, namely either the canonical (Classical) or non-canonical (Alternative). The canonical pathway primarily controls the activity of RelA/p50 and c-Rel/p50 dimers whereas the non-canonical pathway regulates RelB/p52 dimers. But a common feature of both pathways is the utilisation of IκB kinases (IKK) to induce activation of NF-κB in response to external stimuli.

### 1.3.2 Canonical NF-κB signalling

Interactions between cell surface receptors, including TNFR1, TCR, BCR, IL-1R and TLR4, and their cognate ligands induces NF-κB by activating the IKK complex. This is a multi-subunit complex consisting of two serine/threonine kinases, IKK1 (IKKα) and IKK2 (IKKβ), and the regulatory subunit NEMO (IKKγ). Both kinases have a kinase domain, a scaffolding and dimerization domain (SDD) and a NEMO-binding domain (NBD) (Hayden and Ghosh, 2012; Liu et al., 2012). Crystal structures of IKK2 have revealed that the SDD mediates dimerization of the two kinases, which is essential for activation of IKK, and the ULD-SDD region interacts with IκBα and positions it for phosphorylation (Liu et al., 2013; Polley et al., 2013; Xu et al., 2011). Phosphorylation of specific serine residues of the so called “destruction box” by the IKK complex subsequently drives degradation of IκBα, liberating RelA/p50 dimers to enter the nucleus (Hayden and Ghosh, 2012). But for IKK itself to become active, it too requires phosphorylation of serine residues, specifically those within the activation loops of IKK1 (Ser176 and Ser180) and IKK2 (Ser177 and Ser181) (Mercurio et al., 1997). NEMO is crucial for IKK activation since it contains a ubiquitin binding domain which couples the IKK complex to polyubiquitinated signalling components that assemble around receptors once triggered, thereby bringing IKK into contact with upstream kinases (Hayden and Ghosh, 2012).

IKK1 and IKK2 both show high sequence identity, however studies have revealed differences between their roles in canonical NF-κB activation. IKK1−/− cells have normal IκBα degradation however IKK2−/− cells show some
impairment in NF-κB activation (Hu et al., 1999; Li et al., 1999a, 1999c; Takeda et al., 1999; Tanaka et al., 1999). This discrepancy can be accounted for by the ability of IKK1 and IKK2 to form homodimers together with NEMO. However, IKK2 has more potent kinase activity towards IκBα and binds with higher affinity to NEMO than IKK1 (Huynh et al., 2000; Li et al., 1998; May et al., 2002; Mercurio et al., 1997). IKK2 homodimers are better activators of NF-κB than IKK1 homodimers, but the activity of both is sub-optimal compared to IKK1-IKK2 heterodimers (Huynh et al., 2000). Cells with deletion of both IKK1 and IKK2 fail to induce any NF-κB since NEMO alone does not have a kinase domain (Li et al., 2000). But despite NEMO possessing no catalytic activity, knockout of the protein results in a complete block in NF-κB activation, demonstrating that it is essential for IKK activity (Rudolph et al., 2000).

1.3.3 Non-canonical NF-κB signalling

Although IKK1 is an important but not essential component of the canonical pathway, it plays a crucial role in the non-canonical pathway. Activation of TNFRSF members such as TNFR2, BAFF-R, CD27, CD40, FN14, LTβR, OX40 and RANK, can induce IKK1 activity, independently of IKK2 and NEMO (Liang et al., 2006; Senftleben et al., 2001). This occurs through the stabilisation of NIK which then accumulates in the cell as it is continuously synthesised. NIK subsequently phosphorylates and activates IKK1 (Ling et al., 1998; Senftleben et al., 2001). In the absence of stimulus, p100 acts as an IκB protein to sequester RelB in the cytoplasm. By functioning as a scaffold, NIK is thought to recruit IKK1 to p100 (Xiao et al., 2004). Activated IKK1 consequently targets p100 by phosphorylation, in a similar fashion to IκBα, for ubiquitination and proteasomal processing to produce p52 (Senftleben et al., 2001). The active RelB/p52 dimer can then translocate to the nucleus and initiate gene transcription.

1.3.4 Receptor induced NF-κB activation
Multiple receptors can induce NF-κB activation though either the canonical or non-canonical pathways, however some receptors are able to activate both. Activation of the NF-κB pathway by surface receptors is achieved in different ways using different cellular components. The best characterised activator of NF-κB is TNF, which can bind to either TNFR1 or TNFR2 and induce both canonical and non-canonical pathways (Fig. 1.1). In T cells specifically, TCR can also activate canonical NF-κB upon interaction with peptide-MHC (Fig. 1.1).

1.3.4.1 TCR signalling

The interaction between TCR and peptide-MHC triggers a multitude of signalling pathways activating an array of different transcription factors. These drive gene transcription for T cell proliferation, differentiation, and effector functions. Canonical NF-κB is one such pathway induced during T cell activation and relies upon the assembly of the CBM complex, consisting of CARD11 (CARMA1), BCL10 and the paracaspase MALT1. Before a TCR stimulus is received, CARD11 exists in an inactive conformation, unbound to BCL10-MALT1 complexes (Ruland and Hartjes, 2019). Once a T cell is activated, initial proximal TCR signalling alongside CD28 co-stimulation drives activation of PKCθ (Ruland and Hartjes, 2019). PKCθ then phosphorylates the linker region of CARD11, allowing it to interact with BCL10, thereby forming the CBM complex (Matsumoto et al., 2005; Sommer et al., 2005).

Oligomerisation of CARD11 initiates the formation of BCL10 filamentous oligomers bound to MALT1 dimers, establishing a large scaffold necessary for the recruitment of the E3 ligases TRAF6 and LUBAC (Oikawa et al., 2020; Qiao et al., 2013). The addition of linear ubiquitin chains to BCL10 by LUBAC is thought to provide binding sites for NEMO (Dubois et al., 2014; Yang et al., 2016). Both BCL10 and MALT1 are ubiquitinated with K63-linked chains by TRAF6 which are also recognised by NEMO together with TAK1 (Oeckinghaus et al., 2007; Sun et al., 2004; Wu and Ashwell, 2008).
Consequently, the IKK complex is phosphorylated and activated by TAK1 to induce NF-κB activation.

Although MALT1 primarily functions as a scaffold, it also possesses paracaspase activity which can serve to augment activation of NF-κB. Dimerisation of MALT1 bound to BCL10 induces its protease activity, enabling it to cleave A20 and CYLD, both negative regulators of NF-κB (Coornaert et al., 2008). This ensures maximal induction of NF-κB at the point of TCR stimulation.

**1.3.4.2 TNFR1 signalling**

TNFR1 is expressed by most cell types, including T cells, and can interact with both membrane-bound and soluble TNF. TNF exists as a trimer and induces trimerization of TNFR1 after binding (Banner et al., 1993; Vanamee and Faustman, 2018). Although it is not fully understood how TNF binding translates to the cytoplasmic events which follow, it is thought that clustering of the receptor intracellular domains permits recruitment of signalling components. Specifically, TNFR1 contains a cytoplasmic death domain which interacts with the death domain of TRADD (Hsu et al., 1995). TRADD serves as an adaptor protein to recruit TRAF2, using its TRAF2 binding domain, and the serine-threonine kinase RIPK1, through its death domain, to TNFR1 (Hsu et al., 1996a, 1996b). Although RIPK1 can bind directly to TNFR1, this interaction is comparatively weak compared to that with TRAF2. Collectively these proteins form what has been termed “complex I” (Micheau and Tschopp, 2003). Importantly, despite RIPK1 possessing kinase activity, this is not required for NF-κB activation where it primarily functions as a scaffold (Polykratis et al., 2014).

For complex I to drive canonical NF-κB, it becomes cloaked in polyubiquitin chains of different types. Despite having E3 ubiquitin ligase activity itself, TRAF2 recruits cIAP1 and cIAP2 which function to decorate complex I, including RIPK1 and the cIAPs themselves, in K63-, K11- and K48- linked polyubiquitin chains (Bertrand et al., 2008; Dynek et al., 2010; Mahoney et
al., 2008; Varfolomeev et al., 2008; Vince et al., 2009). These branched polyubiquitin chains act as docking sites for LUBAC, another E3 ligase which consists of HOIL-1, HOIP and Sharpin (Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011). LUBAC then catalyses the addition of linear M1 linked ubiquitin chains to TNFR1, TRADD, RIPK1 and NEMO, promoting stabilisation of complex I (Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011; Tokunaga et al., 2009, 2011).

The polyubiquitin network which forms around complex I serves to bring the IKK complex into proximity with the kinase responsible for its activation, TAK1. NEMO is recruited to the complex by binding both K63- and M1-linked ubiquitin chains, but interacts with greater affinity with M1 linked chains (Ea et al., 2006; Hadian et al., 2011; Rahighi et al., 2009; Wu et al., 2006). TAB2 and TAB3 also bind to the K63 linked chains and lead to recruitment and activation of TAK1 (Ea et al., 2006; Kanayama et al., 2004). TAK1 subsequently phosphorylates the activation loops of IKK1 and IKK2 leading to activation of the complex and canonical NF-κB activation (Wang et al., 2001).

1.3.4.3 TNFR2 signalling

Expression of TNFR2 is more restricted than TNFR1, however it is expressed on T cells. TNFR2 preferentially binds to membrane TNF and distinct from TNFR1, it contains a TRAF binding domain but not a death domain. Consequently, TRAF proteins can be recruited directly without the need for TRADD or RIPK1. At the steady state, TRAF3 binds to NIK and utilises TRAF2 to recruit cIAP1 and cIAP2 (Liao et al., 2004; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Activation of the E3 ligase activity of cIAP1/2 leads to K48-linked ubiquitination of NIK and subsequent proteasomal degradation (Vallabhapurapu et al., 2008; Varfolomeev et al., 2007; Zarnegar et al., 2008). As such, constitutive degradation of NIK maintains it at a low level in the cell.
Upon interaction of TNF with TNFR2, TRAF2 is recruited to the membrane, bringing along with it TRAF3, cIAP1 and cIAP2 (Rothe et al., 1994, 1995a, 1995b). K63-linked ubiquitination of cIAP1/2 by TRAF2 permits cIAP1/2 to catalyse K48-linked ubiquitination of TRAF3, targeting TRAF3 for proteasomal degradation (Sun, 2012; Vallabhapurapu et al., 2008). The drop in cellular TRAF3 levels results in less degradation of NIK, allowing it to accumulate and drive non-canonical NF-κB activation.
Figure 1.1 Canonical and non-canonical NF-κB signalling pathways

The canonical NF-κB signalling pathways can be induced by TNFR1 and TCR. Stimulation of TNFR1 and TCR leads to the formation of a ubiquitin scaffold in a TRAF dependent manner. The TAB1/2/TAK1 and IKK complexes are subsequently recruited, allowing phosphorylation and activation of the IKK complex by TAK1. Activated IKK phosphorylates IkB proteins, thus targeting them for proteasomal degradation. RelA/p50 and c-Rel/p50 dimers can then translocate into the nucleus and induce the transcription of genes involved in cell survival, differentiation and inflammation. The non-canonical NF-κB subunits RelB and p100 are also direct targets of canonical NF-κB dimers (Basak et al., 2008; Bren et al., 2001; Lombardi et al., 1995).

The non-canonical NF-κB signalling pathway can be induced by TNFR2. Binding of TNF to TNFR2 leads stabilisation of NIK. NIK phosphorylates and activates IKK1 which subsequently phosphorylates p100, thus targeting it to
the proteasome. Dimers of RelB and p52 are then able to translocate to the nucleus and induce gene transcription. Schematic adapted from (Blanchett et al., 2021).
1.4 Cell death

Although cells have a variety of pathways to ensure survival, including NF-κB; in some scenarios cell death is beneficial for the organism to ensure normal homeostasis. This regulated cell death (RCD) is purposely induced by the cell, unlike accidental cell death (ACD) which is unplanned and occurs as a result of extreme cellular damage. RCD has prominent roles in the immune system, including the death of thymocytes during negative selection and during the reestablishment of immune homeostasis after infection. It is now known that there are many different forms of regulated cell death, including apoptosis and necroptosis, which can occur both in response to internal stress and extracellular signals.

Cells dying via apoptosis display particular morphological characteristics including nuclear and DNA fragmentation, chromatin condensation, cell shrinkage, plasma membrane blebbing and generation of apoptotic bodies (Galluzzi et al., 2018). Apoptosis has long been considered immunogenically silent since, in most cases, it does not result in the release of DAMPs and apoptotic cells are rapidly phagocytosed. It can be induced by both intracellular stress/damage (Intrinsic apoptosis) and extracellular signals (Extrinsic apoptosis). But both forms of apoptosis converge on the activation of executioner caspases: Caspase-3, -6 and -7. Caspases are a family of proteases with crucial roles in apoptosis, so called because they cleave substrates at sites next to aspartate residues. They exist in cells as inactive zymogens termed pro-caspases, which are activated upon cleavage of the pro domain. Once activated, the executioner caspases cleave a large number of substrates, including cytoskeletal and nuclear lamina proteins, thereby driving the distinctive changes in cellular morphology during apoptosis (Fischer et al., 2003). Finally, caspase-3 cleaves DFF/CAD, releasing it from its inhibitor DFF45/ICAD (Enari et al., 1998; Liu et al., 1997). This enables DFF/CAD to dimerise and consequently bind to and cleave DNA (Widlak and Garrard, 2005).
Cells can also die by a more inflammatory form of RCD called necroptosis. This is characterised by classical features of necrosis such as cell swelling, plasma membrane rupture and the release of DAMPs (Grootjans et al., 2017). In contrast to apoptosis, necroptosis does not require the activity of caspases. Given the immunogenic nature of necroptosis, it is thought to play an important role during infections by attracting immune cells to the site of infection.

But why have cells evolved to have multiple cell death pathways? One reason is due to the evolutionary arms race between pathogens and their hosts. Apoptosis is an ancient pathway, having first arisen in early metazoans such as cnidarians, whereas necroptosis is thought to have evolved much later in vertebrates (Tummers and Green, 2022). A number of bacteria, such as *Shigella*, *E. coli*, and *Yersinia*, and viruses, including cytomegalovirus and Epstein barr virus, have been identified which encode proteins to subvert apoptotic and necrototic pathways (Tummers and Green, 2022). Inhibiting cell death pathways is of clear benefit to pathogens, thus promoting their survival, replication and transmission. Pathogens can inhibit multiple cell death pathways at multiple stages via different mechanisms.

1.4.1 **Intrinsic pathways of apoptosis**

Intrinsic apoptosis is primarily induced by intracellular damage and stress signals, including ROS accumulation, DNA damage, nutrient and growth factor deficiency and ER stress (Galluzzi et al., 2018). It is regulated by members of the BCL-2 family, characterised by the presence of BH domains, which can be sub-divided based on whether they have pro- or anti-apoptotic activity (Kale et al., 2018). Together, the Bcl-2 family mediate events which culminate in mitochondrial outer membrane permeabilization (MOMP) and apoptosis (Kale et al., 2018).

Sensing of intracellular damage/stress activates the pro-apoptotic BH3-only proteins (BIM, BAD, BID, NOXA, PUMA, BIK, BMF and HRK). Different
apoptotic triggers can involve the activation of different BH3-only proteins, through either transcriptional induction (e.g. BIM, PUMA, NOXA) or post-translational modification (e.g BID) (Galluzzi et al., 2018; Kale et al., 2018). BH3-only proteins can be classed as either activator (BIM, BID, NOXA and PUMA) or sensitiser (BAD, HRK, BMF). Activator BH3-only proteins migrate to the mitochondrial membrane and bind directly to the pore-forming proteins BAX and BAK, which are comprised of 4 BH domains (Czabotar et al., 2013; Kale et al., 2018). This induces conformational changes in BAX and BAK to form homodimers, leading to dissociation of BH3-only proteins. Oligomerisation of BAK/BAX dimers precedes pore formation in the mitochondrial outer membrane and subsequent release of mitochondrial proteins (Antonsson et al., 2001; Bleicken et al., 2014).

However, other anti-apoptotic proteins of the BCL-2 family reside in the mitochondrial membrane and act to inhibit BAK and BAX activity. BCL-2, BCL-XL, BCL-W, BFL-1/A1 and MCL-1 inhibit oligomerisation of BAK and BAX both directly and indirectly, by binding to activator BH3-only proteins (Galluzzi et al., 2018; Kale et al., 2018). Nonetheless, the anti-apoptotic proteins can also be bound by the sensitiser BH3-only proteins, thereby sequestering them from BAK and BAX (Kale et al., 2018; Yi et al., 2003). Ultimately, progression to MOMP depends on the balance between anti-apoptotic and pro-apoptotic BH3-only proteins at the mitochondrial membrane.

Establishment of MOMP allows proteins such as cytochrome c and DIABLO/SMAC to leak from the mitochondrial intermembrane space into the cytoplasm. Once there, cytochrome c interacts with APAF1 to generate the apoptosome, allowing recruitment and activation of caspase-9 (Li et al., 1997). Active caspase-9 cleaves and activates the executioner caspases, caspase-3 and caspase-7. Simultaneously, Smac/DIABLO promotes caspase activation by inhibiting IAP proteins, in particular XIAP, a potent caspase inhibitor (Du et al., 2000; Verhagen et al., 2000).
1.4.2 **Extrinsic pathways of apoptosis**

Apoptosis can be triggered by cell surface receptors of the TNFR superfamily, which possess an intracellular death domain (DD), and includes TNFR1, Fas (CD95), TRAIL-R1 (DR4; not present in mouse), TRAIL-R2 (DR5), DR3 and DR6. Following ligand binding, these receptors can induce the formation of complexes containing FADD and pro-caspase-8. Dimerisation and auto-cleavage of pro-caspase-8 subsequently leads to formation of active caspase-8 (Oberst et al., 2010). Active caspase-8 can then cleave pro-caspase-3 and -7 to drive apoptosis.

1.4.2.1 **Fas and TRAIL-R pathways**

Fas receptor can induce apoptosis following binding of Fas ligand (FasL). Fas is expressed by a wide range of cell types whereas FasL expression is more restricted to mostly T cells and NK cells (Strasser et al., 2009). Mutations in Fas or FasL in mice (lpr and gld mice), leading to defective Fas induced apoptosis, result in lymphoproliferative disease characterised by an accumulation of CD4⁺CD8⁻B220⁺ T cells in the periphery and presence of auto-antibodies (Cohen and Eisenberg, 1991). There are two forms of FasL: membrane-bound FasL (mFasL) and soluble FasL (sFasL), in which FasL is cleaved from the membrane by matrix metalloproteases. However, only mFasL is thought to be primarily responsible for inducing cell death (Huang et al., 1999; Schneider et al., 1998; Suda et al., 1997). Mice deficient in sFasL are normal whereas mice lacking mFasL develop lymphadenopathy, similar to lpr/gld mice, and have T cells which lack the ability to induce apoptosis via the Fas pathway (O’Reilly et al., 2009).

TRAIL and its receptors can be expressed by a number of immune cell types and is thought to induce apoptosis via a similar mechanism to Fas. Although humans express two TRAIL receptors with death domains, mice only express one (TRAIL-R). In contrast to lpr and gld mice which lack Fas signalling, TRAIL-R deficient mice are phenotypically normal (Diehl et al.,
But combined FasL and TRAIL deficiency causes more severe lymphoproliferative disease (Sedger et al., 2010).

Fas and TRAIL-R induced apoptosis requires formation of the death inducing signalling complex (DISC) at the membrane (Fig. 1.2). Binding of trimeric mFasL to Fas (and TRAIL to TRAIL-R) is thought to lead to receptor oligomerisation (Scott et al., 2009; Wang et al., 2010). The receptor DD is then able to recruit FADD by interacting with its DD (Scott et al., 2009; Wang et al., 2010). This interaction is then thought to reveal the death effector domain (DED) of FADD, which permits it to bind to the DED of pro-caspase-8. Through a series of tandem DED interactions, FADD nucleates a filament containing 6 pro-caspase-8 monomers (Dickens et al., 2012; Hughes et al., 2016; Tummers et al., 2020). It’s currently thought that three filaments can assemble into a triple helix, with all three filaments extending at the same time (Fu et al., 2016). This forms the DISC and enables the dimerization of pro-caspase-8 catalytic domains which subsequently results in cleavage and activation (Fox et al., 2021).

Alongside pro-caspase-8, c-FLIP can also be recruited by FADD and incorporate into the DISC. c-FLIP has a similar structure to pro-caspase-8 but does not have catalytic activity, and as such, it acts as an inhibitor of apoptosis (Irmler et al., 1997). There are 3 forms of c-FLIP generated by alternative splicing: the long (c-FLIP_L), short (c-FLIP_S) and Raji (c-FLIP_R) forms. Unlike humans, c-FLIP_S is not present in mice, although c-FLIP_R is thought to perform a similar role. The incorporation of c-FLIP_{SR} into the DISC, through DEDD interactions, blocks the extension of pro-caspase-8 filaments and the dimerization of pro-caspase-8 catalytic domains, preventing pro-caspase-8 auto-processing and apoptosis (Fox et al., 2021; Krueger et al., 2001). On the other hand, c-FLIP_L can both inhibit or promote apoptosis. Pro-caspase-8 has been shown to have a higher affinity for c-FLIP_L than other pro-caspase-8 monomers, leading to formation of pro-caspase-8/c-FLIP_L heterodimers within the DISC (Boatright et al., 2004). High concentrations of c-FLIP_L are thought to impede the generation of dimers of the pro-caspase-8 catalytic domains, thereby inhibiting apoptosis.
(Fricker et al., 2010; Hughes et al., 2016; Kavuri et al., 2011). However, lower concentrations of c-FLIP$_L$ have been shown to promote the activity of pro-caspase-8 following heterodimerisation and thereby accelerate pro-caspase-8 auto-processing and apoptosis (Boatright et al., 2004; Fricker et al., 2010; Hughes et al., 2016; Micheau et al., 2002). Thus, the concentrations of c-FLIP isoforms within the cell determines the outcome of Fas signalling.

Active caspase-8 subsequently cleaves pro-caspase-3. In type I cells, which includes thymocytes and T cells, this is sufficient to induce apoptosis. However, in type II cells such as hepatocytes, this is not enough to induce apoptosis (Yin et al., 1999). This is because, following FasL (and TRAIL) stimulation, hepatocytes upregulate the expression of XIAP, a caspase inhibitor (Jost et al., 2009). In type II cells, caspase-8 mediated cleavage of BID is required to counter this (Luo et al., 1998). This releases truncated BID (tBID) which inserts into the mitochondrial membrane and activates BAX, subsequently resulting in MOMP (Kuwana et al., 2005). This allows release of SMAC into the cytosol which subsequently inhibits XIAP (Fig. 1.2).
Figure 1.2 Fas/TRAIL-R signalling pathway

The binding of mFasL to Fas or TRAIL to TRAIL-R leads to receptor oligomerisation which permits the recruitment of FADD. If c-FLIP<sub>L</sub> levels are low relative to pro-caspase-8, this precipitates the formation of a DISC consisting of pro-caspase-8 filaments. Pro-caspase-8 cleavage releases active caspase-8 dimers. Caspase-8 subsequently cleaves and activates the executioner caspase-3. For type I cells, this is sufficient to induce apoptosis. For type II cells, the caspase inhibitor XIAP is upregulated which inhibits caspase-3. As such, caspase-8 cleaves Bid to form tBid. The insertion of tBid into the mitochondrial membrane leads to the recruitment and activation of Bax which results in MOMP. SMAC is released from the mitochondrion into the cytosol where it inhibits XIAP.

Alternatively, if c-FLIP<sub>L</sub> levels are high relative to pro-caspase-8, c-FLIP<sub>L</sub> is incorporated into the DISC where it blocks cleavage of pro-caspase-8 to active caspase-8. This results in cell survival.
1.4.2.2 TNFR1 pathway

Following the binding of TNF to TNFR1, rather than inducing apoptosis, the default outcome is activation of pro-survival signalling pathways such as NF-κB (Fig. 1.3). Cell death is tightly regulated by a series of checkpoints. However, disabling these checkpoints genetically and pharmacologically has revealed that TNF can trigger death via formation of cytoplasmic “complex II” (Wang et al., 2008). Complex II can be made up of different components and initiate apoptosis in a manner independent or dependent on RIPK1 kinase activity. After formation of complex I at the membrane, gradual dissociation of TRADD allows it to bind to FADD via its death domain, and further recruit caspase-8 to form “complex IIa”, which drives the activation of caspase-8 in absence of RIPK1 kinase activity (Micheau and Tschopp, 2003; Ting and Bertrand, 2016). Complex IIa mediated cell death is inhibited by the NF-κB dependent expression of pro-survival proteins. This constitutes the late NF-κB dependent checkpoint (Ting and Bertrand, 2016).

Alternatively, destabilisation of complex I permits the additional incorporation of RIPK1 from complex I into complex II to form “complex IIb” (Ting and Bertrand, 2016; Wang et al., 2008). For complex IIb mediated apoptosis, the kinase activity of RIPK1 is required and RIPK1 autophosphorylates at multiple sites. One phosphorylation site of significance is serine 166 and mutating this site to block phosphorylation can prevent TNF induced cell death in vitro and in vivo (Laurien et al., 2020). RIPK1 autophosphorylation is thought to drive a conformational change in RIPK1, allowing recruitment of FADD to complex IIb and leading to more potent induction of apoptosis. Inhibition of complex IIb mediated cell death involves post-translational modifications of RIPK1. This constitutes the early NF-κB independent checkpoint (Ting and Bertrand, 2016).

Although both checkpoints regulate cell death via different mechanisms the checkpoints are somewhat intertwined. c-IAP1/2, LUBAC, TAK1 and IKK are all critical for both TNF induced NF-κB activation and inhibition of RIPK1 dependent cell death. Additionally, many regulators of the early checkpoint,
including cIAP1/2, A20 and CYLD are also themselves NF-κB gene targets. The expression of the components of each checkpoint can vary between different cell types. Thus, work is ongoing to establish how and why TNF induced cell death is regulated differently in different tissues.

### 1.4.2.2.1 Transcription of pro-survival proteins

Canonical NF-κB is rapidly activated following the binding of TNF to TNFR1. This induces the transcription of pro-survival NF-κB gene targets which act to inhibit complex IIa mediated apoptosis. In particular, NF-κB dependent induction of c-FLIP inhibits the activation of caspase-8 within complex IIa (Micheau and Tschopp, 2003; Micheau et al., 2001). As such, when NF-κB induced upregulation of c-FLIP is blocked with the translation inhibitor cycloheximide, c-FLIP levels are rapidly reduced, due to its short half-life, and TNF induced apoptosis ensues (Kreuz et al., 2001). Other proteins including the pro-survival Bcl-2 family members, Bcl-2 and Bcl-xL, and the caspase inhibitor XIAP are also NF-κB targets and could additionally contribute to the inhibition of TNF induced apoptosis. In line with this mechanism, deletion of RelA in mice causes embryonic lethality due to extensive liver hepatocyte apoptosis (Beg et al., 1995). However, RelA knockout mice survive until after birth following deletion of TNF or TNFR1 (Alcamo et al., 2001; Doi et al., 1999). This constitutes the late NF-κB dependent checkpoint (Ting and Bertrand, 2016).

### 1.4.2.2.2 Ubiquitination of complex I

Following the assembly of complex I downstream of TNFR1, it is shrouded in a cloak of polyubiquitin chains of varying linkages by the E3 ubiquitin ligases cIAP1/2 and the LUBAC complex. Depletion of cIAP1 and cIAP2 using IAP antagonists (Smac mimetics) leads to defective K63-linked ubiquitination of RIPK1 within complex I (Bertrand et al., 2008). This subsequently sensitises cell lines to TNF induced, RIPK1 dependent cell death by promoting the formation of complex IIb (Bertrand et al., 2008; Dondelinger et al., 2013; Wang et al., 2008). The regulation of RIPK1 dependent cell death by
ubiquitin occurs independently of NF-κB and forms part of the early NF-κB independent checkpoint (Dondelinger et al., 2013; Ting and Bertrand, 2016; Wang et al., 2008). It has been proposed that ubiquitination of RIPK1 acts to lock it within complex I, preventing it dissociating to form complex IIb.

Ubiquitination of RIPK1 by cIAP1/2 to prevent TNF induced cell death is crucial during development and for tissue homeostasis. cIAP1/2 knockout mice die during embryogenesis (E10.5) due to cardiovascular defects (Moulin et al., 2012). Embryonic survival can be extended following additional deletion of one or two copies of Ripk1. But mice can survive up to birth following deletion of Tnfrsf1a (Moulin et al., 2012). Specific deletion of cIAP1/2 in epidermal keratinocytes causes severe skin inflammation, but deletion of one Ripk1 copy can lessen and delay the disease (Anderton et al., 2017). Thus cIAP1/2 is crucial for preventing TNF induced, RIPK1 dependent cell death in vivo.

Ubiquitination of complex I by cIAP1/2 is required for recruitment of the LUBAC complex, which catalyses the addition of linear ubiquitin chains to complex I. These linear ubiquitin chains also play an essential role in preventing TNF induced cell death. Mice with deletion of any one of the three components of the LUBAC complex exhibit profound abnormalities due to TNF induced cell death. Similarly to cIAP1/2 deficient mice, Hoip or Hoil-1 knockout mice also die during embryogenesis (at E10.5) due to vascularisation defects (Peltzer et al., 2014, 2018). MEFs from Hoip or Hoil-1 knockout mice exhibit an absence of M1-linked ubiquitination of complex I after TNF stimulation, formation of complex IIb and undergo RIPK1 dependent cell death (Peltzer et al., 2014, 2018). Deletion of Tnfrsf1a or ablation of RIPK1 kinase activity can prolong embryonic survival (Peltzer et al., 2014, 2018). Mice with a loss of function mutation in Sharpin, so called cpdm mice, have an absence of Sharpin expression. These mice exhibit a less severe phenotype than Hoil-1 and Hoip deficient mice and survive until birth. This is likely because some linear ubiquitination of complex I remains in absence of Sharpin (Peltzer et al., 2018). However, cpdm mice display severe inflammation of the skin, lungs and liver and a lack of Peyer’s patches (HogenEsch et al., 1993, 1999; Seymour et al., 2007). Deletion of TNF or
TNFR1 or ablation of RIPK1 kinase activity can abrogate the multi-organ inflammation in cpdm mice (Berger et al., 2014; Gerlach et al., 2011; Kumari et al., 2014; Rickard et al., 2014).

Other proteins within the cell act to regulate the ubiquitin network formed by cIAP1/2 and LUBAC which surrounds complex I. The recruitment of LUBAC to complex I also leads to the recruitment of CYLD, which associates with HOIP via SPATA2 (Elliott et al., 2016; Kupka et al., 2016). CYLD is a deubiquitinase which acts to remove ubiquitin chains from complex I components. As such, it negatively regulates NF-κB but is also important for control of TNFR1 induced cell death. Amongst other targets, CYLD has been shown hydrolyse K63- and M1- linked ubiquitin chains from TNFR1, TRADD and RIPK1, thereby acting to destabilise complex I (Draber et al., 2015). This facilitates complex II formation since, in the absence of CYLD, TNF induces less complex IIb formation and apoptosis in Smac mimetic treated cell lines (Dondelinger et al., 2013; Wang et al., 2008). Furthermore, CYLD is thought to deubiquitinate RIPK1 within the necrosome, promoting the activity of RIPK1 and RIPK3 and accelerating necroptosis (Moquin et al., 2013).

A20 is another important regulator of the complex I ubiquitin scaffold. Similarly to CYLD, it functions as a negative regulator NF-κB signalling but in contrast, it also protects cells from TNF induced cell death. A20 is termed a ubiquitin-editing enzyme since it possesses E3 ubiquitin ligase and deubiquitinase activities and has ubiquitin binding domains. Early studies suggested that A20 interferes with RIPK1 recruitment to TNFR1 or catalyses the removal of K63- linked ubiquitin chains and the addition of K48-linked ubiquitin chains to RIPK1, thereby flagging it for proteasomal degradation (He and Ting, 2002; Wertz et al., 2004, 2015). This could thereby reduce the amount of RIPK1 available to integrate into complex II. More recent studies suggest that A20 inhibits TNF induced cell death by interacting with the linear ubiquitin chains on complex I and shielding them from degradation by CYLD (Draber et al., 2015; Priem et al., 2019). A20 deficiency reduces linear ubiquitination of complex I and sensitises MEFs to TNF induced RIPK1 dependent cell death which can be rescued by knockdown of CYLD (Priem
et al., 2019). Consistent with this role for A20 in repression of TNF induced cell death, A20 knockout mice die soon after birth due to severe inflammation of multiple organs, but survival can be extended by ablation of RIPK1 kinase activity (Lee et al., 2000; Newton et al., 2016).

1.4.2.2.3 Phosphorylation of RIPK1

Ubiquitination of complex I subsequently permits the recruitment of multiple kinases, including TAK1 and IKK. These kinases have been shown to have crucial roles for the regulation of TNF induced cell death independently of NF-κB activation and thus contribute to the early NF-κB independent checkpoint (Dondelinger et al., 2013, 2015; Ting and Bertrand, 2016). K63-linked ubiquitin chains are required for recruitment of TAK1 to complex I. Pharmacological inhibition or knockdown of TAK1 in cell lines sensitises them to TNF induced, RIPK1 dependent cell death (Dondelinger et al., 2013; Vanlangenakker et al., 2011). Importantly, complex IIb formation occurred despite intact K63-linked ubiquitination of RIPK1 (Dondelinger et al., 2013). This protective role of TAK1 has also been investigated in vivo. TAK1 knockout mice also die during embryogenesis (at E10.5) but deletion of Tnfrsf1a cannot rescue survival to birth, in contrast to cIAP1/2 knockout mice (Morioka et al., 2012). Nonetheless, specific deletion of TAK1 in enterocytes, keratinocytes and hepatocytes leads to excessive inflammation and cell death which is rescued upon genetic ablation of TNFR1 (Inokuchi et al., 2010; Kajino-Sakamoto et al., 2008; Omori et al., 2006). Thus, TAK1 does appear to be important for protecting against TNF induced cell death in vivo but the contribution of RIPK1 activity to these phenotypes has yet to be explored. One mechanism by which TAK1 may suppress RIPK1 dependent cell death is through direct phosphorylation of RIPK1. TAK1 directly phosphorylates RIPK1 at serine 321 to prevent RIPK1 activation (Geng et al., 2017).

In addition, TAK1 is crucial for activation of the IKK complex at complex I. Deletion of NEMO or both IKK1 and IKK2 sensitises cell lines to TNF induced RIPK1 dependent cell death, independently of NF-κB activation.
IKK1 and IKK2 both directly phosphorylate RIPK1 within complex I to inhibit RIPK1 autophosphorylation and prevent complex IIb formation (Dondelinger et al., 2015). IKK phosphorylation of RIPK1 at serine 25 appears to be particularly important for RIPK1 inhibition since mutation of this residue, to mimic phosphorylation, prevents TNF induced cell death (Dondelinger et al., 2019). Repression of RIPK1 kinase activity by the IKK complex is important normal tissue homeostasis in vivo. Deletion of NEMO, but not NF-κB subunits, in intestinal epithelial cells causes colitis due to TNFR1 induced cell death (Nenci et al., 2007; Vlantis et al., 2016). However, additional ablation of RIPK1 kinase activity can prevent colitis by inhibiting intestinal epithelial cell death (Vlantis et al., 2016). Specific deletion of NEMO or IKK2 in keratinocytes leads to development of a perinatally fatal inflammatory skin disease due to TNFR1 induced cell death (Kumari et al., 2021; Nenci et al., 2006; Pasparakis et al., 2002). But ablation of RIPK1 kinase activity can almost completely prevent skin disease in IKK2 deficient mice (Kumari et al., 2021).

NEMO facilitates the recruitment of IKK1 and IKK2 to complex I. Recently, it's been shown that NEMO also binds to TANK and NAP1 to recruit the kinases IKKε and TBK1 (Lafont et al., 2018a). These kinases are not required for NF-κB activation but function to protect cells from TNF induced cell death. Pharmacological inhibition of IKKε and TBK1 sensitises cell lines to TNF induced RIPK1 dependent cell death by promoting the formation of complex IIb (Lafont et al., 2018a). This occurs because IKKε and TBK1 directly phosphorylate RIPK1 within complex I to prevent RIPK1 activation in a manner which is distinct from IKK1 and IKK2 (Lafont et al., 2018a; Xu et al., 2018). Phosphorylation of RIPK1 by TBK1 is crucial for embryonic development. Knockout of TBK1 in mice causes embryonic lethality, however ablation of RIPK1 kinase activity rescues survival (Xu et al., 2018).

Phosphorylation of RIPK1 within complex I is crucial to prevent TNF induced cell death. But RIPK1 is also phosphorylated within the cytosol to further prevent RIPK1 dependent cell death. TAK1 induces p38 MAPK signalling
which activates the kinase MK2. MK2 directly phosphorylates cytosolic RIPK1 which is thought to prevent it integrating into complex IIb (Dondelinger et al., 2017; Jaco et al., 2017; Menon et al., 2017). Pharmacological inhibition of MK2 in combination with Smac mimetics or IKK inhibitors sensitises cell lines to RIPK1 dependent cell death (Dondelinger et al., 2017; Jaco et al., 2017). Thus, MK2 regulates RIPK1 dependent cell death but by controlling a different pool of RIPK1 to that controlled by IKK.

The ubiquitination of complex I by cIAP1/2 and LUBAC is a prerequisite for the recruitment of TAK1 and IKK. Thus, cells lacking cIAP1/2 or LUBAC also show deficient phosphorylation of RIPK1 by IKK (Dondelinger et al., 2015). As such, it has been questioned whether ubiquitination and phosphorylation of RIPK1 are distinct mechanisms repressing formation of complex IIb or whether ubiquitination just acts to facilitate phosphorylation of RIPK1. In support of the latter, mimicking IKK phosphorylation of RIPK1 by mutating serine 25, can inhibit TNF induced death of cell lines treated with Smac mimetics (Dondelinger et al., 2019). Additionally, mimicking RIPK1 phosphorylation by IKK blocks TNF induced cell death in Sharpin deficient MEFs and rescues the multi-organ inflammation in cpdm mice (Dondelinger et al., 2019). On the other hand, other studies have implicated a direct role of ubiquitin for regulation of RIPK1 dependent cell death. The UBA domain of cIAP1 has been shown to be important for ubiquitination of RIPK1 and also catalyses the addition of K48-linked ubiquitin chains to RIPK1 in complex I, targeting it for proteasomal degradation (Annibaldi et al., 2018). Disrupting the activity of the UBA domain sensitises to TNF induced RIPK1 dependent cell death, without affecting IKK activation (Annibaldi et al., 2018). Furthermore, RIPK1 can be ubiquitinated by other E3 ubiquitin ligases which do not participate in NF-κB activation. MIB2 ubiquitinates RIPK1 to protect cells from RIPK1 dependent cell death without affecting IKK activity (Feltham et al., 2018).

1.4.3 Necroptosis
Necroptosis can be triggered by TNFR1, Fas and TRAIL-R amongst other receptors. This form of cell death involves RIPK3, MLKL and the kinase activity of RIPK1. Following generation of complex II downstream of TNFR1, when caspase-8 activity is absent, RIPK3 is recruited to form the necrosome (Cho et al., 2009; Grootjans et al., 2017; He et al., 2009; Zhang et al., 2009). Autophosphorylated active RIPK1 binds to RIPK3 via RIPK homotypic interaction motif (RHIM) interactions generating an oligomeric complex resembling an amyloid-fibril (Li et al., 2012). RIPK3 subsequently undergoes autophosphorylation and activated RIPK3 then phosphorylates MLKL at serine and threonine residues in the pseudokinase domain, although this is thought to differ between species (He et al., 2009; Murphy et al., 2013). Phosphorylation of MLKL is thought to cause a conformation change to reveal the four-helix bundle (4HB) domain (Hildebrand et al., 2014; Murphy et al., 2013; Zhao et al., 2012). MLKL then oligomerises and translocates to the plasma membrane where its 4HB domain is crucial for membrane permeabilisation, but the exact mechanism by which MLKL executes cell death is still under discussion (Murphy, 2020; Wang et al., 2014).

### 1.4.3.1 Caspase-8 inhibition of necroptosis

Depending on the cellular circumstances, TNF can trigger caspase-8 dependent apoptosis or caspase-8 independent necroptosis. Early studies observed that caspase inhibition could sensitise cell lines to necroptosis, first implicating a role for caspase-8 in the regulation of this death pathway (Holler et al., 2000; Vercammen et al., 1998). The generation of knockout mouse strains demonstrated a critical developmental role of caspase-8 to inhibit necroptosis. Mice deficient in caspase-8, or its adaptor FADD, die during embryogenesis due to defective cardiac development, haemorrhages and impaired vascularisation (Varfolomeev et al., 1998; Yeh et al., 1998). However, upon additional deletion of RIPK3 or MLKL, to block necroptosis, mice did not exhibit these abnormalities and survived to adulthood (Alvarez-Diaz et al., 2016; Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011). Instead, they develop lymphoproliferative disease characterised by accumulation of CD4⁻CD8⁻B220⁺ T cells which resembles lpr/gld mice. Within
complex II formed downstream of TNFR1, pro-caspase-8 forms a heterodimer with c-FLIP<sub>L</sub>, the catalytic activity of which is required to suppress necroptosis (Dillon et al., 2012; Newton et al., 2019; Oberst et al., 2011). Earlier work suggested that cleavage of RIPK1, RIPK3 or CYLD by the pro-caspase-8/c-FLIP<sub>L</sub> heterodimer was responsible for limiting necroptosis (Feng et al., 2007; Lin et al., 1999; O'Donnell et al., 2011). Recently, it was shown that cleavage of RIPK1 appeared to be particularly important. Mice expressing RIPK1 with a mutated caspase-8 cleavage site die during embryogenesis and resemble caspase-8 knockout mice (Lalaoufi et al., 2019; Newton et al., 2019; Zhang et al., 2019). These mice survive to adulthood following additional knockout of Caspase-8 and MLKL but not MLKL alone. Thus, caspase-8 cleavage of RIPK1 restricts both apoptosis and necroptosis.
**Figure 1.3 The TNFR1 signalling pathway**

TNFR1 can either induce NF-κB activation or formation of cell death complexes which trigger apoptosis or necroptosis. The binding of TNF to TNFR1 leads to the recruitment of TRADD which interacts with both TRAF2 and RIPK1 and thus initiates formation of complex I. The IAPs catalyse the addition of K63-linked ubiquitin chains to themselves and RIPK1. LUBAC further decorates complex I with M1-linked ubiquitin chains. The ubiquitin network which forms around complex I allows the recruitment of the kinases TAK1 and IKK. Activation of IKK by TAK1 is required for activation of NF-κB. Both TAK1 and IKK also directly phosphorylate RIPK1 within complex I to inhibit its kinase activity.

Dissociation of TRADD from complex I allows it to interact with FADD and caspase-8. This forms complex IIa which subsequently induces apoptosis. If ubiquitination of complex I is defective, such as when cIAP1/2 or LUBAC are deficient or due to deubiquitination by CYLD, or if phosphorylation of RIPK1 by IKK and TAK1 is impaired, complex I is destabilised. RIPK1 can
incorporate into complex II to form complex IIb, which is a potent inducer of apoptosis. Disruption of caspase-8 activity promotes further recruitment of RIPK3 and MLKL into complex II to form the necrosome which initiates necroptosis. Schematic adapted from (Blanchett et al., 2021).
1.5 Cues from TNFRSF members direct T cell development, homeostasis and differentiation

1.5.1 Thymocyte development

Canonical NF-κB signalling is thought to have important functions during thymocyte development. Before thymocyte selection, the pre-TCR triggers NF-κB in DN3 thymocytes. Mice expressing a T cell specific IκBα superrepressor, to block NF-κB, exhibit a loss of DN3 thymocytes (Voll et al., 2000). This wave of NF-κB activation is thought to promote the survival and proliferation of DN3 thymocytes, facilitating differentiation to DN4 cells (Aifantis et al., 2001; Voll et al., 2000). One NF-κB target particularly important at this stage is the anti-apoptotic Bcl-2 family member Bfl-1/A1 (Mandal et al., 2005).

Once thymocytes reach the DP stage, NF-κB has been implicated to play a role in positive and negative selection. CD8+ thymocytes undergoing selection show an increase in NF-κB activity. When NF-κB is blocked through expression of IκBα superrepressor, both positive and negative selection of CD8+ thymocytes is impaired (Boothby et al., 1997; Hettmann and Leiden, 2000; Jimi et al., 2008; Mora et al., 2001). On the other hand, NF-κB is not activated during selection of CD4+ thymocytes and blocking NF-κB has no effect on CD4+ thymocyte selection (Jimi et al., 2008). Thus, these studies implicated a link between TCR and NF-κB signalling to regulate CD8+ but not CD4+ thymocyte selection.

However, these earlier findings have since been questioned following the generation of mouse strains deficient in upstream NF-κB activators. Mice deficient in IKK1/2 or TAK1 from the DN2 stage have normal DN and DP populations (Liu et al., 2006; Sato et al., 2006; Webb et al., 2016). Additionally, the TCR is not responsible for triggering NF-κB in thymocytes since mice deficient in CARD11, BCL10 or MALT1 exhibit normal thymocyte development (Egawa et al., 2003; Hara et al., 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003; Schmidt-Supprian et al., 2004a). Thus, this
supported the view that TCR induced NF-κB activation is not required for thymocyte selection. Instead, canonical NF-κB signalling appeared to be important for maturation of SP thymocytes following selection. Ablation of NEMO, IKK1/2, TAK1, Hoil-1 or HOIP in DP thymocytes leads to a profound loss of mature SP thymocytes (Schmidt-Supprian et al., 2003; Teh et al., 2016; Wan et al., 2006; Webb et al., 2016, 2019; Xing et al., 2016).

Although IKK, TAK1 and LUBAC facilitate NF-κB activation, they also constitute part of TNFR1 complex I and are important for repression of TNF induced cell death. Indeed, knockout of TNFR1 or TNF in mice lacking IKK or TAK1 rescues SP thymocyte development (Webb et al., 2016; Xing et al., 2016). Thus, in SP thymocytes, canonical NF-κB signalling did not appear to be important for the SP thymocyte maturation programme but instead was specifically protecting mature SP thymocytes from TNF induced cell death. IKK can protect cells from TNF induced cell death by inducing NF-κB activation, to inhibit complex IIa, or by directly phosphorylating and inhibiting RIPK1, to prevent complex IIb formation (Dondelinger et al., 2015). Mice lacking all three canonical NF-κB subunits exhibit normal thymocyte development and SP thymocytes were resistant to TNF induced death in vitro (Webb et al., 2019). Thus, IKK does not protect SP thymocytes from TNF induced cell death via NF-κB activation. However, blocking RIPK1 kinase activity both in vitro and in vivo rescued the survival of mature SP thymocytes (Webb et al., 2019). Therefore, these studies demonstrate that NF-κB activation by IKK (and likely the other complex I components), is not required for thymocyte selection, maturation or survival. Instead, IKK directly regulates RIPK1 activity to protect mature SP thymocytes from TNF induced RIPK1 dependent cell death.

RIPK1 has both pro- and anti-apoptotic roles for developing thymocytes. It is expressed at a low level in DP thymocytes but is upregulated throughout development reaching its highest level in mature SP thymocytes (Webb et al., 2019). On the one hand, it mediates TNF induced cell death of SP thymocytes in absence of IKK. Increasing RIPK1 expression correlates with greater RIPK1 activity and increased sensitivity to TNF induced cell death in
IKK1/2 deficient SP thymocytes (Webb et al., 2019). However, in absence of RIPK1, SP thymocytes exhibit increased sensitivity to TNF induced cell death (Webb et al., 2019). In contrast, SP thymocytes lacking canonical NF-κB subunits or RIPK1 kinase activity are resistant to TNF induced cell death (Dowling et al., 2016; Webb et al., 2019). Thus, RIPK1 protects SP thymocytes from complex IIa mediated cell death via its scaffold function, but independently of NF-κB. However, the physiological relevance of this is unclear since RIPK1 deficient mice have mostly normal thymocyte development (Dowling et al., 2016; Webb et al., 2019).

Collectively, the expression of RIPK1 increases during thymocyte development, reaching a peak in the most mature populations. There are multiple potential explanations for this developmental upregulation of RIPK1. Firstly, this may be required to protect T cells from TNF induced complex IIa mediated cell death before and after thymic export. Secondly, the upregulation of RIPK1 may be important for optimal TNF driven NF-κB activation in SP thymocytes which induces the expression of genes important for T cell function and survival in the periphery (Webb et al., 2019). But consequently, mature SP thymocytes require more stringent RIPK1 control mechanisms in place to inhibit TNF induced RIPK1 dependent cell death.

1.5.2 T cell homeostasis

Although NF-κB is not required for thymocyte development, a number of NF-κB target genes are transcriptionally induced at the mature SP stage (Webb et al., 2016; Xing et al., 2016). It’s now becoming clear that these genes may have important roles for T cell function and survival in the periphery. One validated NF-κB target induced in SP thymocytes is IL-7R, leading to upregulation of surface IL-7R expression following exit from the thymus (Miller et al., 2014; Silva et al., 2014; Webb et al., 2016, 2019). Ablation of IKK2, to partially block NF-κB, or deletion all three canonical NF-κB subunits, causing complete NF-κB blockade, has no effect on thymocyte development but leads to a reduction in peripheral naïve T cells (Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2019). This corresponds with reduced
expression of IL-7R by naïve T cells, leading to impaired peripheral survival and homeostatic proliferation. Thus, NF-κB induced IL-7R upregulation by T cells is crucial for normal homeostasis. The role of IKK for IL-7R induction by T cells partly explains why ablation of TNFR1 in IKK1/2 deficient mice does not rescue peripheral T cells (Webb et al., 2016). The receptor(s) responsible for IL-7R upregulation by new T cells is yet to be fully established. The TCR does not appear to be necessary at this stage (Silva et al., 2014) and mice deficient in CARD11, BCL10 or MALT1 have an intact peripheral T cell compartment (Egawa et al., 2003; Hara et al., 2003; Newton and Dixit, 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003). Both TNF and CD70 are capable of inducing IL-7R upregulation on SP thymocytes in vitro (Silva et al., 2014). However, both TNFR1 and CD27 knockout mice have normal numbers of peripheral T cells (Hendriks et al., 2000; Webb et al., 2016).

RIPK1 has an important role for T cell homeostasis. RIPK1 deficient mice exhibit peripheral T cell lymphopenia, unlike mice expressing kinase dead RIPK1 (Dowling et al., 2016; Webb et al., 2016). Since RIPK1 deficient T cells have lower IL-7R expression, the scaffold function of RIPK1 appears to be important for facilitating NF-κB induced upregulation of IL-7R (Webb et al., 2019). Additionally, treatment of RIPK1 deficient mice with anti-TNF boosts peripheral T cell numbers (Dowling et al., 2016). This indicates that another function of RIPK1 in peripheral T cells is to protect them from TNF induced cell death. Whether mature T cells constitutively require IKK for maintenance of IL-7R expression and repression of RIPK1 dependent cell death remains unknown.

### 1.5.3 Regulatory T cell generation, homeostasis and function

Canonical NF-κB signalling is crucial for the development of regulatory T cells (Tregs) in the thymus. Deletion of TAK1, Sharpin, Hoil-1, HOIP, IKK1 or IKK2 during thymocyte development leads to decreased numbers of thymic and peripheral Tregs (Chen et al., 2015; Park et al., 2016; Sato et al., 2006; Schmidt-Supprian et al., 2003; Teh et al., 2016; Wan et al., 2006). It’s now clear that perturbed NF-κB activation is predominantly responsible for the
Treg defect. Of the NF-κB subunits, c-Rel has crucial functions at multiple stages of Treg development, including for the generation of Treg precursors. Mice deficient in c-Rel exhibit a loss of Tregs and also have fewer CD25⁺FoxP3⁻ and CD25⁻FoxP3⁺ Treg precursors in the thymus (Deenick et al., 2010; Grigoriadis et al., 2011; Isomura et al., 2009; Oh et al., 2017a; Ruan et al., 2009a; Schuster et al., 2019; Visekruna et al., 2010). The induction of c-Rel in CD25⁺FoxP3⁻ precursors is thought to promote survival rather than drive the transcription of genes required for precursor Treg development (Fulford et al., 2021). It remains unclear if the same mechanism is true of CD25⁻FoxP3⁺ Treg precursors. Additionally, c-Rel is required for the differentiation of mature Tregs from Treg precursors. It’s been shown that c-Rel binds to the promoter of FoxP3 where it is thought to permit IL-2 and IL-15 driven transcription (Grigoriadis et al., 2011; Long et al., 2009a).

Following development, Tregs continue to require c-Rel to maintain their suppressive function. Although FoxP3Cre mediated deletion of c-Rel has no effect on the frequency of peripheral Tregs, mice exhibit mild inflammation (Oh et al., 2017a). This appears to be due to impaired function since suppression of conventional T cells by c-Rel deficient Tregs was impaired in colitis and tumour models (Grinberg-Bleyer et al., 2017; Oh et al., 2017a). Nonetheless, RelA appears to be more important than c-Rel for mature Tregs. Treg specific ablation of RelA leads to severe inflammatory and lymphoproliferative disease culminating in impaired survival, despite normal Treg numbers (Messina et al., 2016; Oh et al., 2017a; Ronin et al., 2019; Vasanthakumar et al., 2017). RelA has been shown to be crucial for the generation, stability, and suppressive function of effector Tregs in lymphoid and non-lymphoid tissues. In absence of RelA, Tregs lose FoxP3 expression and become pathogenic, producing inflammatory cytokines such as TNF and IFN-γ (Messina et al., 2016; Ronin et al., 2019). But if both c-Rel and RelA are deficient in Tregs, mice develop a more severe surfy like disease and exhibit significantly reduced survival, accompanied by decreased Treg numbers and suppressive function (Oh et al., 2017a). This phenotype also resembles mice with Treg specific ablation of IKK2 or HOIP, which could not be rescued with TNF blockade (Heuser et al., 2017; Teh et al., 2016). Thus,
c-Rel and RelA act separately and in combination to promote the stability and function of mature Tregs.

The TCR is an important trigger of NF-κB activity in both developing and mature Tregs. Knockout of CARD11, BCL10 or MALT1 leads to defective thymic Treg development, including a reduced number of CD25*FoxP3+ Treg precursors (Brüstle et al., 2017; Medoff et al., 2009; Molinero et al., 2009; Schmidt-Supprian et al., 2004a). FoxP3<sup>Cre</sup> mediated deletion of Bcl10 causes a similar scurvy-like phenotype to mice with combined c-Rel and RelA deficiency, including similar defects in effector Tregs (Rosenbaum et al., 2019; Yang et al., 2019). Thus, these studies implicate TCR as an important trigger of NF-κB for both Treg development and homeostasis.

TCR signalling in thymic Treg progenitors induces the expression of TNFR superfamily members, including OX40, GITR and TNFR2, in a TAK1 and c-Rel dependent manner (Fulford et al., 2021; Mahmud et al., 2014). Signalling through these receptors is subsequently thought to augment the IL-2 dependent induction of FoxP3 expression during Treg maturation. TNFRSF members also have roles for peripheral Tregs. GITR signalling can drive the differentiation of effector Tregs <i>in vitro</i> and <i>in vivo</i> (Vasanthakumar et al., 2017). Furthermore, costimulation of anti-CD3/CD28 or IL-2 treated Tregs with TNFR2, 4-1BB, GITR and DR3 promotes cell division and survival in a RelA dependent manner <i>in vitro</i> (Ricco et al., 2020; Vasanthakumar et al., 2017).

### 1.5.4 T cell activation

It has been well established that NF-κB is crucial during T cell activation. Early studies analysed individual NF-κB subunit knockout mice or mice transplanted with foetal liver cells from knockout mice. These demonstrated distinct roles for NF-κB subunits in activated T cells. T cells lacking RelA, c-Rel or p50 show impaired proliferation after anti-CD3/CD28 stimulation <i>in vitro</i> (Doi et al., 1997; Köntgen et al., 1995; Zheng et al., 2001). Proliferation of T cells lacking c-Rel is impaired due to defective IL-2 production (Köntgen
et al., 1995). Indeed, TCR/CD28 induced c-Rel directly promotes the transcription of IL-2 and the proliferation of c-Rel deficient T cells is rescued if IL-2 is added to cultures (Rao et al., 2003). The proliferative defects in RelA, c-Rel or p50 knockout T cells are not due to increased apoptosis (Doi et al., 1997; Zheng et al., 2001). Nonetheless, in the absence of one NF-κB subunit, there is likely compensatory activity of other subunits in activated T cells (Zheng et al., 2001).

T cells deficient in multiple NF-κB subunits have more profound proliferative defects, demonstrating a degree of redundancy between subunits. Knockout of both RelA and c-Rel leads to failure of T cells to undergo blast transformation (Grumont et al., 2004). This occurs since both subunits are required in a redundant fashion to induce the expression of c-Myc after TCR stimulation. On the other hand, T cells with combined absence of c-Rel and p50 can blast transform but fail to divide normally due to impaired cell cycle entry (Grumont et al., 2004; Zheng et al., 2003). The same is true of T cells expressing p105 which cannot be degraded following IKK activation and which exhibit perturbed nuclear activity of RelA, c-Rel and p50 (Sriskantharajah et al., 2009). This is likely because NF-κB regulates the expression of multiple proteins involved in the cell cycle, including Cyclins D1, D2 and D3, CDK2, CDK6 and E2F3 (Cheng et al., 2003; Guttridge et al., 1999; Huang et al., 2001; Iwanaga et al., 2008; Ledoux and Perkins, 2014).

NF-κB protects T cells from apoptosis following activation. T cells lacking multiple NF-κB subunits or expressing a non-degradable IκBα undergo increased apoptosis following activation (Aune et al., 1999; Boothby et al., 1997; Grumont et al., 2004; Mora et al., 2003; Zheng et al., 2003). It’s thought that NF-κB controls survival of activated T cells by regulating the expression of Bcl-2 family members including Bcl-xL, Bfl1/A1 and Bcl-2 itself (Catz and Johnson, 2001; Chen et al., 2000; Grumont et al., 1999; Lee et al., 1999; Zong et al., 1999). CD28 co-stimulation of T cells induces the expression of anti-apoptotic member Bcl-xL, which was suggested to promote T cell survival (Boise et al., 1995; Okkenhaug et al., 2001). The induction of Bcl-xL occurs in an NF-κB dependent manner and therefore it
was assumed that the impaired upregulation of Bcl-x<sub>L</sub> in T cells with defective NF-kB activation was responsible for increased apoptosis following activation (Khoshnan et al., 2000; Manicassamy et al., 2006; Marinari et al., 2004; Mora et al., 2003; Zheng et al., 2003). However, Bcl-x<sub>L</sub> knockout mice exhibit normal effector and memory T cell responses in vivo, thus disputing a role Bcl-x<sub>L</sub> in activated T cell survival (Zhang and He, 2005).

The pro-apoptotic Bcl-2 family member Bim is upregulated after TCR stimulation and drives the apoptosis of activated T cells (Hildeman et al., 2002; Koenen et al., 2013; Sandalova et al., 2004). NF-kB has been shown to control activated T cell survival by indirectly regulating the expression of Bim. TCR stimulation induces the expression of p73 in an E2F1 dependent manner (Irwin et al., 2000; Lissy et al., 2000; Wan and DeGregori, 2003). p73 then binds to the promoter of Bim, leading to increased Bim expression (Busuttil et al., 2010). Simultaneously, RelA/p50 dimers directly induce transcription of Mdm2 which interacts with p73 (Busuttil et al., 2010). This inhibits the transcriptional activity of p73 and blocks Bim mediated apoptosis. The other pro-apoptotic member Bax can also be transcriptionally induced by p73 (Cianfrocca et al., 2008). However, RelA binds to the Bax promoter to prevent p73 driven upregulation.

In line with the crucial role of NF-kB during T cell activation, defects are also observed in T cells lacking upstream NF-kB activators. Disrupting TCR induced NF-kB activation by knockout of CARD11, BCL10 or MALT1 leads to failure of T cells to upregulate activation markers, and impaired blast transformation and cell cycle entry (Egawa et al., 2003; Hara et al., 2003; Newton and Dixit, 2003; Ruefi-Brasse et al., 2003; Ruland et al., 2001, 2003). This culminates in an almost complete failure of the T cells to divide. Similarly, IKK1 deficient T cells treated with IKK2 inhibitor do not divide after TCR stimulation (Webb et al., 2019). These profound activation defects in absence of functional CBM or IKK complexes reflect a near complete lack of NF-kB activity. On the other hand, IKK2 deficient T cells proliferate normally in response to anti-CD3/CD28 (Schmidt-Supprian et al., 2003). This corresponds with the partial, albeit impaired, NF-kB activity that can occur in
absence of IKK2. However, recall responses to antigen and in vivo responses in EAE models are impaired, which has been attributed to a priming defect (Greve et al., 2007; Schmidt-Supprian et al., 2004b).

The generation of memory T cells requires NF-κB. Mice deficient in IKK2 or c-Rel and p50, expressing non-degradable IκBα, or with defective p105 proteolysis have reduced numbers of memory phenotype cells (Hettmann et al., 2003; Schmidt-Supprian et al., 2003; Sriskantharajah et al., 2009; Webb et al., 2019; Zheng et al., 2003). CARD11 and BCL10 deficient mice also have fewer peripheral memory phenotype cells, demonstrating that generation of these cells requires TCR triggered NF-κB activation (Medoff et al., 2009; Schmidt-Supprian et al., 2004a). One mechanism by which NF-κB regulates memory T cell generation is by controlling the expression of Eomes, a critical transcription factor in memory T cells. TCR induced NF-κB activation is thought to drive the upregulation and maintenance of Eomes and facilitate the establishment of memory (Knudson et al., 2017).

Extrinsic cell death pathways have long been thought to play a role in activated T cell survival. Early studies implicated Fas as the key trigger of cell death upon TCR restimulation (activation induced cell death) in primary T cells and cell lines in vitro (Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Russell et al., 1993). In this settings, both Fas and FasL are upregulated after T cell activation, leading to both autocrine and paracrine FasL induced apoptosis (Brenner et al., 2008; Strasser et al., 2009). TCR restimulation causes the localisation of Fas into lipid rafts to promote its apoptotic activity (Muppidi and Siegel, 2004). Although Bim was subsequently found to be crucial for contraction of T cell responses in vivo, there are some contexts where a role for Fas has been described (Hildeman et al., 2002). Fas has been suggested to be important during chronic infections, which likely reflect scenarios of TCR restimulation. Combined Fas and Bim deficiency leads to greater accumulation of T cells after LCMV or murine herpesvirus infections, compared to absence of either Fas or Bim alone (Hughes et al., 2008; Weant et al., 2008).
Extrinsic apoptosis requires both FADD and caspase-8 (Varfolomeev et al., 1998). However, T cells deficient in FADD or caspase-8 exhibit impaired T cell responses, both in vitro and in vivo, despite a block in extrinsic apoptosis (Osborn et al., 2010; Salmena et al., 2003). Although FADD and caspase-8 deficient T cells activate normally after anti-CD3/CD28 stimulation, they exhibit defective proliferation due to increased cell death (Salmena et al., 2003). Cell death could be blocked with Nec-1 to inhibit RIPK1 or by knockout of RIPK3, thus demonstrating that T cells were sensitive to necroptosis (Bell et al., 2008; Ch’en et al., 2008a, 2011; Osborn et al., 2010). In addition, caspase-8 deficient T cell responses to LCMV in vivo can be rescued by deletion RIPK3 (Ch’en et al., 2011). T cells lacking c-FLIP_{L} have also been shown to undergo Fas induced necroptosis following TCR stimulation (He and He, 2013). These studies demonstrate an important role for caspase-8/c-FLIP_{L} heterodimers for suppression of necroptosis in activated T cells.
1.6 Thesis aims

Canonical NF-κB signalling has long been thought to be important for the development, homeostasis and function of T cells. Due to the redundancy between NF-κB subunits, many earlier studies attempted to understand the role of NF-κB by manipulating the activity of upstream kinases which activate it, such as the IKK complex. Many of the immune defects observed when IKK activity was perturbed were assumed to be due to disrupted NF-κB activation. However more recently it has become clear that the IKK complex has additional functions independently of NF-κB activation, including regulation of TNF induced cell death (Dondelinger et al., 2015). Indeed, thymocyte development relies exclusively on IKK repression of TNF induced cell death rather than NF-κB activation (Webb et al., 2016, 2019). However, after thymic export, it appears that NF-κB may play a more important role in T cells for normal homeostasis and activation (Silva et al., 2014; Webb et al., 2019). Nonetheless, the relative contributions of NF-κB activation and cell death repression by IKK for peripheral T cells remains unclear. As such, our intention was to clarify the role of the IKK complex in different T cell populations. To address this, we used a range of different mouse models lacking different components of the canonical NF-κB and cell death pathways.

As part of our overall goal, our first aim was to investigate the role of IKK signalling in activated T cells. We wanted to understand the nature of the requirement for IKK during T cell activation. Is it solely required for NF-κB activation? If not, does it regulate cell death and are the cell death pathways regulated differently in activated T cells compared to other T cell states?

The second aim was to explicitly understand the relative importance of IKK activation of NF-κB versus repression of extrinsic apoptosis in mature T cell populations. Do T cell populations continually require IKK signalling and is the requirement for IKK the same across different T cell populations? Is IKK regulation of TNF induced cell death important for survival of mature T cells and if so, can IKK also control death induced by other TNFRSF members?
Chapter 2 - Materials and Methods

2.1 Mice

The details of the mice used are listed in Table 2.1. Mice were bred at Charles River laboratories, Manston, UK. Experiments were undertaken at the Comparative Biology Unit, Royal Free Hospital, UCL. Cre-ve littermates were used as experimental controls. All strains are on a C57BL6/J background, except F5 Rag1−/− lkk2fx/fix huCD2Cre R26R{EYFP} strains which are on a mixed H-2b C57BL6/J/129S1/SvImJ background. Genotyping of mice was carried out by Transnetyx, Cordova, Tennessee. Animal procedures were performed in accordance with the UCL Animal Welfare and Ethical Review Board and in line with Home Office regulations.

2.1.1 Generation of conditional knockout mice

The Cre-lox system was used to conditionally delete alleles in mice. Mice with floxed alleles for Ikk1 (Gareus et al., 2007), Ikk2 (Li et al., 2003), Tnfaip3 (Vereecke et al., 2010) and Casp8 (Beisner et al., 2005) were intercrossed with mice expressing Cre recombinase under the control of the human CD2 (HuCD2Cre) (de Boer et al., 2003) or Cd4 (CD4Cre) (Lee et al., 2001) regulatory elements or with mice expressing CreERT2 under the control of the Cd4 regulatory elements (CD4CreERT) (Śledzińska et al., 2013). For some strains, an EYFP Cre reporter allele was inserted in the ubiquitously expressed Rosa26 gene locus (R26R{EYFP}) (Srinivas et al., 2001). Floxed trimeric TpA stop codons upstream of the EYFP gene are removed during active Cre-recombinase expression. Strains were also backcrossed or intercrossed with Tnfrsf1a−/− (Peschon et al., 1998), Tnf−/− (Pasparakis et al., 1996), RIPK1D138N (Newton et al., 2014) strains.

To induce activity of the CreERT driver, mice were administered tamoxifen (Merck). Tamoxifen was dissolved in corn oil (ACROS organics) to a
concentration of 20mg/ml. Mice, including Cre-ve controls, were injected I.P with 2mg tamoxifen for 5 consecutive days. Mice were sacrificed on day 21.

Table 2.1 Mouse strains used in this thesis

<table>
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<th>Name</th>
<th>Reference</th>
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<td>CD45.1</td>
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<td>Tnf−/−</td>
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### 2.2 Media and Buffers

#### Complete media

Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco, Invitrogen), 1% L-Glutamine-Penicillin-Streptomycin solution (Merck) and 0.1% 2-Mercaptoethanol (Merck).

#### PBS-BSA

Dulbecco’s phosphate buffered saline (DPBS), no calcium, no magnesium (Gibco) supplemented with 0.1% (w/v) bovine serum albumin (BSA) and 10 μm EDTA.

#### Fix/Perm Buffer
1 part Fixation/Permeabilization Concentrate (ThermoFisher Scientific) to 3 parts Fixation/Permeabilization diluent (ThermoFisher Scientific) as per manufacturer’s instructions.

Perm/Wash Buffer
1 part Permeabilization Buffer (10X) (ThermoFisher Scientific) to 9 parts distilled water as per manufacturer’s instructions.

### 2.3 Isolation of cells from mouse tissues

The thymus, lymph nodes, spleen and lungs were dissected from mice. The lymph nodes (cervical, brachial, axillary, inguinal and mesenteric) were pooled together, except for the mediastinal lymph nodes which were processed separately. Cell suspensions in complete media were created by pressing tissues through 71 μM diameter nylon mesh (SEFAR NYTAL) with tweezers. Cell suspensions were filtered through 30 μM diameter mesh (Sysmex/Partec CellTrics) and pelleted by centrifugation (1500 rpm for 5 mins at 4°C). Thymus, lymph node and spleen cells were resuspended in complete media and kept on ice. Lung cells were resuspended in 1 ml Ammonium-Chloride-Potassium (ACK) lysis buffer (Gibco) and incubated at room temperature for 3 mins to lyse red blood cells. Lung cell suspensions were then washed with 2 ml complete media, centrifuged (1500 rpm for 5 mins at 4°C), resuspended in complete media and kept on ice. Cell concentrations were determined using an Casy Cell Counter (OMNI Life Science, Germany).

### 2.4 Flow cytometry

#### 2.4.1 Surface staining

For flow cytometric analysis, 0.2-3.5 x 10^6 lymph node, 2-5 x 10^6 thymocyte, 3.5 x 10^6 spleen or 10-20 x 10^6 lung cells were stained with antibody. Cells were incubated with saturated concentrations of antibodies in 100 μl DPBS.
on ice in the dark for 1 hour. Antibodies used for flow cytometry are listed in Table 2.2. At this stage, cells were also stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) at a 1/800 dilution to assess cell viability. Cells were then washed with PBS-BSA and pelleted by centrifugation (1500 rpm for 5 mins at 4°C). If cells did not require staining for intracellular antigens, they were fixed by resuspension in 300 μl IC Fixation Buffer (ThermoFisher Scientific) and incubated on ice in the dark for 20 mins. Cells were then washed with PBS-BSA, pelleted by centrifugation (1500 rpm for 5 mins at 4°C) and resuspended in PBS-BSA.

### 2.4.2 Intracellular staining

Following surface staining, cells were fixed and permeabilised by resuspension in 300 μl Fix/Perm Buffer and incubation on ice in the dark for 30 mins. Cells were then washed in Perm/Wash buffer, pelleted by centrifugation (1500 rpm for 5 mins at 4°C) and resuspended in PBS-BSA. Samples were kept on ice overnight or longer. Before intracellular staining, cells were washed twice with Perm/Wash buffer. Cells were incubated with saturated concentrations of anti-Ki-67 and/or anti-FoxP3 antibodies in 100 μl DPBS at on ice in the dark for 1 hour. Cells were then washed twice with Perm/Wash buffer, pelleted by centrifugation (1500 rpm for 5 mins at 4°C) and resuspended in PBS-BSA. For DNA staining, after permeabilization but immediately prior to flow cytometric analysis, cells were incubated with 5 μg/ml 7-aminoactinomycin D (7-AAD) (Merck) in Perm/Wash buffer for 30 mins at 20°C. The 7-AAD was not washed out of samples prior to acquisition and fluorescence was measured on a linear scale.

**Table 2.2 Antibodies used for flow cytometry**

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2.4.3 Flow cytometric analysis of stained samples

Stained samples were analysed using an LSRFortessa (BD Biosciences). Data analysis and colour compensations were performed using FlowJo V10 software (Becton Dickinson). Data are displayed on log and biexponential displays.

2.5 In vitro cell culture

Thymus or lymph nodes cells were isolated and cell suspensions prepared as described previously and cultured in complete media at 37°C and 5% CO₂. Thymocytes were cultured at 1-2.5 x 10⁶ cells per ml in 24 well plates and lymph node cells were cultured at 1.25-5 x 10⁶ cells per ml in 96 well plates, except for activation experiments. Cells were cultured with varying peptides, recombinant proteins, inhibitors and chemicals listed in Table 2.3. At the end of the culture period, cells were stained and analysed by flow cytometry.

2.5.1 T cell activation

Lymph node cells were labelled with CellTrace Violet (CTV). 20 x 10⁶ cells / ml were incubated with 5 μM CTV in prewarmed DPBS at 37°C for 10 mins. Cells were then washed with ice cold complete media, centrifuged (1500 rpm for 5 mins at 4°C) and resuspended in complete media.

For peptide stimulations of F5 cells, the number of CD8⁺ cells in each sample was first determined by flow cytometry. Lymph node cells containing 2 x 10⁵ CD8⁺ cells were cultured in 200 μl complete media in 96 well round bottom plates for up to 72 hrs. 1 x 10⁵ Rag1⁻⁻ mouse splenocytes were added to each well as a source of APCs, except for TNF⁻⁻ F5 T cell cultures where splenocytes from TNF⁻⁻ mice were used. NP-68 was supplemented to cultures at the concentrations stated. Mean no. divisions (division index), precursor frequency and burst size (proliferation index) were calculated from
cell numbers in each CTV fluorescence peak using FlowJo V10 software and the formulas described in (Roederer, 2011). For cell cycle analysis, the frequency of “post d1” cells is equivalent to the precursor frequency. The frequency of “S + G2/M” cells was calculated by adding the frequency of undivided Ki-67+ 7-AADhi cells to the precursor frequency. The frequency of “G1” cells was calculated by adding the frequency of undivided Ki-67+ 7-AADlo cells to the frequency of “S + G2/M” cells.

For stimulations with anti-CD3 and anti-CD28, 48 well flat bottom plates were coated with 1μg/ml anti-CD3 and 1μg/ml anti-CD28 antibodies in DPBS for 2 hours at 37°C. Plates were then washed with DPBS. The number of TCRβ+ cells in each sample was determined by flow cytometry. Lymph node cells containing 2 x 10⁵ TCRβ+ cells were cultured in 500 μl complete media for 48 hrs.

**Table 2.3 Peptides, recombinant proteins, inhibitors, chemicals, and antibodies used for in vitro cell cultures**

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<tbody>
<tr>
<td>Influenza A NP (366 - 374) Strain A/NT/60/68 (NP-68)</td>
<td>10⁻¹³ to 10⁻⁷ M</td>
<td>Mimotopes Cat# 58255-010</td>
</tr>
<tr>
<td>Recombinant Murine TNF-α</td>
<td>0.0064 to 100 ng/ml</td>
<td>Peprotech Cat# 315-01A-50uG</td>
</tr>
<tr>
<td>FasL, soluble (human) (recombinant) set</td>
<td>Flag-FasL: 0.1 to 100 ng/ml Enhancer: 1 μg/ml</td>
<td>Enzo Life Sciences Cat# ALX-850-014-K102</td>
</tr>
<tr>
<td>SuperKillerTRAIL, Soluble (mouse) (rec.)</td>
<td>0.1 to 100 ng/ml</td>
<td>Adipogen Life Sciences Cat# AG-40T-0004-C020</td>
</tr>
</tbody>
</table>
### Table 1: Compounds and their suppliers

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrostatin-1 (Nec-1)</td>
<td>5 or 10 μM</td>
<td>Santa Cruz Biotechnology Cat# sc-200142</td>
</tr>
<tr>
<td>BI 605906 (IKK2 inhibitor)</td>
<td>0.31 to 20 μM</td>
<td>Tocris Bioscience Cat# 5300</td>
</tr>
<tr>
<td>IKK16</td>
<td>0.063 to 4 μM</td>
<td>Selleck Cat# S2882</td>
</tr>
<tr>
<td>CellTrace Violet Cell Proliferation Kit</td>
<td>5 μM</td>
<td>ThermoFisher Scientific Cat# C34557</td>
</tr>
<tr>
<td>Purified Hamster Anti-Mouse CD3e</td>
<td>1 μg/ml</td>
<td>BD Pharmingen Clone 145-2C11</td>
</tr>
<tr>
<td>Purified Hamster Anti-Mouse CD28</td>
<td>1 μg/ml</td>
<td>BD Pharmingen Clone 37.51</td>
</tr>
</tbody>
</table>

### 2.6 Adoptive transfer of T cells and influenza infection

Lymph nodes cells from donor F5 strains were isolated and cell suspensions prepared as described previously. The number of CD8+ TCRβ+ cells in each sample was determined by flow cytometry. Cells were washed twice with DPBS. Lymph node cells containing 1 x 10^6 CD8+ TCRβ+ cells in 200 μl DPBS were injected I.V. via the tail vain into host CD45.1 mice. Mice were challenged with 100 hemagglutinating (HA) units of influenza A virus (A/NT/60-68; H3N2) by I.V or I.N administration. Virus was injected I.V. simultaneously with donor cells. For I.N virus administration, mice were lightly anaesthetised with isoflurane. Virus in 30 μl DPBS was pipetted in droplets onto mouse nose. Mice were sacrificed after 7 days and tissues were analysed by flow cytometry.

### 2.7 Intraperitoneal injection of anti-TNF antibody

To block TNF in vivo, mice were administered anti-TNF antibody (InVivoMAb anti-mouse TNFα, Clone XT3.11, Bio X cell). Mice were injected with 0.5mg...
anti-TNF antibody three times a week for two weeks (days 0, 2, 4, 7, 9, 11). Control mice were injected with PBS alone. Mice were sacrificed on day 14.

2.8 Analysis of RNA sequencing dataset

2.8.1 Description of dataset

Lymph node cells were isolated from F5 Rag1\(^{-/-}\) Ik2\(^{-fx/fx}\) HuCD2\(^{Cre}\) R26R\(^{EYFP}\) Cre -ve and Cre +ve mice. Cells were cultured with NP-68, necrostatin-1 and Rag1\(^{-/-}\) splenocytes for 24 hrs. After the culture period, CD8\(^{+}\) TCR\(^{hi}\) cells were sorted, mRNA was extracted using the Isolate II RNA mini kit (Bioline) and samples were sequenced using an Illumina Genome Analyser IIx. This generated paired-end reads. The experiment was repeated 4 times, each on different days. These experiments were performed by Louise Webb and Alessandro Barbarulo.

2.8.2 Bioinformatics analysis

R (version 4.0.3) and RStudio (version 1.3.1093) were used for RNA sequencing data analysis using a workflow adapted from (Chen et al., 2016; Law et al., 2018). This analysis was performed by myself. The subread spice-aware aligner (Liao et al., 2013b) of the Rsubread package was used to align the sequencing reads to the mm10 mouse reference genome. The number of fragments overlapping each gene was determined using featureCounts (Liao et al., 2014) with the built in NCBI RefSeq gene annotations, including Entrez Gene IDs, for the mm10 genome. Differential expression analysis was conducted using the edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) packages. Gene symbols were mapped to Entrez Gene IDs using the org.Mm.eg.db package (Carlson, 2020). Genes which could not be mapped to gene symbols were removed from the analysis. The edgeR filterByExpr function was used to filter out genes with less than 0.34 counts per million reads in 3 or more samples. The library sizes for each sample were normalised using the edgeR calcNormFactors
function with uses the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010). Robust empirical Bayes generalised linear models extended with quasi-likelihood methods (Lun et al., 2016; Lund et al., 2012; McCarthy et al., 2012; Phipson et al., 2016) were used to estimate the biological and technical variation in gene expression between samples. Quasi-likelihood F-tests (Lund et al., 2012) were used to assess differential expression of genes between samples. P-values were adjusted by the Benjamini-Hochberg method to control the false discovery rate (Benjamini and Hochberg, 1995).

The limma plotMDS and plotMD functions were used to create multi-dimensional scaling (MDS) and mean-difference (MD) plots. For gene-set testing, the Molecular Signatures Database (MSigDB) Hallmark gene sets mapped to mouse ortholog genes were downloaded from the Walter and Eliza Hall Institute for Medical Research Bioinformatics webpage (https://bioinf.wehi.edu.au/MSigDB/index.html, downloaded on 29/11/2021). The limma roast function with 9,999 rotations was used to test the “HALLMARK_TNFA_SIGNALING_VIA_NFKB” gene set. The outcome of this gene set test was plotted using the limma barcodeplot function. To produce the heatmap, genes were first selected with differential expression significantly above a 1.2 fold change threshold using the edgeR glmTreat function. A heat map of the log counts per million read values of the top 50 differentially expressed genes was created using the limma coolmap function. The ggplot2 (Wickham, 2016) and ggrepel (Slowikowski et al., 2021) packages were used to produce the volcano plot. Expression of individual genes between Cre -ve and Cre +ve samples was plotted as normalised fragments per kilobase exons per million mapped fragments (nFPKM).

2.9 Statistical analysis

Statistical analysis and preparation of figures were performed in GraphPad Prism version 9 for macOS. Calculations of cell numbers for different cell
populations were performed in Microsoft Excel for Mac version 16.54. Two-way statistical comparisons were made using unpaired two tailed Welch's t-test. Three or more way statistical comparisons were made using one-way ANOVA followed by the Šidák multiple comparisons test. ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001
Chapter 3 - The IKK complex controls survival of T cells during activation

3.1 Introduction

It is well established that NF-κB signalling plays a critical role during T cell activation. After binding peptide-MHC, the TCR can directly trigger NF-κB activation through assembly of the CBM complex. Indeed, TCR induced NF-κB is essential for normal T cell proliferation. Deletion of any of the CBM complex components, leads to impaired upregulation of T cell activation markers, blast transformation and cell cycle progression (Egawa et al., 2003; Hara et al., 2003; Newton and Dixit, 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003). Manipulation of NF-κB subunit expression in T cells has shown individual and overlapping roles for different subunits during T cell activation. Both RelA and c-Rel have important roles for blast transformation through the induction of c-Myc expression (Grumont et al., 2004). The production of IL-2 by activated T cells heavily relies on c-Rel dependent transcription (Köntgen et al., 1995; Liou et al., 1999; Rao et al., 2003). Mice deficient in c-Rel also lack memory T cells (Webb et al., 2019; Zheng et al., 2003). Ablation of p105 or impaired TCR induced proteolysis to form p50 leads to impaired cell cycle progression (Grumont et al., 2004; Sriskantharajah et al., 2009). But mice with combined deletion of both c-Rel and NF-κB1 have more severe cell cycle defects (Zheng et al., 2003). This is consistent with NF-κB having an important role in regulating the expression of cell cycle proteins (Guttridge et al., 1999; Hinata et al., 2003; Iwanaga et al., 2008).

NF-κB is thought to be important for survival of activated T cells. The expression of Bcl-2 family members is known to be regulated by NF-κB (Catz and Johnson, 2001; Chen et al., 2000; Lee et al., 1999; Zong et al., 1999). Early studies suggested that TCR and CD28 driven NF-κB dependent Bcl-xL upregulation was required to protect T cells from cell death after activation (Khoshnan et al., 2000; Manicassamy et al., 2006; Marinari et al., 2004;
Mora et al., 2003). Instead, regulation of the pro-apoptotic protein Bim is important during T cell activation (Hildeman et al., 2002). Following TCR stimulation, the expression of p73 is increased in an E2F dependent manner and subsequently drives apoptosis (Lissy et al., 2000; Wan and DeGregori, 2003). Concurrently, NF-κB promotes the expression of Mdm2 which blocks the interaction between p73 and the Bim promoter; thereby inhibiting Bim mediated apoptosis (Busuttil et al., 2010; Wan and DeGregori, 2003). RelA itself has also been suggested to bind to the Bax promoter to prevent p73 induced transcription (Cianfrocca et al., 2008).

Given the large degree of redundancy between NF-κB subunits, others have attempted to further understand the role of NF-κB by disrupting the function of upstream activators, such as the IKK complex. IKK1 deficient T cells do not proliferate normally in response to anti-CD3/CD28, whereas proliferation of IKK2 deficient T cells is intact, presumably reflecting the differing roles of IKK1 and IKK2 for activation of the non-canonical and canonical NF-κB pathways (Chen et al., 2015; Schmidt-Supprian et al., 2003). On the other hand, T cells require IKK2 for normal recall responses to antigen immunisation (Greve et al., 2007; Schmidt-Supprian et al., 2004b). But inactivation of both IKK1 and IKK2, leading to complete NF-κB blockade, inhibits T cell proliferation entirely, further highlighting the crucial role of NF-κB during T cell activation (Webb et al., 2019).

Since the earlier studies implicating a role for IKK signalling during T cell activation, new functions of the IKK complex have come to light. Indeed, the IKK complex is capable of phosphorylating proteins other than the IkB proteins, thereby regulating the activity of other targets independently of NF-κB activation. Proteins now established to be IKK targets are involved in different cellular processes including autophagy, metabolism, cell cycle regulation, differentiation and apoptosis (Antonia et al., 2021). Recently, it’s been shown that the IKK complex regulates the survival of SP thymocytes independently of NF-κB (Webb et al., 2019). Whether this function of the IKK complex is important in activated T cells is unknown.
It’s evident that NF-κB has essential roles during the early stages of T cell activation. Ablation of multiple canonical NF-κB subunits or the IKK complex leads to a complete failure of T cells to divide (Grumont et al., 2004; Webb et al., 2019). As a result, this has impeded exploration of canonical NF-κB in proliferating T cells. Furthermore, although both models have been used for the purpose of understanding canonical NF-κB in activated T cells, they are also likely to exhibit significant disruption to the non-canonical NF-κB pathway. The expression of RelB and NF-κB2 is largely reliant on canonical NF-κB dependent transcription, including in thymocytes (Basak et al., 2008; Bren et al., 2001; Lombardi et al., 1995; Webb et al., 2019). IKK1 is required for optimal activation of the canonical NF-κB pathway but is also crucial for induction of the non-canonical pathway (Senftleben et al., 2001). Consequently, simultaneous disruption of both NF-κB pathways may obscure specific functions of canonical NF-κB in activated proliferating T cells.

Our aim was to understand the role of canonical NF-κB signalling during the later stages of T cell activation. To do this, we perturbed IKK activity in T cells, by ablating IKK2, and assessed the impact of IKK2 deficiency on antigen specific T cell responses both in vitro and in vivo. In this way, we could impair activation of the canonical NF-κB pathway without impacting the non-canonical pathway. But since canonical NF-κB signalling is not completely blocked in absence of IKK2, we predicted that cell division can take place, enabling investigation of canonical NF-κB in activated proliferating T cells (Schmidt-Supprian et al., 2003).
3.2 Results

3.2.1 IKK2 signalling is required for normal T cell proliferation

NF-κB signalling is essential during the early events of T cell activation. Consequently, to understand the role of NF-κB during the later stages of T cell activation, models with complete NF-κB blockade are not appropriate. Instead, we used mice with conditional deletion of *Ikk2*, resulting in impaired canonical NF-κB signalling but leaving the non-canonical NF-κB pathway intact. Floxed *Ikk2* (*Ikk2*fx/fix) alleles (Li et al., 2003) were deleted by Cre-recombinase under the control of the human CD2 (huCD2) promoter and locus control region within an inserted minigene cassette (huCD2iCre) (de Boer et al., 2003; Zhumabekov et al., 1995). Previously, this cassette has been found to induce Cre-recombinase expression in T cells as early as the double negative 2 stage of development in the thymus (de Boer et al., 2003). To identify cells which have expressed active Cre-recombinase, we also used an EYFP Cre reporter allele inserted in the ubiquitously expressed *Rosa26* gene locus (*R26R*EYFP hereon) (Srinivas et al., 2001). Floxed trimeric TpA stop codons upstream of the EYFP gene are removed during active Cre-recombinase expression.

To facilitate antigen specific stimulation of *Ikk2* deficient T cells, *Ikk2*fx/fix HuCD2iCre R26REYFP mice were crossed with TCR transgenic F5 *Rag1*−/− mice to generate F5 *Rag1*−/− *Ikk2*fx/fix HuCD2iCre R26REYFP (termed F5 *Ikk2*ΔTCD2) mice as detailed by Silva et al., 2014. Mice of this strain produce CD8+ T cells with the F5 TCR which recognises peptide 366-374 of influenza virus nucleoprotein (NP-68) in the context of H-2Db MHC class I (Mamalaki et al., 1993). The lack of *Rag1* expression prevents recombination of endogenous *Tcr* alleles resulting in exclusive expression of the F5 TCRαβ chains but also leads to an absence of B cells. Thymocyte development in F5 *Ikk2*ΔTCD2 mice is comparable to F5 Cre-ve (F5 CTRL) mice, however there are fewer peripheral T cells. This is due to a failure of new F5 *Ikk2*ΔTCD2 T cells to upregulate IL-7R, a functional canonical NF-κB gene target required by T cells for normal peripheral homeostasis (Silva et al., 2014).
To investigate whether canonical NF-κB signalling is important in T cells during activation, we stimulated F5 *Ikk2ΔTCD2* T cells *in vitro*. Cells were CTV labelled and cultured with peptide for 72 hrs. CTV profiles showed that a considerable fraction of the F5 *Ikk2ΔTCD2* T cells remained undivided (Fig. 3.1A). F5 *Ikk2ΔTCD2* T cells apparently underwent fewer divisions compared to F5 CTRL T cells at matched peptide concentrations, except at the highest concentration of peptide used (Fig. 3.1B). Additional characterisation of F5 *Ikk2ΔTCD2* T cell proliferation over time revealed that the average number of divisions within the culture plateaued after 48 hrs (Fig. 3.1C). This occurred since only ~40% of the precursor population had committed to cell division compared to ~90% for F5 CTRL T cells. Nonetheless, those F5 *Ikk2ΔTCD2* precursor T cells which were able to commit to division could proliferate in a similar manner to F5 CTRL T cells after 48 and 72 hrs since the burst size was similar.

Therefore, this data suggests that intact canonical NF-κB signalling is required by T cells to commit to a proliferative burst after stimulation but is not a continued requirement of proliferating T cells.
Figure 3.1 IKK2 signalling is required for normal T cell proliferation

Lymph node cells were isolated from F5 Rag1<sup>−/−</sup> lkk2<sup>flox/flox</sup> HuCD2<sup>Cre</sup> R26R<sup>ERT</sup> Cre<sup>+</sup> mice (F5<sub>Ik</sub>2<sup>ΔT<sub>CD2</sub></sup>) and Cre<sup>−</sup>ve controls (F5 CTRL). Cells were labelled with CTV and stimulated with NP-68. The cultures were analysed by flow cytometry.

(A) T cells were stimulated with 10<sup>−9</sup> M NP-68 for 72 hrs. Histograms show the relative CTV fluorescence by live T cells.

(B) T cells were stimulated with between 0 and 10<sup>−7</sup> M NP-68 for 72 hrs. The mean number of divisions was calculated from the numbers of T cells in each CTV peak.

(C) T cells were stimulated with 10<sup>−9</sup> M NP-68 and analysed after 0, 24, 48 and 72 hrs. The number of T cells in each CTV peak was used to calculate the mean number of divisions, precursor frequency and burst size.

Data are representative of 1 (B) or 4 (A) independent experiments or are pooled from 4 independent experiments (C). Error bars indicate SD.
3.2.2 T cell priming and cell cycle progression is normal in activated F5 Ikk2ΔT<sup>CD2</sup> T cells

Since the proliferation defect observed in F5 Ikk2ΔT<sup>CD2</sup> T cells appeared to reflect a failure to commit to cell division, we asked whether this was due to impaired TCR triggering. Acute activation of the TCR causes rapid upregulation of the activation markers CD69 and CD25 on the cell surface. After 24 hrs, F5 Ikk2ΔT<sup>CD2</sup> T cells upregulated both CD69 and CD25 in a similar manner to F5 CTRL T cells, however at the lower peptide concentrations (10<sup>-10</sup>-10<sup>-11</sup> M NP-68) there was a small decrease in the percentage of T cells expressing either marker (Fig. 3.2A). CD44, a marker upregulated by T cells as they differentiate from naïve to effector/memory cells, also shows comparable expression to F5 CTRL T cells. Importantly, when F5 Ikk2ΔT<sup>CD2</sup> T cells were stimulated with 10<sup>-9</sup> M NP-68, the peptide concentration at which the proliferation defect is most evident, almost all cells had upregulated both CD69 and CD25 (Fig. 3.2B). Therefore, there was no evidence that TCR triggering was defective in the absence of IKK2.

Shortly after receiving a TCR stimulus and following successful TCR triggering, T cells prepare for cell division by growing in size, as a result of increased protein synthesis, in a process termed blast transformation. Canonical NF-κB induced c-Myc expression is crucial for normal T cell blast transformation (Grumont et al., 2004). Analysis of F5 Ikk2ΔT<sup>CD2</sup> T cells by flow cytometry 24 hrs after peptide stimulation showed a similar increase in forward and side scatter to F5 CTRL T cells (Fig. 3.2C). The expression of myc at the mRNA level was also normal, altogether suggesting that the blast transformation event is not disrupted in absence of IKK2 (Fig. 3.2D).

Since acute T cell priming proceeded normally in F5 Ikk2ΔT<sup>CD2</sup> T cells after stimulation, we asked whether the proliferation defect was instead due to a failure to enter and progress through the cell cycle. T cells with defective canonical NF-κB signalling fail to cycle normally after stimulation (Grumont et al., 2004; Zheng et al., 2003). Indeed, NF-κB is known to regulate the expression of genes involved in the cell cycle, although many of these
targets have not been validated in T cells. Both IKK1 and IKK2 have also been suggested to directly restrain activity of Aurora A and facilitate progression through mitosis (Blazkova et al., 2007; Irelan et al., 2007; Prajapati et al., 2006). Most F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} T cells apparently fail to divide after stimulation, implying a possible cell cycle block during interphase. Genes, such as \textit{Ccdn2} (cyclin D2), \textit{Cdk6} and \textit{Cdkn1a} (p21), with critical roles during this phase of the cell cycle also have promoters containing NF-κB binding sites (Hinata et al., 2003; Huang et al., 2001; Iwanaga et al., 2008). At 24 hrs post stimulation, the expression of most NF-κB target genes active early in the cell cycle was normal. However, expression of \textit{Ccnd2} and \textit{Cdkn1a} were significantly reduced (Fig. 3.2E). Nonetheless, there is likely considerable redundancy between different cyclin and CDK proteins. To understand if F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} T cells enter the cell cycle normally, we evaluated the transition of cells between cell cycle phases. CTV labelled F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} T cells were stimulated with NP-68 and at different time points, Ki67 expression, cellular DNA content and CTV dye dilution were measured. This enabled the identification of T cells in G1 and S+G2/M phases and cells which had completed division (post d1) (Fig. 3.2F). A similar proportion of the F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} T cell precursor population had progressed through the cell cycle phases and went on to divide (Fig. 3.2G). Up to 30hrs after stimulation, the fraction of F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} precursors in G1 and S+G2/M phases was analogous to F5 CTRL precursors. But by 38hrs, ~80% of F5 CTRL precursors had passed through each phase and ultimately divided, whereas only ~30% of F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} precursors had. Collectively this data suggested that F5 \textit{Ik}k2\textsuperscript{Δ}T\textsuperscript{CD2} T cells can progress through the cell cycle normally after stimulation, but the majority of them do not appear to do.
Figure 3.2 T cell priming and cell cycle progression is normal in activated F5 Ikk2ΔT<sup>CD2</sup> T cells

Lymph node cells were isolated from F5 Ikk2ΔT<sup>CD2</sup> and F5 CTRL mice. Cells were stimulated with NP-68 for 24 hrs. The cultures were analysed by flow cytometry.

(A) T cells were stimulated with between 0 and 10<sup>-7</sup> M NP-68. Plots show the percentage of T cells expressing CD69, CD25 and CD44.

(B) T cells were stimulated with 10<sup>-9</sup> M NP-68. Bar plot shows the percentage of T cells expressing both CD25 and CD69.

(C) Density plots are of side scatter (SSC) vs forward scatter (FSC) for live T cells from indicated strains after culture with or without 10<sup>-9</sup> NP-68.

(D, E) Analysis of RNA-seq data. Gene expression displayed as normalised fragments per kilobase exons per million fragments (nFPKM).

(F) Gating for cell cycle analysis. Lymph node cells from F5 CTRL mice were labelled with CTV and stimulated with 10<sup>-9</sup> M NP-68 for 38 hrs. Density plot of 7-AAD vs Ki-67 for live T cells. Cells in G1 (Ki-67<sup>+</sup> 7-AAD<sup>lo</sup>) and S+G2/M (Ki-67<sup>+</sup> 7-AAD<sup>hi</sup>) phases are gated. Histogram shows relative CTV fluorescence by live T cells. Cells with undiluted CTV are gated “d0” and cells with diluted CTV are gated “Post d1”.

(G) Lymph node cells were isolated from F5 CTRL and F5 Ikk2ΔT<sup>CD2</sup> mice. Cells were labelled with CTV and stimulated with 10<sup>-9</sup> M NP-68 over a 62 hr period. The percentage of precursor T cells which had progressed though the given cell cycle stage at each time point was determined using the gating described in (F).

Data is representative of 1 (A), 4 (C) or 6 (G) independent experiments or pooled from 4 (B) independent experiments. Error bars indicate SD.
3.2.3 F5 Ikk2ΔT<sup>CD2</sup> T cells undergo cell death following activation

Our analysis suggested that F5 Ikk2ΔT<sup>CD2</sup> T cells exhibited some manner of cell cycle defect, since fewer cells committed to cell division. However, contrary to this, both TCR dependent triggering of T cells and subsequent blast transformation appeared normal in F5 Ikk2ΔT<sup>CD2</sup> T cells. Altered cell death regulation could also explain the observed phenotype, were dividing cells specifically susceptible to cell death in the absence of IKK2. However, since only viable cells are considered in the analysis of proliferation, any impact of cell death would not be immediately apparent. We therefore analysed cell viability amongst all cells in cultures of activated T cells to determine whether cell death responses were indeed altered. During apoptosis, cells undergo DNA fragmentation, resulting in a loss of cellular DNA and the appearance of cells with “sub-diploid” DNA content. Analysing DNA content of stimulated F5 Ikk2ΔT<sup>CD2</sup> T cells revealed an increased proportion of cells within the sub-diploid peak after 24 hrs compared to F5 CTRL T cells (Fig. 3.3A). After 38hrs, the proportion of sub-diploid F5 Ikk2ΔT<sup>CD2</sup> T cells had increased further. Analysis of sub-diploid F5 Ikk2ΔT<sup>CD2</sup> T cells over 62 hrs revealed maximal death occurred as early as 30hrs after stimulation (Fig. 3.3B). We confirmed the increased death of activated F5 Ikk2ΔT<sup>CD2</sup> T cells by using an amine-reactive dye that can bind intracellular proteins when cell membrane integrity is lost during most forms of cell death. Quantifying cell death using this dye mirrored the pattern of death seen by measuring cellular DNA content (Fig 3.3C).

Apoptosis can be induced in cells via the intrinsic or the extrinsic pathways. Canonical NF-κB signalling is thought to control the intrinsic apoptotic pathway by regulating expression of Bcl-2 family members (Catz and Johnson, 2001; Lee et al., 1999; Zong et al., 1999). Before activation, expression of Bcl-2 family genes in naive F5 Ikk2ΔT<sup>CD2</sup> T cells is normal (Silva et al., 2014). Therefore, we examined whether regulation of these genes is changed in F5 Ikk2ΔT<sup>CD2</sup> T cells after activation. The majority of Bcl-2 family members showed normal expression 24 hrs post stimulation with the
exception of modest decreases in Bcl2l1 (BCL-xL), Bcl2a1 (BFL-1/A1) and Bax, and an increase in the expression of Bmf (Fig. 3.3D).
Figure 3.3 F5 Ikk2ΔT\textsuperscript{CD2} T cells undergo cell death following activation

Lymph node cells were isolated from F5 Ikk2ΔT\textsuperscript{CD2} and F5 CTRL mice. Cells were stimulated with 10^{-8}/10^{-9} NP-68 over a 62 hr period. The cultures were analysed by flow cytometry.

(A) Density plots are of Ki-67 vs 7-AAD for T cells at 24 and 38 hrs. The gated Ki-67\textsuperscript{-} 7-AAD\textsuperscript{lo} T cells were identified as having sub-diploid DNA content. Numbers indicate the percentage of cells with sub-diploid DNA content.

(B) The percentage of T cells with sub-diploid DNA content at each time point over 62 hrs.

(C) The percentage of T cells staining positive with an amine-reactive dye at each time point over 62 hrs.

(D) Analysis of RNA-seq data. Gene expression displayed as normalised fragments per kilobase exons per million fragments (nFPKM).

Data is representative of 6 (A) independent experiments or pooled from 6 (B, C) independent experiments. Error bars indicated SEM (B, C) or SD (D).
3.2.4 Death of activated F5 Ikk2ΔTC02 T cells is RIPK1 kinase dependent

As there appeared to be no substantial change in the regulation of the intrinsic apoptotic pathway which could account for the death of activated F5 Ikk2ΔTC02 T cells, we wanted to determine whether regulation of extrinsic cell death pathways was perturbed. Earlier studies have shown the IKK complex to be crucial in protecting developing thymocytes from TNFR1 induced cell death by repression of RIPK1 kinase activity, independently of NF-κB activation (Dondelinger et al., 2015; Webb et al., 2016, 2019). Therefore, we wanted to test whether activated F5 Ikk2ΔTC02 T cells were dying due to a failure to control RIPK1 kinase activity. To test this, F5 Ikk2ΔTC02 T cells were stimulated with peptide in the presence of necrostatin-1 (Nec-1), an inhibitor of RIPK1 kinase activity. Since the kinase activity of RIPK1 is dispensable for NF-κB activation, Nec-1 has no effect on this pathway (Degterev et al., 2008; Polykratis et al., 2014). When added to cultures of activated T cells, Nec-1 could almost completely rescue F5 Ikk2ΔTC02 T cells from cell death, particularly 38 hrs after stimulation (Fig. 3.4A). Although cell death was increased in IKK2 deficient T cells, it was possible that increased cell death and an apparent proliferation defect amongst activated F5 Ikk2ΔTC02 T cells reflected two independent functions of IKK2. Therefore, we analysed cell cycle progression of activated F5 Ikk2ΔTC02 T cells in the presence of Nec-1. Significantly, blocking RIPK1 kinase activity resulted in an apparent increase in the proportion of precursor cells which had progressed through G1 phase and subsequently divided 38 hrs post stimulation (Fig. 3.4B).

We next wanted to identify the cell surface receptor responsible for inducing RIPK1 dependent cell death in activated F5 Ikk2ΔTC02 T cells. Members of the TNFR superfamily are capable of inducing RIPK1 activity, in particular TNFR1. Since regulation of RIPK1 by the IKK complex is known to occur downstream of TNFR1, and blockade of TNFR1 signalling could almost completely rescue death of IKK1/2 deficient thymocytes, we hypothesised that TNFR1 was responsible for activating RIPK1 in activated F5 Ikk2ΔTC02 T
cells (Dondelinger et al., 2015; Webb et al., 2016). To test this, we generated TNF deficient F5 \( \text{Ikk2}\Delta^T\text{CD2} \) mice (F5 \( \text{Ikk2}\Delta^T\text{CD2} \text{Tnf}^{-/-} \)). Absence of TNF signalling could rescue F5 \( \text{Ikk2}\Delta^T\text{CD2} \) T cells from cell death completely, up to 24 hrs post stimulation, and gave a partial rescue from death after 38 hrs (Fig. 3.4C). The addition of Nec-1 to the culture gave an additional rescue of F5 \( \text{Ikk2}\Delta^T\text{CD2} \text{Tnf}^{-/-} \) T cells from cell death after 38hrs.

Altogether, this data indicates that IKK2 is required to protect activated T cells from RIPK1 dependent cell death, which can be induced by TNF.
Figure 3.4 Death of activated F5 Ilk2ΔTCD2 T cells is RIPK1 kinase dependent

Lymph node cells were isolated from the indicated strains. Cells were stimulated with NP-68 over a 62 hr period with or without Necrostatin-1. The cultures were analysed by flow cytometry.

(A) T cells were stimulated with 10^{-8}/10^{-9} M NP-68. At each time point, the percentage of T cells with sub-diploid DNA content was determined.

(B) Cells were labelled with CTV and stimulated with 10^{-9} M NP-68 over a 62 hr period. The percentage of precursor T cells which had progressed through the given cell cycle stage at each time point was determined using the gating described in (3.2F).

(C) T cells were stimulated with 10^{-8} M NP-68. At each time point, the percentage of T cells with sub-diploid DNA content was determined.

Data is pooled from 6 (A) independent experiments or is representative of 1 (C) or 6 (B) independent experiments. Error bars indicate SEM.
3.2.5 Impaired response of F5 Ikk2ΔT^CD2 T cells to influenza infection in vivo

To validate a role for IKK2 in protecting activated T cells from cell death in vivo, we used a flu challenge model. F5 T cells were adoptively transferred to congenic CD45.1 C57Bl6/J mice which were subsequently infected with H3N2 influenza A virus. After 7 days, at the peak of the T cell response, the mice were sacrificed and the F5 T cells in the peripheral organs were analysed (Fig. 3.5A). Firstly, to validate the model, we analysed the F5 T cell response in different peripheral organs after intravenous (I.V) or intranasal (I.N) virus administration. Both routes of virus administration induced a robust F5 T cell response in the lymph nodes and spleen, characterised by extensive expansion of transferred F5 T cells. While the magnitude of the F5 response was greatest following I.V virus administration (Fig. 3.5B), only I.N virus administration induced a substantial F5 T cell response in the lung.

Using this model, we first asked whether IKK2 was required for normal CD8^+ T cell responses. Previous work has suggested that IKK2 deficient T cell responses to antigen are impaired in vivo (Greve et al., 2007; Schmidt-Supprian et al., 2004b). Therefore, we analysed the F5 Ikk2ΔT^CD2 T cell response 7 days after I.V viral challenge. There was a large reduction in the percentage of F5 Ikk2ΔT^CD2 T cells in the spleen compared to F5 CTRL T cells, as distinguished by the congenic marker CD45.2 (Fig. 3.5C). The ratio of CD44^hiCD62L^+ (T_cm) vs CD44^hiCD62L^- (T_em) T cells did not change, however. This was consistent with a profound decrease in the number of peripheral F5 Ikk2ΔT^CD2 T cells, reflecting a decrease in the number of both T_cm and T_em cells (Fig. 3.5D, E). Although the majority of naive F5 Ikk2ΔT^CD2 T cells expressed EYFP (reporting Cre-recombinase activity) before adoptive transfer, a small proportion of them did not, suggesting an absence of Cre activity in these cells (Fig. 3.5F). IKK2 is required by F5 T cells for induction of IL-7R (Silva et al., 2014). Therefore, we analysed IL-7R levels on EYFP negative donor T cells, prior to transfer, to ask whether a failure to activate EYFP reporter expression also correlated with a failure to delete Ikk2. EYFP negative cells had normal level of IL-7R, compared to EYFP positive F5
Ikk2ΔT<sup>CD2</sup> T cells, suggesting these cells had not induced complete deletion of Ikk2 (Fig. 3.5F). When we analysed EYFP expression in responding T cells from F5 Ikk2ΔT<sup>CD2</sup> donors, we did on some occasions observe a substantial reduction in the percentage of EYFP positive F5 Ikk2ΔT<sup>CD2</sup> T cells 7 days post infection (Fig. 3.5G). Since F5 Ikk2ΔT<sup>CD2</sup> T cells failed to expand in response to influenza infection, the few rare EYFP negative cells, that represent cells escaping Cre mediated Ikk2 deletion, would be at a competitive advantage compared with EYFP positive cells. This was highlighted by the increased representation of escapants from a small percentage amongst the initial F5 Ikk2ΔT<sup>CD2</sup> donor population. As such, to measure the responses of F5 Ikk2ΔT<sup>CD2</sup> T cells, we enumerated only those that were EYFP positive.
Figure 3.5 Impaired response of F5 *Ikk2ΔT<sup>CD2</sup>* T cells to influenza infection in vivo

(A) Schematic detailing experiment setup. 1 x 10⁶ T cells from F5 mice were adoptively transferred to CD45.1 mice. CD45.1 mice were simultaneously administered influenza virus intravenously (I.V) or intranasally (I.N). Mice were sacrificed after 7 days and the tissues were analysed by flow cytometry.

(B) Bar plots show the number of F5 CTRL T cells recovered from the indicated tissues of CD45.1 mice after no infection (n=2) or after influenza administration I.V (n=2) or I.N (n=2).

(C) The phenotype of splenic T cells from CD45.1 mice recovered 7 days after adoptive transfer of T cells from F5 CTRL or F5 *Ikk2ΔT<sup>CD2</sup>* mice and I.V influenza administration. Density plots are of CD45.2 vs CD45.1 by live CD8<sup>+</sup> T cells (top row) and CD62L vs CD44 by gated CD8<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>+</sup> T cells (Bottom row). Numbers indicate percentage of cells in each gate.

(D) Bar plot shows the number of CD8<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>+</sup> F5 T cells (EYFP<sup>+</sup> for F5 *Ikk2ΔT<sup>CD2</sup>*) recovered from the lymph nodes and spleen of CD45.1 mice.

(E) Bar plots show the number of F5 T cells (EYFP<sup>+</sup> for F5 *Ikk2ΔT<sup>CD2</sup>*) with a T<sub>cm</sub> (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and T<sub>em</sub> (CD44<sup>hi</sup>CD62L<sup>lo</sup>) phenotype.

(F) Histograms showing relative fluorescence of EYFP (left) and IL-7R (right) by live T cells from F5 mice before adoptive transfer. Left histogram is of live F5 *Ikk2ΔT<sup>CD2</sup>* T cells. Right histogram is of live F5 CTRL (filled grey), F5 *Ikk2ΔT<sup>CD2</sup>* EYFP<sup>-ve</sup> (black line) and F5 *Ikk2ΔT<sup>CD2</sup>* EYFP<sup>+</sup> (red line) T cells. Number indicates percentage of cells in each gate.

(G) Scatter plot showing the percentage of F5 *Ikk2ΔT<sup>CD2</sup>* T cells that expressed EYFP recovered from the spleens of CD45.1 mice 7 days after adoptive transfer.

Data in D, E and G is pooled from 4 independent experiments with 11 CD45.1 mice receiving F5 CTRL T cells and 12 CD45.1 mice receiving F5 *Ikk2ΔT<sup>CD2</sup>* T cells in total. Error bars indicate SD.
3.2.6 IKK2 is required for normal T cell responses to influenza infection in the lung

Although our data suggested an impaired T cell response by F5 \( \text{Ikk}2\Delta T^{CD2} \) T cells to influenza after I.V administration, this is not the natural site of viral infection. Upon viral infection in the lung, dendritic cells uptake antigen and migrate to the draining mediastinal lymph nodes (Hamilton-Easton and Eichelberger, 1995). Here, they cross-prime T cells which subsequently divide and travel back to the lung to exert their effector functions (Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2008). Hence, we wanted to confirm that the T cell response was also compromised in tissues important during influenza infection. To test this, we investigated the response of F5 \( \text{Ikk}2\Delta T^{CD2} \) T cells to influenza virus administered through the I.N route. After 7 days, there was a large decrease in the percentage of F5 \( \text{Ikk}2\Delta T^{CD2} \) T cells in the spleen and a similar reduction in the number of cells obtained from the lymph nodes and spleen to I.V challenged mice (Fig. 3.6A). This encompassed decreases in the numbers of both \( T_{cm} \) and \( T_{em} \) T cells. Analogous to the spleen, there was also a substantial reduction of F5 \( \text{Ikk}2\Delta T^{CD2} T_{cm} \) and \( T_{em} \) cells in the draining mediastinal lymph nodes (Fig. 3.6B). In the lung, there was a large population of F5 CTRL \( T_{em} \) cells, which was significantly diminished in absence of IKK2 (Fig. 3.6C).

Therefore, F5 T cell responses were diminished in absence of IKK2 independent of the route of influenza administration.
Figure 3.6 IKK2 is required for normal T cell responses to influenza infection in the lung

1 x 10^6 T cells from F5 mice were adoptively transferred to CD45.1 mice. CD45.1 mice were administered influenza virus intranasally (I.N). Mice were sacrificed after 7 days and the tissues were analysed by flow cytometry.

(A) The phenotype of T cells from the periphery of CD45.1 mice. Density plots are of CD45.2 vs CD45.1 by live CD8^+ T cells (top row) and CD62L vs CD44 by gated CD8^+CD45.2^+CD45.1^- F5 T cells (Bottom row) from the spleen. Numbers indicate percentage of cells in each gate. Bar plots show total numbers of CD8^+CD45.2^+CD45.1^- F5 T cells (EYFP^+ for F5 Ikk2ΔT<sup>CD2</sup>) recovered from the lymph nodes and spleen. This is subdivided into the numbers of CD44<sup>hi</sup> CD62L<sup>hi</sup> (T<sub>em</sub>) and CD44<sup>hi</sup> CD62L<sup>lo</sup> (T<sub>cm</sub>) F5 T cells.

(B) The phenotype of T cells from the mediastinal lymph nodes of CD45.1 mice. Density plots are of CD45.2 vs CD45.1 by live CD8^+ T cells (top row) and CD62L vs CD44 by gated CD8^+CD45.2^+CD45.1^- F5 T cells (Bottom row). Numbers indicate percentage of cells in each gate. Bar plots show total numbers of CD8^+CD45.2^+CD45.1^- F5 T cells (EYFP^+ for F5 Ikk2ΔT<sup>CD2</sup>). This is subdivided into the numbers of CD44<sup>hi</sup> CD62L<sup>hi</sup> (T<sub>em</sub>) and CD44<sup>hi</sup> CD62L<sup>lo</sup> (T<sub>cm</sub>) F5 T cells.

(C) The phenotype of T cells from the lungs of CD45.1 mice. Density plots are of CD45.2 vs CD45.1 by live CD8^+ T cells (top row) and CD62L vs CD44 by gated CD8^+CD45.2^+CD45.1^- F5 T cells (Bottom row). Numbers indicate percentage of cells in each gate. Bar plots show total numbers of CD8^+CD45.2^+CD45.1^- F5 T cells (EYFP^+ for F5 Ikk2ΔT<sup>CD2</sup>). This is subdivided into the numbers of CD44<sup>hi</sup> CD62L<sup>hi</sup> (T<sub>em</sub>) and CD44<sup>hi</sup> CD62L<sup>lo</sup> (T<sub>cm</sub>) F5 T cells.

Data is from 1 experiment with 2 CD45.1 mice receiving F5 CTRL T cells and 3 CD45.1 mice receiving F5 Ikk2ΔT<sup>CD2</sup> T cells in total. Error bars indicate SD.
3.2.7 Ablation of RIPK1 kinase activity rescues the response of F5
Ikk2ΔTCD2 T cells to influenza challenge

F5 Ikk2ΔTCD2 T cells show a profoundly compromised response to influenza virus. Since activated F5 Ikk2ΔTCD2 T cells are sensitive to RIPK1 dependent cell death in vitro, we next asked whether the impaired F5 Ikk2ΔTCD2 T cell responses to influenza was due to a failure to repress RIPK1 kinase activity. To test this in vivo, we bred the F5 Ikk2ΔTCD2 strain with an allele of Ripk1 with a D138N mutation (Newton et al., 2014). This mutation blocks the kinase activity of RIPK1 but does not disrupt its scaffold role during canonical NF-κB induction (Polykratis et al., 2014). In absence of RIPK1 kinase activity, there was a substantial, though incomplete, restoration of the F5 Ikk2ΔTCD2 T cell response as shown by an increased percentage of F5 Ikk2ΔTCD2RIPK1D138N T cells in the spleen (Fig. 3.7A) and a modest rescue of peripheral T cell numbers (Fig. 3.7B). The expression of RIPK1D138N appeared to partly rescue both F5 Ikk2ΔTCD2 Tcm and Tem populations (Fig. 3.7C).

Viral infections, such as influenza, are known to elicit a rapid increase in TNF production by monocytes, DCs and by responding T cells. Hence, we wanted to determine whether TNF induction of TNFR1 signalling was responsible for triggering RIPK1 dependent cell death of F5 Ikk2ΔTCD2 T cells during anti-viral responses. To address this, we crossed F5 Ikk2ΔTCD2 mice with Tnfrsf1a−/− mice. Deletion of Tnfrsf1a had no effect on the responses of F5 CTRL T cells. But loss of TNFR1 signalling could partially rescue the peripheral F5 Ikk2ΔTCD2 T cell response, including both Tcm and Tem cells (Fig. 3.7A-C). However, TNFR1 deletion did not rescue F5 Ikk2ΔTCD2 T cell responses to the same extent as RIPK1 kinase dead.

Together, these data suggest that IKK2 is required for normal anti-viral responses by protecting T cells from TNF induced, RIPK1 dependent cell death.
**A**

CD8^+ T cells

CD45.1^+ F5 T cells

**B**

Total

F5 T cell no.

**C**

T~cm~ cells

T~em~ cells

F5 T cell no.
Figure 3.7 Ablation of RIPK1 kinase activity rescues the response of F5
Ikk2ΔT<sup>CD2</sup> T cells to influenza challenge

1 x 10<sup>6</sup> T cells from F5 mice of the indicated strains were adoptively transferred to CD45.1 mice. CD45.1 mice were simultaneously administered influenza virus intravenously (i.V). Mice were sacrificed after 7 days and the tissues were analysed by flow cytometry.

(A) The phenotype of T cells from the spleens of CD45.1 mice. Density plots are of CD45.2 vs CD45.1 by live CD8<sup>+</sup> T cells (top row) and CD62L vs CD44 by gated CD8<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>+</sup> T cells (Bottom row). Numbers indicate percentage of cells in each gate.

(B) Bar plot shows the total numbers of CD8<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>+</sup> F5 T cells (EYFP<sup>+</sup> for F5 Ikk2ΔT<sup>CD2</sup>) of the indicated strains recovered from the lymph nodes and spleen of CD45.1 mice. “+” indicates presence and “-” indicates absence of gene.

(C) Bar plots show the total numbers of CD44<sup>hi</sup> CD62L<sup>hi</sup> (T<sub>em</sub>) and CD44<sup>hi</sup> CD62L<sup>lo</sup> (T<sub>c</sub>) F5 T cells (EYFP<sup>+</sup> for F5 Ikk2ΔT<sup>CD2</sup>) of the indicated strains recovered from the lymph nodes and spleen of CD45.1 mice. “+” indicates presence and “-” indicates absence of gene.

Data is pooled from 4 independent experiments. The number of CD45.1 mice receiving T cells from each F5 mouse strain is as follows: CTRL (n=10); Tnfrsf1a<sup>−/−</sup> (n=6); Ikk2ΔT<sup>CD2</sup> (n=12); Ikk2ΔT<sup>CD2</sup>Tnfrsf1a<sup>−/−</sup> (n=5); Ikk2ΔT<sup>CD2</sup>RIPK1<sup>ΔD138N</sup> (n=6). Error bars indicate SD.
3.3 Discussion

The role of the IKK complex during T cell activation has typically been thought of in terms of canonical NF-κB activation. Since canonical NF-κB signalling is essential for acute activation of T cells, complete blockade of IKK signalling renders T cells unresponsive to stimulation (Webb et al., 2019). Here, we show that IKK2 deficient T cells, with impaired IKK activity, can respond to cognate peptide but fail to proliferate normally in vitro. Expansion of IKK2 deficient T cells after viral challenge in vivo was also profoundly diminished. This supports earlier findings that IKK2 is important for antigen induced T cell proliferation (Greve et al., 2007; Schmidt-Supprian et al., 2004b). The proliferation defect of IKK2 deficient T cells is not as severe as T cells with complete IKK inactivation, which is likely because IKK2 ablation does not induce a total block in canonical NF-κB activation (Webb et al., 2019). Previous work has suggested that complexes of IKK1 and NEMO can trigger some NF-κB activity in T cells in absence of IKK2 (Schmidt-Supprian et al., 2003). Nonetheless, the proliferation impairment appears to be lost when IKK2 deficient T cells are stimulated with high peptide concentrations. IKK2 is also dispensable for proliferation of T cells after anti-CD3/CD28 and PMA/ionomycin stimulation (Schmidt-Supprian et al., 2003). Therefore, strong stimuli may more efficiently induce the activity of sub-optimal IKK1 homodimeric complexes which can compensate for IKK2 in activated T cells.

The impaired proliferation of IKK2 deficient T cells after antigen stimulation has previously been attributed to a defect in T cell priming (Greve et al., 2007; Schmidt-Supprian et al., 2004b). However, we have shown this not to be the case. Even after almost all T cells had upregulated activation markers, they failed to proliferate normally in absence of IKK2. Therefore, acute signalling downstream of the TCR was intact. IKK2 was also dispensable as T cells exited quiescence and blast transformed. Although NF-κB is important for c-Myc dependent blast transformation, T cells only failed to blast upon almost complete NF-κB blockade (Grumont et al., 2004). Thus,
there was sufficient activation of NF-κB by IKK1 homodimeric complexes for c-Myc dependent T cell blasting in absence of IKK2.

Cell cycle progression in T cells lacking IKK2 was normal and, as such, did not account for the apparent proliferation defect. Although NF-κB regulates the expression of genes with vital roles in G1 phase, the expression of most of these genes in stimulated IKK2 deficient T cells was unchanged. There was a small reduction in the expression of Cyclin D2, but Cyclin D3 was expressed normally and is thought to be able to compensate for Cyclin D2 (Satyanarayana and Kaldis, 2009). There was also decreased expression of the cell cycle inhibitor p21, however p21 deficient T cells show accelerated proliferation after stimulation (Balomenos et al., 2000; Santiago-Raber et al., 2001). Indeed, we show that IKK2 deficient T cells which enter the cell cycle go on to divide normally, in line with the observation that IKK2 is not a continued requirement for proliferation of activated T cells.

Instead, we show that intact IKK signalling is required for normal T cell proliferation by protecting activated T cells from cell death. Although T cells can die by different mechanisms, our data strongly indicates that activated T cells were sensitised to apoptosis in the absence of IKK2, since death was characterised by DNA fragmentation which does not occur during necrosis or necroptosis (Ch’en et al., 2008b; Matteucci et al., 1999). However, other studies have suggested that activated IKK2 deficient T cells do not undergo increased cell death (Schmidt-Supprian et al., 2003, 2004b). IKK2 deficient T cells were not more sensitive to cell death after anti-CD3/CD28 or PMA/ionomycin stimulation in vitro (Schmidt-Supprian et al., 2003). But under these conditions, efficient activation of IKK1 homodimeric complexes may compensate to ensure normal T cell survival. Also, immunisation of IKK2 deficient mice with KLH and CFA did not result in increased T cell apoptosis (Schmidt-Supprian et al., 2004b). However, in this study, annexin-V positive T cells were measured at early time points, without consideration of antigen specificity. Our data strongly indicates that antigen stimulated T cells undergo apoptosis in absence of normal IKK signalling.
Classically, canonical NF-κB has been thought to control survival of T cells by regulating expression of Bcl-2 family members (Khoshnan et al., 2000; Mora et al., 2003; Zheng et al., 2003). However, the expression of most Bcl-2 family members is normal in activated IKK2 deficient T cells. Although expression of the anti-apoptotic genes BCL-xL and BFL-1/A1 was lower, these are not thought to be important for activated T cell survival. T cells from mice deficient in either BCL-xL or BFL-1/A1 show normal survival after activation and in vivo memory responses are unaffected (Schenk et al., 2017; Tuzlak et al., 2017; Zhang and He, 2005). As the expression of the majority of Bcl-2 family members was not affected by IKK2 deletion in naïve (Silva et al., 2014) or activated T cells, this indicates that they either do not require canonical NF-κB for their maintenance/induction in mature T cells or that sufficient residual NF-κB remained for normal regulation. Nonetheless, there is evidence that the IKK complex can regulate Bcl-2 family members by phosphorylation and our data cannot rule out differing post-translational control in absence of IKK2 (Sandow et al., 2012; Yan et al., 2013).

Although intrinsic apoptotic pathways appeared normal, we found evidence that IKK2 was controlling the survival of activated T cells by protecting them from RIPK1 dependent extrinsic cell death processes. Our data suggests that dividing T cells lacking IKK2 were exquisitely sensitive to RIPK1 dependent cell death in vitro. Blocking RIPK1 activity in vitro and in vivo could almost completely restore normal IKK2 deficient T cell responses to antigen. The IKK complex is known to directly phosphorylate and inhibit the activity of RIPK1 downstream of TNFR1, independently of NF-κB (Dondelinger et al., 2015). Regulation of RIPK1 by the IKK complex has been found to be crucial for normal thymocyte development (Webb et al., 2019). Our data strongly indicates that this regulatory circuit is also present in activated T cells and is important for T cell survival.

Additionally, sensitivity to RIPK1 dependent death almost entirely accounted for the defective proliferation of T cells in absence of IKK2. Blocking RIPK1 kinase activity restored a normal cell cycle profile. Thus, increased sensitivity to RIPK1 dependent cell death specifically amongst dividing cells was killing
T blasts and removing cells from the cell cycle and proliferation analyses which only considered viable cells. Therefore, undivided T cells were inevitably overrepresented in these analyses since they were not subject to RIPK1 dependent cell death.

Repression of RIPK1 dependent cell death by the IKK complex was particularly crucial once T cells had begun to divide. One possible explanation for this could be the altered regulation of extrinsic cell death machinery. In particular, the abundance and activity of RIPK1 substantially increases in the 48 hrs after stimulation \textit{in vitro} (Ch’en et al., 2008b). The expression of RIPK1 is also developmentally regulated in thymocytes. As thymocytes mature, the expression of RIPK1 increases, which corresponds with increased sensitivity to TNF induced cell death in absence of the IKK complex (Webb et al., 2019). The increased RIPK1 expression, and its potential to induce death, during T cell activation may therefore require more stringent control mechanisms in place to prevent cell death. The point at which IKK2 deficient T cells start to proliferate could reflect the point at which control of RIPK1 becomes insufficient, tipping the balance towards cell death.

Although RIPK1 is a potent inducer of cell death in activated T cells with aberrant IKK activity, it is also thought to have a pro-survival role. RIPK1 deficient T cells proliferate less after anti-CD3/CD28 stimulation, in part, due to increased sensitivity to apoptosis (Dowlings et al., 2016). In this study, increased apoptosis was apparent at very early time points after stimulation (16-24 hrs) but was absent in T cells with kinase dead RIPK1. This indicates that the scaffold function of RIPK1, which promotes NF-\(\kappa\)B activation and subsequent transcription of pro-survival proteins, is crucial during early T cell activation. Therefore, our data suggests that activated T cells balance this requirement for RIPK1 with the potential death inducing effects of RIPK1 kinase activity later on, by utilising the IKK complex to hold it in check.

Our data suggests that TNF is an important trigger of RIPK1 dependent cell death in activated IKK2 deficient T cells. Ablation of TNF production rescued death of activated T cells lacking IKK2 \textit{in vitro}. TNF appeared to be inducing
death of activated IKK2 deficient T cells through TNFR1 since deletion of
TNFR1 rescued in vivo IKK2 deficient T cell responses. This suggests that in
activated T cells, similarly to thymocytes, the IKK complex is regulating
RIPK1 activity downstream of TNFR1 (Webb et al., 2016, 2019). Blocking
TNF signalling also rescued IKK2 deficient T cells from apoptosis at early
timepoints of culture, around 16-24 hrs, which was not rescued by inhibiting
RIPK1 kinase. This suggests TNF was not limiting in culture to initiate cell
death. Thus, the sensitivity of dividing cells to RIPK1 dependent cell death at
~24 hrs onward was not simply mediated by the kinetics of TNF production in
the culture. Additionally, these data imply that at early timepoints, IKK2 is
protecting T cells from TNF induced, RIPK1 independent death mediated by
complex IIa. The cell death triggered by this pathway is known to be
antagonised by NF-κB induced expression of pro-survival proteins, such as
c-FLIP (Kreuz et al., 2001; Micheau and Tschopp, 2003). Therefore, the
impaired canonical NF-κB activity in activated IKK2 deficient T cells may also
be affecting regulation of this pathway.

Alongside TNF, our data suggests that there are other triggers of RIPK1
dependent cell death in activated T cells. Blocking TNFR1 signalling did not
rescue IKK2 deficient T cell responses in vitro and in vivo to the same extent
as inactivating RIPK1 kinase activity. Moreover, inhibiting RIPK1 activity in
vitro gave an additional rescue to IKK2 deficient T cell responses in absence
of TNF. We suspect that other death receptors of the TNFR superfamily may
be inducing death of activated T cells lacking IKK2. Both Fas and TRAIL
receptors are expressed during T cell activation and are capable of triggering
RIPK1 dependent necroptosis after inactivation of caspase-8, cIAP1/2 or
LUBAC (Geserick et al., 2009; Holler et al., 2000; Lafont et al., 2017). But a
role for the IKK complex in restraining RIPK1 dependent death downstream
of these receptors in T cells has so far not been reported. Others have
suggested that the TCR directly triggers RIPK1 dependent necroptosis in
caspase-8/FADD deficient T cells after stimulation (Ch’en et al., 2008b; Feng
et al., 2019; Osborn et al., 2010). However, there is currently no evidence
that the TCR itself can directly induce the formation of RIPK1 containing cell
death complexes. Either way, it’s clear that there isn’t a single receptor
responsible for triggering RIPK1 dependent cell death in activated IKK2 deficient T cells.

Collectively, our data shows that the IKK complex is important for canonical NF-κB activation, but that T cells with impaired IKK activity do not display activation defects consistent with blocked NF-κB induction. Instead, intact IKK signalling is required for normal T cell responses to antigen almost entirely for protection against RIPK1 dependent cell death.
Chapter 4 - IKK signalling fine tunes the regulation of RIPK1 in activated T cells

4.1 Introduction

The IKK complex, composed of the kinases IKK1 and IKK2, and the regulatory subunit NEMO, is a critical regulator of NF-κB activation. Despite significant homology between the two kinases, IKK2 has long been known to be essential for optimal activation of canonical NF-κB. Early studies showed that IKK2 knockout mice die during embryogenesis due to hepatocyte apoptosis, thus resembling the phenotype of RelA knockout mice (Beg et al., 1995; Li et al., 1999b, 1999c; Tanaka et al., 1999). Indeed, MEFs from IKK2 knockout mice exhibit significantly diminished IκBα phosphorylation and nuclear NF-κB activity in response to various stimuli (Li et al., 1999b, 1999c; Tanaka et al., 1999). Ablation of IKK2 in T cells has no effect on thymocyte development but does cause a modest reduction in peripheral naïve T cells (Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2019). This occurs since impaired NF-κB activation leads to defective IL-7R upregulation by new T cells and aberrant peripheral homeostasis (Silva et al., 2014).

IKK induced NF-κB signalling is known to regulate the expression of pro-survival proteins which protect cells from TNF induced cell death. Complex IIa, consisting of TRADD, FADD and caspase-8, can form downstream of TNFR1 and initiate apoptosis (Micheau and Tschopp, 2003). But simultaneously, TNF induces NF-κB dependent transcription of proteins which antagonise complex IIa mediated apoptosis, such as c-FLIP, which functions to prevent caspase-8 processing (Kreuz et al., 2001; Micheau and Tschopp, 2003; Micheau et al., 2001). This constitutes the late NF-κB dependent cell death checkpoint (Ting and Bertrand, 2016).

Additionally, the IKK complex plays a crucial role in the early NF-κB independent cell death checkpoint. Impaired ubiquitylation or phosphorylation of RIPK1 leads to destabilisation of complex I downstream
of TNFR1 (Dondelinger et al., 2013, 2015; Gerlach et al., 2011; Ikeda et al., 2011; Peltzer et al., 2018; Ting and Bertrand, 2016). RIPK1 kinase activity subsequently drives the formation of complex IIb, consisting of TRADD, FADD, RIPK1 and caspase-8, which initiates apoptosis (Wang et al., 2008). The IKK complex directly phosphorylates and inhibits RIPK1 activity to block complex IIb formation (Dondelinger et al., 2015, 2019). But the IKK complex may also be fine-tuning the regulation of the complex IIb mediated death pathway by inducing the transcription of NF-κB gene targets involved in TNFR1 signalling. One such example of this could be cIAP1 and cIAP2. Both are NF-κB gene targets and are important for K63-linked ubiquitylation of complex I, thus promoting its stability (Bertrand et al., 2008; Stehlik et al., 1998; Varfolomeev et al., 2008; You et al., 1997). In the absence of cIAP1 and cIAP2, TNF triggers RIPK1 dependent cell death due to destabilisation of complex I (Moulin et al., 2012).

The precise quantitative and qualitative nature of IKK kinase activity required to inhibit RIPK1 downstream of TNFR1 is poorly understood, but appears to vary depending on the cell type. For some tissues, IKK2 appears crucial for control of RIPK1 kinase activity. Mice with keratinocyte specific deletion of IKK2 display severe skin inflammation resulting from extensive cell death which can be abrogated by deletion of TNFR1 or by kinase dead RIPK1 (Kumari et al., 2021; Pasparakis et al., 2002). Deletion of IKK2 in synovial fibroblasts is also sufficient to sensitive them to TNF induced RIPK1 dependent cell death (Armaka et al., 2018). On the other hand, IKK2 seems dispensable for inhibition of RIPK1 in other cell types, including in T cells. Only ablation of both IKK1 and IKK2 sensitises MEFs to RIPK1 dependent cell death (Dondelinger et al., 2015). Similarly, thymocyte development is normal in mice lacking IKK1 or IKK2 but combined deletion of IKK1 and IKK2 induces a block in SP thymocyte development due to TNF induced RIPK1 dependent cell death (Chen et al., 2015; Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2016, 2019). Additionally, there is no indication of extensive RIPK1 dependent cell death of naïve T cells in IKK1 or IKK2 deficient mice, since they are largely lymphoreplete (Chen et al., 2015; Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2019).
contrast, my new studies of activated IKK2 deficient T cells suggested that the threshold of IKK activity required to repress RIPK1 changes following activation, since IKK2 deficiency alone is sufficient to sensitise activated T cells to TNF induced RIPK1 dependent cell death.

In this chapter, my objective was to better understand how the requirement for IKK activity to repress RIPK1 kinase changes after T cell activation. Furthermore, we investigated why activated T cells may be sensitive to RIPK1 dependent cell death in absence of IKK2. We hypothesised that IKK2 may be required for the normal expression of canonical NF-κB gene targets, some of which may in turn be required for optimal control of RIPK1 kinase activity by the IKK complex. To address this, we analysed the transcriptome of activated IKK2 deficient T cells.
4.2 Results

4.2.1 Naïve F5 \( \text{Ikk}2\Delta^{\text{CD2}} \) T cells are resistant to TNF induced cell death

Although there is no evidence of extensive T cell death in IKK2 deficient mice, it was possible that F5 T cells have altered development compared with polyclonal mice, so it was important to check that the sensitivity of F5 blasts did not simply reflect broader differences of the F5 strain to polyclonal mice. Therefore, we tested whether naïve F5 \( \text{Ikk}2\Delta^{\text{CD2}} \) T cells were also sensitive to RIPK1 dependent cell death by culturing them with increasing concentrations of TNF. Like F5 CTRL T cells, naïve F5 \( \text{Ikk}2\Delta^{\text{CD2}} \) T cells were not sensitive to TNF induced cell death (Fig. 4.1A). However, we wanted to confirm that the resistance of naïve F5 \( \text{Ikk}2\Delta^{\text{CD2}} \) T cells to TNF induced cell death was due to ongoing restraint of RIPK1 rather than an absence of components of the cell death machinery. To do this, we specifically blocked the activity of IKK2 recruited to TNFR1, assumed to be inhibiting RIPK1 activation, using a pharmacological inhibitor. In the presence of IKK2 inhibitor (IKK2i), naïve F5 CTRL T cells were rendered susceptible to TNF induced cell death (Fig. 4.1B). Cell death was RIPK1 kinase dependent because T cells were completely rescued from death with Nec-1.

Together, this data suggests that before activation, F5 \( \text{Ikk}2\Delta^{\text{CD2}} \) T cells are resistant to RIPK1 dependent cell death.
Figure 4.1 Naïve F5 Ikk2ΔCD2 T cells are resistant to TNF induced cell death

(A) Lymph node cells were isolated from F5 CTRL and F5 Ikk2ΔCD2 mice and cultured with different doses of TNF for 24 hrs. Naïve T cell viability was determined by flow cytometry using a LIVE/DEAD dye.

(B) Lymph node cells were isolated from F5 CTRL mice and cultured with different doses of TNF for 24 hrs with or without PBS, 10 μM IKK2i and Nec-1. Naïve T cell viability was determined by flow cytometry using a LIVE/DEAD dye.

Data is pooled from 1-2 independent experiments. Error bars indicate SEM.
4.2.2 Transcriptomic analysis of activated F5 Ilk2ΔTCD2 T cells

Sensitisation of F5 Ilk2ΔTCD2 T cells to RIPK1 dependent cell death during activation implied there is an alteration in the mechanisms controlling RIPK1 kinase activity following activation. We hypothesised that impaired canonical NF-κB signalling in activated F5 Ilk2ΔTCD2 T cells altered expression of genes required for optimal regulation of RIPK1. To explore this, we analysed the transcriptome of activated F5 Ilk2ΔTCD2 T cells. We took advantage of an unpublished historical RNA sequencing experiment undertaken in the lab. This dataset consisted of four biological replicates each of F5 CTRL T cells and F5 Ilk2ΔTCD2 T cells stimulated with peptide for 24 hrs. This time point was chosen because most T cells had become activated by 24 hrs and at this timepoint, F5 Ilk2ΔTCD2 T cells were beginning to show earliest indications of sensitivity to RIPK1 dependent cell death. Firstly, we performed quality control steps to verify that sequencing data from each sample was sufficient of quality and depth. The library sizes were similar between replicates of each condition and between the two conditions, indicating that a similar number of reads had been mapped to the mouse genome (Fig. 4.2A). The 21% of genes with absent or very little expression across samples were removed since these were unlikely to be having any significant biological impact (Fig. 4.2B). The library sizes were normalised to adjust for any non-biological effects on samples occurring during sequencing. However, the scaling factors were close to 1 indicating that very little normalisation needed to be applied (Fig. 4.2C). To gain an insight into how much variation in gene expression between samples could be explained by Ilk2 expression, we performed unsupervised clustering. Calculating the leading logFC of the 500 genes with the greatest expression changes between samples revealed that experimental replicates clustered over dimension 2 rather than dimension 1 (Fig. 4.2D). Instead, the samples appeared to separate over dimension 1 according to the date on which the T cells were stimulated. Thereby, the majority of gene expression variation between samples was not due to an absence of Ilk2, but was introduced by external factors. To account for this batch effect, the date of stimulation was included as a factor during differential expression analysis.
A

Library size (x 10^6)

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B

Density

Log(cpm)

Raw

Filtered

C

Un-normalised

Log(cpm)

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D

Sample

Date of stimulation

Leading logFC dim 1 vs Leading logFC dim 2

F5 CTRL, F5 Ikk2

Leading logFC dim 1

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Figure 4.2 Transcriptomic analysis of activated F5 \( Ikk2\Delta T^{CD2} \) T cells

Analysis of historical lab RNA sequencing experiment. T cells from F5 CTRL and F5 \( Ikk2\Delta T^{CD2} \) mice were stimulated with NP-68 for 24 hrs.  
(A) Bar plot showing the library size (number of mapped reads) of the 4 F5 CTRL sample replicates (black bars) and 4 F5 \( Ikk2\Delta T^{CD2} \) sample replicates (red bars) 
(B) Line graphs showing the density distribution of log(counts per million; cpm) values for each sample before (Raw) and after filtering (Filtered). The dotted black line indicates the log(cpm) threshold (cpm=0.34) below which genes were removed.  
(C) Boxplots showing the distribution of log(cpm) values for each sample before (top) and after (bottom) TMM normalisation  
(D) Multi-dimensional scaling plots of the leading log fold changes (logFCs) between pairs of samples over the 1\(^{st}\) and 2\(^{nd}\) dimensions which explain the greatest proportions of data variability. The leading logFC is defined as the average logFC of the 500 genes with the greatest differences between sample pairs. The sample type (left plot) or date of stimulation (right plot) is overlayed.
4.2.3 Gene expression in activated F5 T cells was altered in absence of Ikk2

The clustering analysis of samples indicated that Ikk2 deletion had impacted the expression of other genes in activated F5 T cells. Next, we undertook differential expression analysis using EdgeR to identify which specific genes had changed. A significant number of genes had increased or decreased expression in activated F5 Ikk2ΔTCD2 T cells compared to F5 CTRL T cells (Fig. 4.3A). To identify expression changes that may be most biologically relevant, we focused on those genes showing the largest log fold changes. In validation of our model, the expression of Ikbkb (Ikk2) was reduced while expression of Gt(ROSA)26Sor (ROSA26) was increased in activated F5 Ikk2ΔTCD2 T cells (Fig. 4.3B). Classical NF-κB targets such as Nfkbia, Nfkbie, Il2 and Tnfaip3 were decreased in expression. Other genes involved in T cell differentiation and function, including Irf4, Cxcr5, Cd44, Icam1 and Gadd45b also had lower expression in activated F5 Ikk2ΔTCD2 T cells. In contrast, some genes associated with T cell activation had increased expression, such as Cd5 and Ctl4a.
Figure 4.3 Gene expression in activated F5 T cells was altered in absence of *Ikk2*

(A) MD plot showing the logFC vs the log(cpm) for each gene in the dataset after differential expression analysis. Comparing F5 *Ikk2ΔTCD2* to F5 CTRL groups, genes with non-significant changes (black dots) and genes significantly increased (red dots; n=291) or decreased (blue dots; n=228) in expression are highlighted.

(B) Heatmap showing relative expression of the top 50 differentially expressed genes between the F5 CTRL and F5 *Ikk2ΔTCD2* groups with fold changes significantly greater than 1.2. Log(cpm) values for each sample are adjusted by the mean and SD across samples for each gene to calculate the z-score. Increasing shades of red represents increasing relative expression and increasing shades of blue represents decreasing relative expression. Genes are ordered based on the correlation between expression changes.
4.2.4 Some canonical NF-κB targets showed normal expression in activated F5 *Ikk2ΔT<sup>CD2</sup>* T cells

IKK2 is important for canonical NF-κB activation but earlier work has shown there to be a significant level of residual NF-κB activity in activated IKK2 deficient T cells (Schmidt-Supprian et al., 2003). Since some well-established NF-κB target genes had lower expression in absence of IKK2, we wanted to understand the extent to which canonical NF-κB signalling was impaired in activated F5 *Ikk2ΔT<sup>CD2</sup>* T cells. To investigate this, we tested whether a set of 200 defined NF-κB target genes had collectively changed in expression by using the *roast* function contained within the limma package. Expression of this NF-κB gene signature was significantly lower in activated F5 *Ikk2ΔT<sup>CD2</sup>* T cells versus F5 CTRL T cells with 75 genes having decreased expression, confirming that absence of IKK2 resulted in an NF-κB activation defect (Fig. 4.4A).

Previous studies have also identified a number of NF-κB target genes in CD8<sup>+</sup> SP thymocytes, many of which are thought to be important for peripheral T cell survival and function (Webb et al., 2016, 2019). We asked whether expression of these genes in activated F5 T cells required IKK2. Although some genes were reduced in expression in activated F5 *Ikk2ΔT<sup>CD2</sup>* T cells, the expression of other genes such as *Nfkb1*, *Bcl3*, *Traf1*, *Birc2* and *Birc3* was unchanged (Fig. 4.4B). The non-canonical NF-κB subunits *Relb* and *Nfkb2* were expressed normally and, since IKK2 is not required for activation of this pathway, suggests that non-canonical signalling was intact. The expression of IL-7R is reduced in naïve F5 *Ikk2ΔT<sup>CD2</sup>* T cells and expression remained lower after activation (Silva et al., 2014). Together, this data provides evidence of a canonical NF-κB activation defect in activated F5 *Ikk2ΔT<sup>CD2</sup>* T cells, although there was heterogeneity in the response since some NF-κB target genes did not require IKK2 for normal expression.
A

NF-κB target genes

p=0.00885

B

Nfkbia (IκBα) ★★★

Traf1

Birc2 (cIAP1)

Birc3 (cIAP2)

Bcl3

Nfkb1

Nfkbia (IκBα)

Nfkbie (IκBε)

Bcl3

Birc3 (cIAP2)

Bcl3

nRPMK

F5 CTRL

F5 ikk2T107CD2

Enrichment NF-κB target genes p=0.00885

Down

Up

Study

F5 CTRL

F5 ikk2T107CD2

-6e+00

-2e-01

-1e-01

-8e-02

-4e-02

9e-02

4e-02

2e-01

3e-01

3e+00

0

2.9

-
Figure 4.4 Some canonical NF-κB targets showed normal expression in activated F5 \( Ikk2ΔT^{CD2} \) T cells

(A) Barcode plot showing enrichment of the MsigDB gene set “HALLMARK_TNFA_SIGNALING_VIA_NFKB” in F5 \( Ikk2ΔT^{CD2} \) vs F5 CTRL groups. All genes are ordered from left to right on the x-axis by increasing logFC; genes most downregulated in F5 \( Ikk2ΔT^{CD2} \) are positioned on the left and genes most upregulated in F5 \( Ikk2ΔT^{CD2} \) are on the right. The black vertical bars indicate the positions of the genes within the gene set. The worm shows the relative enrichment of the vertical bars. The p-value of the roast test for the gene set is indicated.

(B) Bar plots showing gene expression (normalised fragments per kilobase exons per million fragments; nFPKM) of indicated genes between F5 \( Ikk2ΔT^{CD2} \) and F5 CTRL T cells as determined by RNA-seq.
4.2.5 The expression of Tnfaip3 is significantly reduced in activated F5 Ikk2ΔT<sup>CD2</sup> T cells

A number of adaptors and kinases facilitate signal transduction downstream of TNFRSF members to activate NF-κB. But simultaneously, many of these intracellular signalling components also have crucial roles in protecting cells from death by inhibiting RIPK1 kinase activity by various mechanisms. As F5 Ikk2ΔT<sup>CD2</sup> T cells were sensitised to RIPK1 dependent cell death during activation, we questioned whether the impaired canonical NF-κB activity had affected the regulation of TNFRSF signalling pathways. Therefore, we analysed the expression of TNFRSF signalling intermediates and known regulators of RIPK1. Expression of the E3 ligases, cIAP1, cIAP2 and constituents of the LUBAC complex was normal (Fig. 4.5). Kinases such as Chuk (IKK1) and Map3k7 (TAK1) and their respective scaffolds Ikbkg (NEMO) and Tab2/Tab3 were also unchanged in expression. Gene expression of other proteins not required for NF-κB induction, but which have been implicated in RIPK1 control, including Ikbke, Tbk1, Mapkapk2 was also unaffected. Although the vast majority of TNFRSF signalling components showed normal expression, some proteins were significantly altered in their expression. There was a small increase in the expression of Ripk3 and minor decreases in the expression of Cflar and Mib2. However, there was a profound ~3.5 fold decrease in the expression of Tnfaip3 in activated F5 Ikk2ΔT<sup>CD2</sup> T cells. Tnfaip3 was also one of the most significant differentially expressed genes identified.
Figure 4.5 The expression of Tnfaip3 is significantly reduced in activated F5 Ikk2ΔTCD2 T cells

Volcano plot showing statistical significance (\(-\log_{10}(\text{adjusted P value})\)) vs log(fold change) of genes between F5 Ikk2ΔTCD2 and F5 CTRL groups. Horizontal dashed line indicates adjusted p value equal to 0.05. Vertical dashed lines indicate logFC equal to 1 or -1. Red points represent genes with adjusted p value greater than 0.05 and logFC less than -1 or greater than 1. Genes encoding proteins involved in regulation of TNFRSF signalling and RIPK1 are labelled.
4.3 Discussion

Intact IKK signalling is required to protect activated T cells from RIPK1 dependent cell death. Here, we demonstrate that naïve and activated T cells have distinct qualitative requirements of IKK signalling for survival. We show that F5 naïve IKK2 deficient T cells are completely resistant to TNF induced cell death. This is consistent with earlier observations that IKK2 is redundant for thymocyte survival (Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2019). But acute pharmacological inhibition of IKK2 does sensitise naïve T cells to TNF induced cell death. Deletion of both IKK1 and IKK2 in thymocytes leads to a developmental block at the mature SP stage due to TNF induced RIPK1 dependent cell death (Webb et al., 2016, 2019). This shows that in SP thymocytes and naïve T cells, IKK1 homodimeric complexes are sufficient to restrain RIPK1 kinase activity in absence of IKK2, despite possessing relatively weaker kinase activity than IKK1/2 heterodimeric complexes (Huynh et al., 2000; Schmidt-Supprian et al., 2003). However, inhibiting IKK2 in normal IKK complexes appears inadequate for control of RIPK1 after TNF stimulation. Blocking IKK2 in IKK1/2 heterodimers is likely to result in more profound inhibition of IKK activity than genetic ablation of IKK2, which permits IKK1 homodimerisation to compensate to some extent. Therefore, it is likely a threshold of IKK activity is required to successfully repress RIPK1.

Control of RIPK1 was normal in naïve T cells lacking IKK2. Thus, IKK2 deficient T cells become sensitised to RIPK1 dependent cell death during activation. This indicates that the regulation of RIPK1 is in some way altered following activation such that the activity of IKK1 homodimeric complexes is no longer sufficient for control of RIPK1 kinase activity. Therefore, the regulation of RIPK1 during T cell activation appears to be subject to active tuning by some mechanism. Our hypothesis was that IKK2 induced NF-κB dependent changes in gene expression feedback to control the threshold of IKK signalling required to repress RIPK1 activity.
TNF was one trigger of RIPK1 dependent cell death in activated IKK2 deficient T cells and the stability of complex I downstream of TNFR1 is a critical checkpoint to prevent RIPK1 integration into complex IIb and subsequent induction of apoptosis. However, at the transcriptomic level, we saw no changes in the expression of complex I components including TAK1, LUBAC and cIAP1/2 in activated IKK2 deficient T cells that could account for the changes in IKK dependent regulation of RIPK1. Thus, perhaps impaired IKK activity was affecting the post-translational regulation of complex I stability.

Broader analysis of gene expression changes in IKK2 deficient blasts revealed a set of established NF-κB targets was reduced, including substantial decreases in the expression of the negative regulators of NF-κB, Nfkbia, Nfkbie and Tnfaip3. This is in line with previous data demonstrating an NF-κB activation defect in CD3/CD28 stimulated T cells lacking IKK2 (Schmidt-Supprian et al., 2003). Although this suggests canonical NF-κB is impaired in activated IKK2 deficient T cells, our data suggests this block was only partial. Several known NF-κB target genes identified in SP thymocytes do not appear to require IKK2 to maintain normal expression in activated T cells. Expression of Nfkb2, RelB, Bcl3 and Traf1 are all diminished in IKK1/2 deficient SP thymocytes (Webb et al., 2016) but were expressed normally in activated IKK2 deficient T cells. This suggests quantitative differences in how NF-κB induces different gene targets. This could reflect differences in the number of NF-κB binding sites in promoters of different genes. The promoters of both Nfkbia and Tnfaip3 have clusters of several NF-κB binding sites and consequently, transcriptional expression is thought to be more sensitive to changes in nuclear NF-κB concentration compared to genes with fewer NF-κB binding sites (Giorgetti et al., 2010). Additionally, IKK1 and IKK2 can regulate gene expression in different ways and expression of some genes may be more reliant on such mechanisms than others. For instance, IKK1 (but not IKK2) is known to possess a nuclear localisation sequence and can influence gene expression by modifying histone proteins (Anest et al., 2003; Yamamoto et al., 2003).
IKK2 appears to be required for normal expression of c-FLIP in activated T cells, consistent with the fact that Cflar is a known NF-κB target (Micheau et al., 2001). However, Cflar does not appear to be regulated by NF-κB in SP thymocytes, suggesting differing regulation between T cell differentiation states (Webb et al., 2016, 2019). This modest reduction in c-FLIP expression does appear to correspond with the point at which activated F5 IKK2 deficient T cells show some sensitivity to TNF induced RIPK1 independent cell death. Since c-FLIP regulates complex Ila mediated apoptosis, this could suggest that IKK2 is regulating this pathway through c-FLIP expression in activated T cells (Kreuz et al., 2001; Micheau and Tschopp, 2003). In line with this, c-FLIP\textsubscript{L} deficient T cells are more sensitive to cell death within 24 hrs after TCR stimulation, before cell division had taken place (He and He, 2013).

Many of the genes differentially expressed in activated IKK2 deficient T cells are not direct NF-κB targets. Canonical NF-κB signalling is important for the regulation of other transcription factors and therefore IKK2 ablation may impact the expression of genes indirectly. The transcription factor Irf4 shows reduced expression in activated IKK2 deficient T cells (Grumont and Gerondakis, 2000). IRF4 is crucial for sustaining effector CD8\textsuperscript{+} T cell proliferation and function (Grusdat et al., 2014; Man et al., 2013; Raczkowski et al., 2013; Yao et al., 2013). Thus, the wider impacts of IKK2 deficiency on the transcriptome of activated T cells may explain why blocking RIPK1 activity does not completely rescue F5 IKK2 deficient T cell responses \textit{in vivo}, but does provide a better rescue of \textit{in vitro} cultures.

IKK2 was responsible for normal regulation of many genes in activated T cells. Tnfaip3, which encodes for A20, appeared to be a prime candidate for control of RIPK1 in T cells. Firstly, expression of Tnfaip3 was profoundly reduced in activated IKK2 deficient T cells. It was one of the most significant differentially expressed genes identified and is known to be an NF-κB gene target (Krikos et al., 1992). Indeed, expression of Tnfaip3 was reduced to the same extent as in IKK1/2 deficient SP thymocytes (Webb et al., 2016). Therefore, IKK2 appeared to be particularly important for normal expression
of \textit{Tnfaip3}. Secondly, the expression of A20 is dynamically regulated during T cell activation. A20 is constitutively expressed by T cells, but is rapidly degraded by MALT1 and the proteasome after TCR stimulation, to ensure maximal NF-κB induction (Coornaert et al., 2008; Düwel et al., 2009; Tewari et al., 1995). Restoration of A20 expression requires NF-κB mediated \textit{Tnfaip3} transcription (Coornaert et al., 2008). As such, at the protein level, A20 is likely to be extremely low in activated IKK2 deficient T cells.

In conclusion, many NF-κB target genes were expressed normally in activated IKK2 deficient T cells, but the expression of a subset was perturbed, perhaps reflecting quantitative differences in NF-κB required for their regulation. We speculate that the threshold of IKK activity required to repress RIPK1 changes in activated T cells by a transcriptional mechanism. This could alter the substrate processing by IKK, either by saturating IKK activity with increased downstream substrate levels e.g. RIPK1 or Casp8 or by reducing the efficiency with which IKK is able to interact with RIPK1 and thereby repress it’s activity. A failure to efficiently form or maintain complex I could certainly alter such efficiencies, resulting in an increase in the threshold of IKK activity necessary to repress RIPK1.
Chapter 5 - T cells require A20 for optimal control of RIPK1 by the IKK complex

5.1 Introduction

A20 is a key regulator of inflammation, functioning to both dampen down pro-inflammatory NF-κB signalling and to promote cellular survival. It has multiple domains with differing ubiquitin modifying and binding activities. In particular, the OTU domain possesses deubiquitylase activity, the ZnF4 domain can act as an E3 ubiquitin ligase and bind branched ubiquitin chains, and the ZnF7 domain can bind to linear ubiquitin chains. Such is the importance of A20 for tissue homeostasis that A20 knockout mice die soon after birth due to excessive organ inflammation (Lee et al., 2000).

The role of A20 has been best characterised downstream of TNFR1. It's rapidly upregulated after activation of canonical NF-κB and mediates its activities at the level of complex I. The early studies investigating how A20 negatively regulates NF-κB signalling proposed that it utilised its OTU domain to remove K63-linked ubiquitin chains from RIPK1 and TNFR1 and subsequently catalysed the addition of K48-linked ubiquitin chains to RIPK1 with the ZnF4 domain, to target it for proteasomal degradation (Wertz et al., 2004, 2015). However, mutating the OTU domain to ablate the deubiquitylase activity of A20 has no effect on NF-κB activity (De et al., 2014). Instead, it's thought that the ZnF7 domain of A20 is responsible for regulating NF-κB signalling by permitting A20 to bind to linear M1 ubiquitin chains which decorate complex I (Tokunaga et al., 2012; Verhelst et al., 2012). Although it is not clear how A20 binding to linear ubiquitin shuts down NF-κB, it has been hypothesised that A20 competes with the IKK complex for ubiquitin binding at complex I or that A20 impedes phosphorylation and subsequent activation of the IKK complex by TAK1 (Draher et al., 2015; Skaug et al., 2011; Verhelst et al., 2012).
In addition to its role restricting NF-κB signalling, A20 can protect cells from TNF induced cell death. Indeed, ablation of A20 in MEFs, hepatocytes, IECs, NK cells and macrophages sensitises them to TNF induced cell death (Catrysse et al., 2016; Polykratis et al., 2019; Priem et al., 2019; Vereecke et al., 2010; Vetters et al., 2019). The ZnF7 domain appears to play a prominent role mediating A20’s protective function. It has been shown that A20 binds to M1-ubiquitin chains, which surround complex I, through the ZnF7 domain (Draber et al., 2015). In this way, A20 stabilises complex I by preventing CYLD mediated degradation of M1-linked ubiquitin chains and subsequently inhibits complex IIb mediated apoptosis (Draber et al., 2015; Priem et al., 2019; Yamaguchi and Yamaguchi, 2015). In addition, the ZnF7 domain appears to be important for restricting necroptosis in macrophages (Polykratis et al., 2019). Other work has shown that deubiquitylation of RIPK3 by the A20 OTU domain, prevents formation of the necrosome and necroptotic cell death (Onizawa et al., 2015).

In T cells, unlike other cell types, A20 is constitutively expressed at a high level (Tewari et al., 1995). The role of A20 in T cells has been explored through the generation of conditional knockout mouse strains. Previous work has suggested that A20 regulates the development of NKT cells and regulatory T cells. A20 deficient mice show impaired NKT cell development but exhibit greater numbers of regulatory T cells (Drennan et al., 2016; Fischer et al., 2017a). Indeed, A20 appears to restrict generation of regulatory T cells in the thymus (Fischer et al., 2017a). During T cell activation, the expression of A20 is dynamically regulated. A20 modulates TCR induced NF-κB activation through its deubiquitylase activity (Düwel et al., 2009). TCR stimulation causes rapid degradation of A20 as a result of cleavage by Malt1 and proteasomal degradation (Coornaert et al., 2008; Düwel et al., 2009). This ensures maximal NF-κB induction at the point of stimulation. The expression of A20 is then restored in a manner dependent on NF-κB transcription (Coornaert et al., 2008). A20 has been suggested to moderate T cell responses and, in absence of A20, T cells exhibit increased proliferation and cytokine production in vitro (Giordano et al., 2014; Just et al., 2016; Onizawa et al., 2015). However, ablation of A20 in T cells leads to
impaired T cell responses in vivo in models of EAE and GVHD (Fischer et al., 2017b; Onizawa et al., 2015). Establishment of antigen specific T cell memory after Listeria infection is also impaired (Just et al., 2016). This appears to be due to increased sensitivity to cell death. Indeed, A20 has been proposed to play an important role protecting activated T cells from both apoptosis and RIPK3 dependent necroptosis (Just et al., 2016; Onizawa et al., 2015). Additionally, A20 has been suggested to promote survival of activated T cells by regulating autophagy (Matsuzawa et al., 2015). Nonetheless, whether A20 is important for regulation of RIPK1 kinase activity in T cells remains unclear.

Sensitisation of activated IKK2 deficient T cells to RIPK1 dependent cell death is accompanied by reduced expression of Tnfaip3, the gene encoding A20. We hypothesised that induction/maintenance of A20 during T cell activation is important for T cell survival by facilitating optimal control of RIPK1 by the IKK complex. We tested this hypothesis by analysing the phenotype and functional responses of T cells lacking A20 expression.
5.2 Results

5.2.1 T cells are resistant to TNF induced cell death in absence of A20

First, we asked if A20 was essential for protecting T cells against RIPK1 dependent cell death. We examined the sensitivity of thymocytes and peripheral T cells from mice with T cell specific deletion of A20 (Tnfaip3ΔTCD4) to TNF induced cell death. SP thymocytes are sensitised to RIPK1 dependent cell death in absence of IKK1/2 (Webb et al., 2019). Death amongst A20 deficient CD4+ and CD8+ SP thymocyte subpopulations was higher after culture for 24 hrs in absence of exogenous TNF, but the fraction of dead cells did not change with increasing TNF concentration (Fig. 5.1A). Similarly, death of naïve CD4+ and CD8+ T cells from Tnfaip3ΔTCD4 mice was independent of TNF, even at supra-physiological concentrations (Fig. 5.1B).

Together, this data showed that A20 alone was not required to protect thymocytes and naïve T cells from RIPK1 dependent cell death.
Figure 5.1 T cells are resistant to TNF induced cell death in absence of A20

(A) Thymocytes were isolated from *Tnfaip3ΔTCD4* and Cre -ve (WT) mice and cultured with different doses of TNF for 24 hrs. Viability (% dead cells) of SP thymocyte subsets was determined by flow cytometry using a LIVE/DEAD dye.

(B) Lymph node cells were isolated from *Tnfaip3ΔTCD4* and Cre -ve (WT) mice and cultured with different doses of TNF for 24 hrs. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.

Graphs show the means of 2 independent experiments. Error bars indicate SEM.
5.2.2 A20 regulates RIPK1 kinase activity in T cells

Although A20 deficiency did not sensitive T cells to TNF induced cell death, a potential role for A20 in the control of RIPK1 activity could be obscured by other protective mechanisms functional at different stages of the TNFR1 signalling pathway. Phosphorylation of RIPK1 by the IKK complex is a key check point preventing the formation of cell death complex IIb (Dondelinger et al., 2015). Previous data has shown increased sensitivity of A20 deficient MEFs to TNF induced cell death after IKK blockade (Priem et al., 2019). Therefore, we asked whether disruption of IKK activity could uncover a role for A20 in T cells for protection against RIPK1 dependent cell death. To do this, we stimulated A20 deficient T cells with TNF for 24 hrs in the presence of increasing concentrations of two different IKK inhibitors. The IKK2 inhibitor BI605906 (IKK2i) was used to specifically block IKK2 activity without affecting the function of IKK1. In absence of TNF, increasing concentrations of IKK2i had no effect on cell death of naïve CD4+ and CD8+ T cells from Cre-ve (WT) mice (Fig. 5.2A). Addition of TNF to the culture caused an increase in the percentage of dead cells, but only at the highest concentrations of IKK2i. TNF induced cell death was completely rescued by blocking RIPK1 kinase activity with Nec-1, suggesting it was RIPK1 dependent. In contrast, T cells from Tnfaip3ΔTCD4 mice appeared to be sensitive to TNF induced cell death at lower concentrations of IKK2i compared to T cells from WT mice. At the highest concentrations of IKK2i, almost all T cells had died from TNF induced cell death. In addition, there was also evidence that CD8+ T cells, and CD4+ T cells to a lesser extent, were dying with increasing IKK2i concentrations even in absence exogenous TNF. Nonetheless, Nec-1 completely rescued A20 deficient T cells from TNF induced cell death, indicating the additional death was RIPK1 dependent, but also reduced death in absence of TNF.

We further investigated the protective role of A20 against TNF induced cell death using IKK16, an inhibitor which targets both IKK1 and IKK2, thereby causing a complete block in IKK activity. At high concentrations, IKK16 appears to trigger non-IKK specific cell death of almost all T cells from WT
mice in absence of TNF, not observed in other IKK deficient settings, suggesting off target effects of the inhibitor. Nevertheless, consistent with a complete IKK blockade, high concentrations of IKK16 were also potent at sensitising T cells to TNF induced cell death (Fig. 5.2B). Addition of Nec-1 to cultures rescued survival of T cells in the presence of TNF back to the background levels observed with IKK16 alone, also suggesting that TNF induced death was entirely RIPK1 dependent. In contrast, T cells from Tnfaip3ΔTCD4 mice were exquisitely sensitive to TNF induced death, even at the lowest concentration of IKK16 used. As seen using IKK2i, A20 deficient T cells more readily died in the presence of IKK16, even without adding TNF to the culture. Inhibiting RIPK1 kinase activity with Nec-1 gave an almost complete rescue of T cells from TNF induced death.

A20 deficient T cells appeared more sensitive to TNF induced death upon IKK inhibition. To better quantify the level of IKK activity required to protect T cells from TNF induced death, we calculated the 50% lethal dose (LD50) concentrations of IKK16 in the presence and absence of TNF. Compared to WT naïve CD4+ T cells cultured without TNF, in the presence of TNF, the LD50 of IKK16 was approximately halved (Fig. 5.2C). Addition of Nec-1 raised the IKK16 LD50 to the level seen in the absence of TNF, confirming the RIPK1 dependence of cell death. In contrast, even without TNF present, the IKK16 LD50 for naïve CD4+ T cells from Tnfaip3ΔTCD4 mice was comparable to that of TNF stimulated T cells from WT mice. Furthermore, TNF addition to A20 deficient T cells substantially reduced the LD50 of IKK16. Collectively, naïve CD4+ T cells from Tnfaip3ΔTCD4 mice showed a ~4 fold reduction in the IKK16 LD50 concentration upon TNF stimulation, compared to a ~2 fold reduction for WT cells.

For naïve CD8+ T cells, the IKK16 LD50 concentrations for TNF unstimulated and stimulated cells from WT mice were similar to those for naïve CD4+ T cells (Fig. 5.2C, D). But there was a significant decrease in the IKK16 LD50 concentration of TNF stimulated T cells from Tnfaip3ΔTCD4 mice compared to unstimulated counterparts. However, A20 deficient T cells treated with both TNF and Nec-1 required more IKK16 to induce a comparable amount of cell death as the unstimulated control. So although there was a ~3 fold decrease
in the IKK16 LD50 concentration of TNF stimulated A20 deficient T cells versus unstimulated cells, this decrease was larger compared to TNF stimulated cells lacking RIPK1 activity. Therefore, this suggests that in both naïve CD4\(^+\) and CD8\(^+\) T cells, A20 deficiency necessitates greater IKK activity in order to successfully restrain RIPK1 kinase activity and protect T cells from TNF induced cell death. Furthermore, these data suggest that a critical function of A20 in T cells is to facilitate control of RIPK1 kinase activity.
Figure 5.2 A20 regulates RIPK1 kinase activity in T cells

(A) Lymph node cells were isolated from \textit{Tnfaip3}Δ\textsubscript{CD4} and Cre -ve (WT) mice and cultured with different concentrations of IKK2i for 24 hrs with or without PBS, 20 ng/ml TNF and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.

(B) Lymph node cells were isolated from \textit{Tnfaip3}Δ\textsubscript{CD4} and Cre -ve (WT) mice and cultured with different concentrations of IKK16 for 24 hrs with or without PBS, 20 ng/ml TNF and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.

(C) IKK16 LD50 values for naïve CD4\textsuperscript{+} T cells from experiment shown in (B) (left). Ratio of IKK16 LD50 for PBS treated cultures to IKK16 LD50 for TNF treated cultures with and without Nec-1 (right).

(D) IKK16 LD50 values for naïve CD8\textsuperscript{+} T cells from experiment shown in (B) (left). Ratio of IKK16 LD50 for PBS treated cultures to IKK16 LD50 for TNF treated cultures with and without Nec-1 (right).

Data is pooled from 2 (A, B) and 3 (C, D) independent experiments. Error bars indicate SEM (A, B) and 95% confidence intervals (C, D).
5.2.3 Impaired CD8$^+$ SP thymocyte development in mice with combined A20 and IKK2 deficiency

Acute pharmacological inhibition of the IKK complex revealed that A20 was important for protection of T cells from RIPK1 dependent cell death. To further validate a role for A20 in vivo for control of RIPK1 activity in T cells, we first analysed T cell development in $Tnfaip3\Delta T^{CD4}$ mice. In this strain, Cre expression is driven by the CD4 promoter and therefore A20 is deleted from the DP stage of thymocyte development. Normal SP thymocyte development requires active repression of RIPK1 activity by the IKK complex (Webb et al., 2019). However, $Tnfaip3\Delta T^{CD4}$ mice had a similar percentage of SP thymocytes subpopulations to WT mice (Fig. 5.3A). The numbers of DP and immature HSA$^{hi}$CD62L$^{lo}$ and mature HSA$^{lo}$CD62L$^{hi}$ SP thymocyte populations were normal (Fig. 5.3B). This is consistent with previous studies and suggests that thymocyte development is normal in these mice (Fischer et al., 2017a; Onizawa et al., 2015).

Since activity of the IKK complex was likely sufficient to ensure normal thymocyte development in $Tnfaip3\Delta T^{CD4}$ mice, we next asked if A20 was important in thymocytes deficient in IKK2 expression in which IKK function is suboptimal. First, we confirmed that IKK2 deficiency had no effect on thymocyte development, as indicated by previous reports (Schmidt-Supprian et al., 2003; Silva et al., 2014; Webb et al., 2019). Therefore, we analysed the thymocyte subpopulations of mice with T cell specific deletion of IKK2 ($Ikk2\Delta T^{CD4}$). The percentage and numbers of DP thymocytes and both immature HSA$^{hi}$CD62L$^{lo}$ and mature HSA$^{lo}$CD62L$^{hi}$ CD4$^+$ SP populations was normal (Fig. 5.3A, B). Although the number of immature HSA$^{hi}$CD62L$^{lo}$ CD8$^+$ SP thymocytes was similar to WT mice, there was a decrease in the number of mature HSA$^{lo}$CD62L$^{hi}$ CD8$^+$ SP
thymocytes (Fig. 5.3B). This was consistent with a reduction in the fraction of CD8+ SP thymocytes with low HSA expression (Fig. 5.3C). Together, this data suggests that although A20 and IKK2 are not required for CD4 lineage development in the thymus, combined deletion of both caused impaired development of CD8+ SP thymocytes at the mature stage.
A

CD4+ SP thymocytes

CD8+ SP thymocytes

B

DP thymocytes

HSA\textsuperscript{lo} CD4+ SP thymocytes

HSA\textsuperscript{hi} CD4+ SP thymocytes

HSA\textsuperscript{lo} CD8+ SP thymocytes

HSA\textsuperscript{hi} CD8+ SP thymocytes

C

CD4+ SP thymocytes

CD8+ SP thymocytes

% HSA\textsuperscript{lo}
Figure 5.3 Impaired CD8⁺ SP thymocyte development in mice with combined A20 and IKK2 deficiency

Flow cytometric analysis of thymocytes from Cre -ve (WT, n=15), Tnfaip3ΔTCD4 (n=6), Ikk2ΔTCD4 (n=4) and Tnfaip3.Ikk2ΔTCD4 (n=11) mice. (A) Density plots are of CD4 vs CD8 by live thymocytes (top row) and HSA vs CD62L by gated CD4⁺ SP thymocytes (middle row) and CD8⁺ SP thymocytes (bottom row). Numbers indicate percentage of cells in each gate. (B) The total number of the specified thymocyte subsets from the indicated strains. (C) The percentage of CD4⁺ and CD8⁺ SP thymocytes with low HSA expression. Data is pooled from 9 independent experiments. Error bars indicate SD.
5.2.4 Mice deficient in A20 and IKK2 have profound T cell lymphopenia

Next, we wanted to understand if combined deficiency of A20 and IKK2 affected peripheral T cell homeostasis. *Tnfaip3ΔT*<sup>CD4</sup> mice had a normal number of peripheral naïve (CD44<sup>lo</sup>CD25<sup>-</sup>) CD4<sup>+</sup> T cells, however there was a small reduction in the number of naïve CD8<sup>+</sup> T cells (Fig. 5.4A, B). *Ikk2ΔT*<sup>CD4</sup> mice also had a normal peripheral naïve CD4<sup>+</sup> T cell compartment, but fewer naïve CD8<sup>+</sup> T cells (Fig. 5.4A, B). The phenotype in *Ikk2ΔT*<sup>CD4</sup> mice was consistent with previous reports and is thought to be due to defective IL-7R upregulation (Silva et al., 2014; Webb et al., 2019). These mice had a greater decrease in peripheral naïve CD8<sup>+</sup> T cells numbers than seen in *Tnfaip3ΔT*<sup>CD4</sup> mice. However, combined ablation of both A20 and IKK2 expression in mice caused profound peripheral T cell lymphopenia with a substantial reduction in both CD4<sup>+</sup> and CD8<sup>+</sup> naïve T cells (Fig. 5.4A, B). The loss of naïve T cells appeared greatest amongst CD8<sup>+</sup> T cells compared to CD4<sup>+</sup> T cells. Therefore, these data show that compound deletion of both *Tnfaip3* and *Ikk2* results in significantly compromised peripheral naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cell homeostasis, suggesting a strong genetic interaction between *Tnfaip3* and *Ikk2*.
Mice deficient in A20 and IKK2 have profound T cell lymphopenia

Figure 5.4 Mice deficient in A20 and IKK2 have profound T cell lymphopenia

Flow cytometric analysis of lymph nodes and spleens from Cre -ve (WT, n=15), Tnfaip3ΔTCD4 (n=6), Ikk2ΔTCD4 (n=4) and Tnfaip3.Ikk2ΔTCD4 (n=11) mice.

(A) Density plots are of CD4 vs CD8 by live lymph node cells (top row) and CD25 vs CD44 by TCR+CD4+ cells (middle row) and TCR+CD8+ cells (bottom row). Numbers indicate percentage of cells in each gate.
(B) The total number of naïve (CD44loCD25-) CD4+ and CD8+ T cells from the lymph nodes and spleens of the indicated strains.

Data is pooled from 9 independent experiments. Error bars indicate SD.
5.2.5 Defective regulatory T cell development and homeostasis in mice deficient for A20 and IKK2

Canonical NF-κB signalling is known to be crucial for the thymic development and peripheral homeostasis of FoxP3⁺CD25⁺ regulatory T cells (Tregs) (Isomura et al., 2009; Long et al., 2009b; Oh et al., 2017b; Ruan et al., 2009b). Given the opposing functions of A20 and IKK2 upon NF-κB activation, we wanted to understand how combined A20 and IKK2 deletion affected Treg development and homeostasis. *Tnfaip3Δ* CD4 mice had an increased percentage and number of FoxP3⁺CD25⁺ Tregs in the thymus and periphery (Fig. 5.5A-D), as previously reported, which is thought to result from increased NF-κB induced differentiation of Tregs from precursors (Fischer et al., 2017a). In contrast, there was a decrease in the percentage and number of FoxP3⁺CD25⁺ Tregs in *Ikk2Δ* CD4 mice (Fig. 5.5A-D), in line with previous studies and consistent with defective canonical NF-κB signalling (Schmidt-Supprian et al., 2003). Mice deficient in A20 and IKK2 also had a reduction in the number of FoxP3⁺CD25⁺ Tregs in the thymus and periphery (Fig. 5.5B, D). Compared to *Ikk2Δ* CD4 mice, the number of thymic FoxP3⁺CD25⁺ Tregs in *Tnfaip3.Ikk2Δ* CD4 mice was similar, suggesting that the additional loss of A20 had no effect on Treg development. However, in the periphery, there was a small increase in the number of FoxP3⁺CD25⁺ Tregs in *Tnfaip3.Ikk2Δ* CD4 mice compared to *Ikk2Δ* CD4 mice (Fig. 5.5D). Together, this data suggests that mice with deficiency in both A20 and IKK2 had impaired FoxP3⁺CD25⁺ Treg development and homeostasis, although this appeared to be mostly driven by the lack of IKK2 activity.
A

CD4+ SP thymocytes

WT  Trnfap3ΔCD4  Ikk2ΔCD4  Trnfap3.Ikk2ΔCD4

2.6  7.2  0.4  1.4

B

Thymic FoxP3+ Treg

Cell no.

WT  Trnfap3ΔCD4  Ikk2ΔCD4  Trnfap3.Ikk2ΔCD4

10^6  10^5  10^4

C

CD4+ T cells

WT  Trnfap3ΔCD4  Ikk2ΔCD4  Trnfap3.Ikk2ΔCD4

14  19  1  25

D

Peripheral FoxP3+ Treg

Cell no.

WT  Trnfap3ΔCD4  Ikk2ΔCD4  Trnfap3.Ikk2ΔCD4

10^7  10^6  10^5

155
Figure 5.5 Defective regulatory T cell development and homeostasis in mice deficient for A20 and IKK2

Flow cytometric analysis of thymocytes, lymph nodes and spleens from Cre-ve (WT, n=15), *Tnfaip3ΔT^CD4^* (n=6), *Ikk2ΔT^CD4^* (n=4) and *Tnfaip3.Ikk2ΔT^CD4^* (n=11) mice.

(A) Density plots of FoxP3 vs CD25 by live CD4^+^ SP thymocytes. Numbers indicate percentage of cells in each gate.

(B) The total number of thymic FoxP3^+^CD25^+^ Tregs from the indicated strains.

(C) Density plots of FoxP3 vs CD25 by live lymph node TCR^+^CD4^+^ cells. Numbers indicate percentage of cells in each gate.

(D) The total number of FoxP3^+^CD25^+^ Tregs from the lymph nodes and spleens of the indicated strains.

Data is pooled from 9 independent experiments. Error bars indicate SD.
5.2.6 Reduced CD4+ memory T cells in mice with A20 and IKK2 deficiency

As homeostasis of peripheral naïve T cells was perturbed in \textit{Tnfaip3.Ikk2ΔT^{CD4}} mice, we next asked if the same was true of effector and memory compartments. To address this, we analysed the memory-phenotype populations of unchallenged naïve mice. Previous work has shown canonical NF-κB signalling to be important for the generation of CD4+ memory cells (Webb et al., 2019; Zheng et al., 2003). \textit{Tnfaip3ΔT^{CD4}} mice had an increased number and percentage of CD4+ CD44^{hi}CD62L^{hi} central memory T cells (T_{cm} cells) but a normal number of CD4+ CD44^{hi}CD62L^{lo} effector memory T cells (T_{em} cells) (Fig. 5.6A, B). \textit{Ikk2ΔT^{CD4}} mice had a reduced number of both CD4+ memory populations. Mice with combined deletion of both A20 and IKK2 had fewer CD4+ T_{cm} and T_{em} cells. However, compared to \textit{Ikk2ΔT^{CD4}} mice, there was a further decrease in CD4+ T_{cm} cells but an increase in the CD4+ T_{em} population.

Examination of the CD8+ memory compartments also revealed differences between the strains. Both \textit{Tnfaip3ΔT^{CD4}} and \textit{Ikk2ΔT^{CD4}} mice had a reduced number of CD8+ CD44^{hi}CD122^{+}CD62L^{hi} virtual memory T cells (T_{vm} cells) (Fig. 5.6D). Although there was an increased number of CD8+ T_{em} cells in \textit{Tnfaip3ΔT^{CD4}} mice, there was a reduced number and percentage in \textit{Ikk2ΔT^{CD4}} mice (Fig. 5.6C, D). However, \textit{Tnfaip3.Ikk2ΔT^{CD4}} mice had normal numbers of both CD8+ T_{vm} and T_{em} cells.

Together, this data suggests that individual and combined loss of A20 and IKK2 has differing impacts on memory CD4+ and CD8+ T cell populations.
A

CD4⁺ CD25⁺ T cells

WT  Tnfaip3ΔCD4  Ikk2ΔCD4  Tnfaip3.Ikk2ΔCD4

3.8  6.2  1.1  4.0

B

CD4⁺ Tcm cells  CD4⁺ Tem cells

WT  Tnfaip3ΔCD4  Ikk2ΔCD4  Tnfaip3.Ikk2ΔCD4

C

CD8⁺ CD44hi T cells

WT  Tnfaip3ΔCD4  Ikk2ΔCD4  Tnfaip3.Ikk2ΔCD4

43  31  60  75

D

CD8⁺ Tvm cells  CD8⁺ Tem cells

WT  Tnfaip3ΔCD4  Ikk2ΔCD4  Tnfaip3.Ikk2ΔCD4
Figure 5.6 Reduced CD4$^+$ memory T cells in mice with A20 and IKK2 deficiency

Flow cytometric analysis of lymph nodes and spleens from Cre-ve (WT, n=15), *Tnfaip3*ΔT$^{CD4}$ (n=6), *Ikk2*ΔT$^{CD4}$ (n=4) and *Tnfaip3*.*Ikk2*ΔT$^{CD4}$ (n=11) mice.

(A) Density plots are of CD62L vs CD44 by live lymph node TCR$^+$CD4$^+$CD25$^-$ cells. Numbers indicate percentage of cells in each gate.

(B) The total number of CD4$^+$ T$^{cm}$ (CD44$^{hi}$CD62$^{hi}$) and T$^{em}$ (CD44$^{hi}$CD62L$^{lo}$) cells from the lymph nodes and spleens of the indicated strains.

(C) Density plots are of CD62L vs CD122 by live lymph node TCR$^+$CD8$^+$CD44$^{hi}$ cells. Numbers indicate percentage of cells in each gate.

(D) The total number of CD8$^+$ T$^{vm}$ (CD122$^{+}$CD62L$^{hi}$) and T$^{em}$ (CD62L$^{lo}$) cells from the lymph nodes and spleens of the indicated strains.

Data is pooled from 9 independent experiments. Error bars indicate SD.
5.2.7 Genetic inhibition of RIPK1 kinase activity restored normal CD8+ SP thymocyte development in mice with combined A20 and IKK2 deficiency.

Since our in vitro data suggested that A20 deficiency altered the regulation of RIPK1 in T cells, we next asked whether the impaired CD8+ SP thymocyte development in Tnfaip3.Ikk2ΔTCD4 mice was due to a failure to control RIPK1 kinase activity. To address this, we introduced the D138N RIPK1 kinase inactivating mutation into Tnfaip3.Ikk2ΔTCD4 mice (Tnfaip3.Ikk2ΔTCD4RIPK1D138N). In common with the parental Tnfaip3.Ikk2ΔTCD4 strain, Tnfaip3.Ikk2ΔTCD4RIPK1D138N mice had normal numbers of both CD4+ SP thymocytes and immature HSAhiCD62Llo CD8+ SP thymocytes (Fig. 5.7A, B). However, the RIPK1D138N mutation rescued the number of mature HSAloCD62Lhi CD8+ SP thymocytes in Tnfaip3.Ikk2ΔTCD4 mice to the normal range observed in WT controls (Fig. 5.7B). Rescue was also reflected by a restoration of the normal ratio of HSAlo CD8+ thymocytes (Fig. 5.7A, C).

Therefore, these data suggest that the combined loss of A20 and IKK2 in mature HSAloCD62Lhi CD8+ SP thymocytes and the consequent loss of numbers was the result of a RIPK1 kinase dependent cell death event.
Figure 5.7 Ablation of RIPK1 activity restored normal CD8+ SP thymocyte development in mice with combined A20 and IKK2 deficiency.

Flow cytometric analysis of thymocytes from Cre-ve (WT, n=15), Tnfaip3.IkkoΔCD4+ (n=11) and Tnfaip3.IkkoΔCD4RIPK1D138N (n=8) mice.

(A) Density plots are of CD4 vs CD8 by live thymocytes (top row) and HSA vs CD62L by gated CD4+ SP thymocytes (middle row) and CD8+ SP thymocytes (bottom row). Numbers indicate percentage of cells in each gate.

(B) The total number of the specified thymocyte subsets from the indicated strains.

(C) The percentage of CD4+ and CD8+ SP thymocytes with low HSA expression from the indicated strains.

Data is pooled from 12 independent experiments. Error bars indicate SD.
5.2.8 RIPK1$^{D138N}$ rescued peripheral T cell homeostasis in mice lacking both A20 and IKK2.

Next, we wanted to understand the contribution of RIPK1 dependent cell death to the perturbed naive T cell homeostasis in $Tnfaip3.Ikk2Δ^{TCD4}$ mice. Enumerating the composition of peripheral T cell compartments of $Tnfaip3.Ikk2Δ^{TCD4}RIPK1^{D138N}$ mice revealed a rescue of peripheral T cell numbers as compared with $Tnfaip3.Ikk2Δ^{TCD4}$ mice, almost completely restoring naïve CD4$^+$ and CD8$^+$ T cell numbers to within normal range (Fig. 5.8A, B). These data suggest that the profound peripheral T cell lymphopenia in $Tnfaip3.Ikk2Δ^{TCD4}$ mice was due to a failure to control RIPK1 kinase activity in the absence of just the IKK2 kinase subunit of the IKK complex.

IL-7R expression level on naïve T cells is also known to play an important role in regulating peripheral T cell homeostasis. New T cells exiting the thymus require canonical NF-κB signalling for normal upregulation of IL-7R (Silva et al., 2014). Since A20 is a negative regulator of NF-κB signalling, A20 deficient T cells have been shown to exhibit enhanced NF-κB signalling in TCR stimulated thymocytes and mature T cells (Fischer et al., 2017a; Giordano et al., 2014). Therefore, we first asked whether A20 deficiency also influences the NF-κB dependent expression of IL-7R by peripheral naïve T cells. Naïve CD4$^+$ and CD8$^+$ T cells in $Tnfaip3Δ^{TCD4}$ mice had normal IL-7R expression (Fig. 5.9). But it was possible that IL-7R was already at maximal levels on WT naïve T cells, potentially obscuring any impact of A20 deletion. Therefore, we also examined whether A20 deficiency effected IL-7R expression by naïve T cells also lacking IKK2, for whom NF-κB signalling was likely suboptimal. Naïve T cells from $Ikk2Δ^{TCD4}$ mice had reduced IL-7R expression, with slightly lower levels on CD8$^+$ compared to CD4$^+$ T cells, as shown previously (Silva et al., 2014; Webb et al., 2019). Naïve CD8$^+$ T cells from both $Tnfaip3.Ikk2Δ^{TCD4}$ and $Tnfaip3.Ikk2Δ^{TCD4}RIPK1^{D138N}$ exhibited similar loss of IL-7R expression as T cells from $Ikk2Δ^{TCD4}$ mice. However, IL-7R expression by naïve CD4$^+$ T cells from $Tnfaip3.Ikk2Δ^{TCD4}$ mice was even lower than observed on T cells from $Ikk2Δ^{TCD4}$ mice. This difference appeared to be due to loss of peripheral T cells in $Tnfaip3.Ikk2Δ^{TCD4}$ mice,
since rescuing cell survival by introduction of RIPK1^{D138N} also restored IL-7R levels to those of IKK2 deficient T cells. Therefore, this data suggests that A20 expression does not affect peripheral T cell homeostasis through modulation of IL-7R expression. Instead, the primary function of A20 for normal T cell homeostasis is to control RIPK1 kinase activity.
Figure 5.8 RIPK1<sup>D138N</sup> rescued peripheral T cell homeostasis in mice lacking both A20 and IKK2.

Flow cytometric analysis of lymph nodes and spleens from Cre-ve (WT, n=15), Tnfaip3<sup>-</sup>Ikk2Δ<sup>CD4</sup> (n=11) and Tnfaip3<sup>-</sup>Ikk2Δ<sup>CD4</sup>RIPK1<sup>D138N</sup> (n=8) mice.

(A) Density plots are of CD4 vs CD8 by live lymph node cells (top row) and CD25 vs CD44 by TCR<sup>+</sup>CD4<sup>+</sup> cells (middle row) and TCR<sup>+</sup>CD8<sup>+</sup> cells (bottom row). Numbers indicate percentage of cells in each gate.

(B) The total number of CD4<sup>+</sup> and CD8<sup>+</sup> naïve T cells from the lymph nodes and spleens of the indicated strains.

Data is pooled from 12 independent experiments. Error bars indicate SD.
Figure 5.9 A20 deficiency in naïve T cells has no effect on IL-7R expression

Flow cytometric analysis of lymph node cells from Cre -ve (WT, n=15), Tnfaip3ΔTCD4 (n=6), Ikk2ΔTCD4 (n=4), Tnfaip3.Ikk2ΔTCD4 (n=11) and Tnfaip3.Ikk2ΔTCD4.RIPK1D138N (n=8) mice. Bar plots show the IL-7R MFI by live naïve CD4+ (left) and CD8+ (right) T cells of the indicated strains as a fraction of the IL-7R MFI by Cre -ve (WT) or RIPK1D138N controls analysed at the same time.

Data is pooled from 12 independent experiments. Error bars indicate SD.
5.2.9 Inhibition of RIPK1 kinase activity had no effect on Treg development and homeostasis in *Tnfaip3.Ikk2ΔT*<sup>CD4</sup> mice

Next, we asked whether the ablation of RIPK1 kinase activity was sufficient to rescue the impaired FoxP3*+CD25* Treg development and homeostasis in mice with combined A20 and IKK2 deletion. However, enumerating FoxP3*+CD25* Tregs in *Tnfaip3.Ikk2ΔT*<sup>CD4</sup>RIPK1<sup>D138N</sup> mice did not reveal an increase in numbers of thymic or peripheral FoxP3* Tregs compared to *Tnfaip3.Ikk2ΔT*<sup>CD4</sup> mice (Fig. 5.10A-D). This suggested that a lack of RIPK1 kinase control in FoxP3*CD25* Tregs was not sufficient to explain the developmental and homeostatic defects seen in *Tnfaip3.Ikk2ΔT*<sup>CD4</sup> mice. Thereby, other IKK2 functions, most likely canonical NF-κB activation, were also important for FoxP3*CD25* Tregs.
A

CD4^+ SP thymocytes

WT

Trnfai3

Ikkt2AtCD4

Trnfai3

Ikkt2AtCD4

RIPK1D138N

2.6

1.4

1.4

B

Thymic FoxP3' Treg

WT

Trnfai3.Ikkt2AtCD4

Trnfai3.Ikkt2AtCD4 RIPK1D138N

C

CD4^+ T cells

WT

Trnfai3

Ikkt2AtCD4

Trnfai3

Ikkt2AtCD4 RIPK1D138N

14

25

5

D

Peripheral FoxP3' Treg

WT

Trnfai3.Ikkt2AtCD4

Trnfai3.Ikkt2AtCD4 RIPK1D138N

Cell no.
**Figure 5.10 Inhibition of RIPK1 kinase activity had no effect on Treg development and homeostasis in *Tnfaip3.Ikk2ΔTCD4* mice**

Flow cytometric analysis of thymocytes, lymph nodes and spleens from Cre-ve (WT, n=15), *Tnfaip3.Ikk2ΔTCD4* (n=11) and *Tnfaip3.Ikk2ΔTCD4RIPK1D138N* (n=8) mice.

(A) Density plots are of FoxP3 vs CD25 by live CD4+ SP thymocytes. Numbers indicate percentage of cells in each gate.

(B) The total number of thymic FoxP3+CD25+ Tregs from the indicated strains.

(C) Density plots are of FoxP3 vs CD25 by live lymph node TCR+CD4+ cells. Numbers indicate percentage of cells in each gate.

(D) The total number of FoxP3+CD25+ Tregs from the lymph nodes and spleens of the indicated strains.

Data is pooled from 12 independent experiments. Error bars indicate SD.
5.2.10 Disruption of CD4\(^+\) memory subsets in *Tnfaip3.Ikk2\(\Delta T\)\(^{CD4}\) RIPK1\(^{D138N}\) mice

Since there was a reduction in the CD4\(^+\) memory T cell populations in mice lacking both A20 and IKK2, we asked whether this contraction of compartments could be explained by RIPK1 dependent cell death processes. Examining the number of T\(_{cm}\) and T\(_{em}\) in *Tnfaip3.Ikk2\(\Delta T\)\(^{CD4}\)RIPK1\(^{D138N}\) mice, revealed a modest rescue in the number of CD4\(^+\) T\(_{cm}\) cell numbers. However, there were still substantially fewer CD4\(^+\) T\(_{cm}\) cells when compared to WT mice (Fig. 5.11A, B). Examination of the T\(_{em}\) compartment suggested there was a further decrease in the number of CD4\(^+\) T\(_{em}\) cells in *Tnfaip3.Ikk2\(\Delta T\)\(^{CD4}\)RIPK1\(^{D138N}\) mice compared to *Tnfaip3.Ikk2\(\Delta T\)\(^{CD4}\) mice. In contrast to CD4\(^+\) memory compartments, the size of CD8\(^+\) memory T cell compartments were unchanged after introduction of RIPK1\(^{D138N}\).

Together, these data suggest that of the CD4\(^+\) and CD8\(^+\) memory T cell populations, there was some evidence that A20 and IKK2 were required to control RIPK1 kinase activity in CD4\(^+\) T\(_{cm}\) cells. However, it appeared that a lack of RIPK1 kinase regulation was not the main cause for the defective CD4\(^+\) memory T cell populations in *Tnfaip3.Ikk2\(\Delta T\)\(^{CD4}\) mice.
A

CD4+ CD25- T cells

WT
Tnfaip3
Ikk2ΔCD4
ΔT
CD4
RIPK1D138N

B

CD4+ Tcm cells

CD4+ Tem cells

WT
Tnfaip3,ikk2ΔTCD4
ΔT
CD4
RIPK1D138N

C

CD8+ CD44hi T cells

WT
Tnfaip3
Ikk2ΔTCD4
ΔT
CD4
RIPK1D138N

D

CD8+ Tvm cells

CD8+ Tem cells

WT
Tnfaip3,ikk2ΔTCD4
ΔT
CD4
RIPK1D138N

Cell no.
Figure 5.11 CD4+ memory T cells populations were altered in Tnfaip3.Ikk2ΔTCD4 mice after introduction of RIPK1D138N

Flow cytometric analysis of the lymph nodes and spleens from Cre -ve (WT, n=15), Tnfaip3.Ikk2ΔTCD4 (n=11) and Tnfaip3.Ikk2ΔTCD4RIPK1D138N (n=8) mice.

(A) Density plots of CD62L vs CD44 by live lymph node TCR+CD4+CD25- cells. Numbers indicate percentage of cells in each gate.

(B) The total number of CD4+ Tcm (CD44hiCD62Lhi) and Tem (CD44hiCD62Llo) cells from the lymph nodes and spleens of the indicated strains.

(C) Density plots of CD62L vs CD122 by live lymph node TCR+CD8+CD44hi cells. Numbers indicate percentage of cells in each gate.

(D) The total number of CD8+ Tvm (CD122hiCD62Lhi) and Tem (CD62Llo) cells from the lymph nodes and spleens of the indicated strains.

Data is pooled from 12 independent experiments. Error bars indicate SD.
5.2.11 TNF is the trigger for RIPK1 dependent death in mature CD8+ SP thymocytes lacking both A20 and IκK2

*Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice have impaired CD8\textsuperscript{+} SP thymocyte development due to defective RIPK1 kinase regulation. TNF binding to TNFR1 is known to be capable of driving RIPK1 kinase activity if normal signalling is disrupted. Previous work has shown TNF to be largely responsible for death of SP thymocytes in mice deficient in IκK1 and IκK2, which also lack RIPK1 control (Webb et al., 2016, 2019). Therefore, we asked whether TNF was inducing RIPK1 dependent death of mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice. To test this, we first blocked TNF signalling in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice by treating them with neutralising anti-TNF antibody for 14 days. Blockade of TNF signalling in this way restored normal CD8\textsuperscript{+} SP thymocyte development by completely rescuing the number of mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes compared to PBS treated *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice (Fig. 5.12A, B).

Next, we wanted to confirm that TNF was inducing death of mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice through specific ligation of the receptor TNFR1. Hence, we examined thymocyte development in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} Tnfrsf1a\textsuperscript{-/-} mice. Deletion of TNFR1 completely rescued the number of mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice (Fig. 5.12A, C) and restored normal proportions of mature and immature CD8\textsuperscript{+} SP thymocytes. However, we also note that the other SP thymocyte subpopulations in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} Tnfrsf1a\textsuperscript{-/-} were also slightly increased.

Since absence of TNFR1 rescued mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} mice, we wanted to test whether TNF is the sole ligand in the thymus and if not, whether RIPK1\textsuperscript{D138N} would have an additional rescue. Therefore, we introduced RIPK1\textsuperscript{D138N} into *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} Tnfrsf1a\textsuperscript{-/-} mice. The mature HSA\textsuperscript{lo}CD62L\textsuperscript{hi} CD8\textsuperscript{+} SP thymocyte population was rescued in *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} Tnfrsf1a\textsuperscript{-/-}RIPK1\textsuperscript{D138N} mice, which also showed a similar number to *Tnfaip3*Ikk2ΔΤ\textsuperscript{CD4} Tnfrsf1a\textsuperscript{-/-} mice.
Therefore, this data suggests that TNFR1 signalling was solely responsible for triggering RIPK1 dependent cell death of mature CD8$^+$ SP thymocytes in mice with combined A20 and IKK2 deficiency.
A

**Trnaip3.Ikk2ΔCD4**

<table>
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<tr>
<th></th>
<th>WT</th>
<th>+ PBS</th>
<th>+ anti-TNF</th>
<th>Tnfrsf1aΔ</th>
<th>RIPK1D138N</th>
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<tr>
<td>Total</td>
<td></td>
<td></td>
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<td>CD8</td>
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<td>CD4+ SP  thymocytes</td>
<td>76</td>
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<td>19</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>CD8+ SP  thymocytes</td>
<td>53</td>
<td>40</td>
<td>24</td>
<td>37</td>
<td>42</td>
</tr>
</tbody>
</table>

B

**Cell no.**

- **DP thymocytes**
  - WT: 10^6
  - PBS treated Trnaip3.Ikk2ΔCD4: 10^6
  - anti-TNF treated Trnaip3.Ikk2ΔCD4: 10^6

- **HSA^hi CD4+ SP thymocytes**
  - WT: 10^6
  - PBS treated Trnaip3.Ikk2ΔCD4: 10^6
  - anti-TNF treated Trnaip3.Ikk2ΔCD4: 10^6

- **HSA^lo CD4+ SP thymocytes**
  - WT: 10^6
  - PBS treated Trnaip3.Ikk2ΔCD4: 10^6
  - anti-TNF treated Trnaip3.Ikk2ΔCD4: 10^6

- **HSA^hi CD8+ SP thymocytes**
  - WT: 10^6
  - PBS treated Trnaip3.Ikk2ΔCD4: 10^6
  - anti-TNF treated Trnaip3.Ikk2ΔCD4: 10^6

- **HSA^lo CD8+ SP thymocytes**
  - WT: 10^6
  - PBS treated Trnaip3.Ikk2ΔCD4: 10^6
  - anti-TNF treated Trnaip3.Ikk2ΔCD4: 10^6

C

**Cell no.**

- **DP thymocytes**
  - WT: 10^6
  - Trnaip3.Ikk2ΔCD4: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ RIPK1D138N: 10^6

- **HSA^hi CD4+ SP thymocytes**
  - WT: 10^6
  - Trnaip3.Ikk2ΔCD4: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ RIPK1D138N: 10^6

- **HSA^lo CD4+ SP thymocytes**
  - WT: 10^6
  - Trnaip3.Ikk2ΔCD4: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ RIPK1D138N: 10^6

- **HSA^hi CD8+ SP thymocytes**
  - WT: 10^6
  - Trnaip3.Ikk2ΔCD4: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ RIPK1D138N: 10^6

- **HSA^lo CD8+ SP thymocytes**
  - WT: 10^6
  - Trnaip3.Ikk2ΔCD4: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ: 10^6
  - Trnaip3.Ikk2ΔCD4 Tnfrsf1aΔ RIPK1D138N: 10^6

**Note:**

- **CD62L**
- **HSA**
- **CD4**
- **CD8**
- **SP**
- **WT**
- **PBS treated**
- **anti-TNF treated**
- **Tnfrsf1aΔ**
- **RIPK1D138N**

**Significance:**

- **✱✱✱**
- **✱✱**
- **✱**
- **ns**
Figure 5.12 TNF is the trigger of RIPK1 dependent death in mature CD8+ SP thymocytes lacking both A20 and IKK2

*Tnfaip3.Ikk2ΔT<sup>CD4</sup>* mice were injected intraperitoneally with anti-TNF antibody or PBS for 2 weeks. Thymocytes from treated mice and untreated Cre -ve (WT, n=15), *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* (n=11), *Tnfaip3.Ikk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>−/−</sup>* (n=9) and *Tnfaip3.Ikk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>−/−</sup>RIPK1<sup>D138N</sup>* (n=8) mice were analysed by flow cytometry.

(A) Density plots are of CD4 vs CD8 by live thymocytes (top row) and HSA vs CD62L by gated CD4<sup>+</sup> SP thymocytes (middle row) and CD8<sup>+</sup> SP thymocytes (bottom row). Numbers indicate percentage of cells in each gate.

(B) The total number of the specified thymocyte subsets from untreated WT (n=3), PBS treated *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* (n=2) and TNF treated *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* mice (n=3).

(C) The total number of the specified thymocyte subsets from untreated indicated strains.

Data is pooled from 1 (B) or 12 (C) independent experiments. Error bars indicate SD.
5.2.12 Blockade of TNF signalling partially rescued the peripheral T cell lymphopenia in mice with combined A20 and IKK2 deficiency

*Tnfaip3.ikk2ΔTCD4* mice have profound peripheral T cell lymphopenia due to defective RIPK1 kinase regulation. Since our data suggested that the TNF/TNFR1 axis alone was inducing death of mature CD8+ SP thymocytes lacking A20 and IKK2, we next wanted to test whether TNF was also responsible for inducing death of naïve T cells in *Tnfaip3.ikk2ΔTCD4* mice. Treatment of *Tnfaip3.ikk2ΔTCD4* mice with anti-TNF for 14 days led to an increase in both naïve CD4+ and CD8+ T cells compared to PBS treated mice. However, naïve T cell numbers were not restored to WT levels, remaining substantially lower (Fig. 5.13A, B). Since treatment with blocking mAb was only for a short period, it may have been unrealistic to expect full restoration of naïve T cell numbers. Therefore, we further assessed the role of TNF signalling by analysing the phenotype of *Tnfaip3.ikk2ΔTCD4 Tnfrsf1a−/−* mice. Naïve T cell compartments in this strain were also partially restored by loss of TNFR1 expression. But restoration of naïve compartments by germline deletion of TNFR1 was no better than observed following anti-TNF treatment of *Tnfaip3.ikk2ΔTCD4* mice. To assess whether RIPK1 activity might be triggered by other receptors, we analysed *Tnfaip3.ikk2ΔTCD4 Tnfrsf1a−/−RIPK1D138N* mice. These mice had an increased number of naïve CD4+ and CD8+ T cells compared to *Tnfaip3.ikk2ΔTCD4 Tnfrsf1a−/−* mice.

Together, these data suggest that TNFR1 was only partially responsible for triggering RIPK1 dependent cell death of naïve T cells lacking A20 and IKK2, implying the activity of another RIPK1 trigger signal.
A

**Tnfaip3**.Ik**k2Δ**CD4

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>+ PBS</th>
<th>+ anti-TNF</th>
<th>Tnfrsf1a&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Tnfrsf1a&lt;sup&gt;−/−&lt;/sup&gt; RIPK1&lt;sup&gt;D138N&lt;/sup&gt;</th>
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<td>CD4+ T cells</td>
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<tr>
<td>CD8+ T cells</td>
<td>28</td>
<td>7</td>
<td>10</td>
<td>7</td>
<td>17</td>
</tr>
</tbody>
</table>

B

**Naive CD4**+ T cells

- WT
- PBS
- anti-TNF
- Tnfrsf1a<sup>−/−</sup>
- Tnfrsf1a<sup>−/−</sup> RIPK1<sup>D138N</sup>

- **Naive CD8**+ T cells

- WT
- PBS
- anti-TNF
- Tnfrsf1a<sup>−/−</sup>
- Tnfrsf1a<sup>−/−</sup> RIPK1<sup>D138N</sup>
**Figure 5.13 Blockade of TNF signalling partially rescued the peripheral T cell lymphopenia in mice with combined A20 and IKK2 deficiency**

*Tnfaip3.Ikk2ΔT<sup>CD4</sup>* mice injected intraperitoneally with anti-TNF antibody (n=3) or PBS (n=3) for 2 weeks. Lymph nodes and spleens from treated mice and untreated Cre-ve (WT, n=15), *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* (n=11), *Tnfaip3.Ikk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>a/-</sup> (n=9) and *Tnfaip3.Ikk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>a/-</sup>RIPK1<sup>D138N</sup> (n=8) mice were analysed by flow cytometry. 

(A) Density plots are of CD4 vs CD8 by live lymph node cells (top row) and CD25 vs CD44 by TCR<sup>+</sup>CD4<sup>+</sup> cells (middle row) and TCR<sup>+</sup>CD8<sup>+</sup> cells (bottom row). Numbers indicate percentage of cells in each gate.

(B) The total number of CD4<sup>+</sup> and CD8<sup>+</sup> naïve T cells from the lymph nodes and spleens of the treated and untreated indicated strains. Data is pooled from 1 (treated) or 12 (untreated) independent experiments. Error bars indicate SD.
5.2.13 Loss of A20 expression sensitises activated IKK2 deficient T cells to RIPK1 dependent cell death

During activation, F5 *Ikk2ΔT<sup>CD2</sup>* T cells became sensitised to RIPK1 dependent cell death, which coincided with a reduction in A20 expression. Since our results suggested that A20 is important for control of RIPK1 kinase activity in T cells, we next directly tested whether loss of A20 expression was responsible for sensitising IKK2 deficient T cells to RIPK1 dependent cell death during activation. To do this, we stimulated CTV labelled T cells deficient in A20 and/or IKK2 with anti-CD3/CD28. Cell viability was analysed after 24 hrs, once the T cells had upregulated T cell activation markers, and 48 hrs, the point at which the T cells were dividing. Deletion of A20 and/or IKK2 in activated T cells altered cell viability and subsequently impacted the proportion of T cells which could divide (Fig. 5.14A). After 24 hrs, viability of T cells from *Tnfaip3ΔT<sup>CD4</sup>* mice was normal (Fig. 5.14B). CD4<sup>+</sup> T cells from *Ikk2ΔT<sup>CD4</sup>* mice also showed normal viability but there was evidence of decreased viability amongst CD8<sup>+</sup> T cells at this early time point. Cell death appeared to be RIPK1 independent since the addition of Nec-1 did not improve viability of cultures. In contrast, viability of CD4<sup>+</sup> and CD8<sup>+</sup> T cells deficient in both A20 and IKK2 was significantly reduced in a RIPK1 dependent manner, since kinase dead RIPK1<sup>D138N</sup> could substantially rescue the T cells from death in culture. But deletion of *Tnfrsf1a* appeared not to improve viability of T cells from *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* mice, though there was a similar rescue upon further introduction of RIPK1<sup>D138N</sup>. After 48 hrs, T cells from *Tnfaip3ΔT<sup>CD4</sup>* mice exhibited reduced viability, that appeared to be RIPK1 independent, since the addition of Nec-1 failed to rescue cells from death (Fig. 5.14B). In contrast, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Ikk2ΔT<sup>CD4</sup>* mice showed decreased viability that could be substantially restored by the addition of Nec-1. T cells lacking both A20 and IKK2 exhibited the greatest levels of cell death following activation. As was true of IKK2 deficient T cells, cell death appeared to be RIPK1 dependent, since introduction of RIPK1<sup>D138N</sup> restored viability of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Tnfaip3.Ikk2ΔT<sup>CD4</sup>* mice to levels observed in control cultures of WT T cells. The absence of *Tnfrsf1a* had no effect on the viability of T cells lacking
A20 and IKK2, as observed at 24 hrs. Nonetheless, RIPK1\textsuperscript{D138N} could rescue survival of T cells from \textit{Tnfaip3.Ikk2ΔT}^{CD4}\textit{Tnfrsf1a}⁻/⁻ mice. Taken together, these data show that genetic ablation of \textit{Tnfaip3} from IKK2 deficient T cells results in a similar phenotype of RIPK1 dependent cell death as observed in T cells lacking only IKK2, suggesting that loss of A20 in both settings is responsible for cell death.
A

CD4+ T cells

CD8+ T cells

CTV

B

CD4+ T cells

CD8+ T cells

Cell viability after 24 hrs (%)

Cell viability after 48 hrs (%)

WT

Tnfaip3ΔCD4

Tnfaip3ΔCD4 + Nec-1

Ikk2ΔCD4

Ikk2ΔCD4 + Nec-1

Tnfaip3 Δ

Ikk2ΔCD4

RIPK1D138N

Tnfaip3 Δ

Ikk2ΔCD4

Tnfrsf1a-/-

RIPK1D138N

ns

✱✱✱

ns

✱✱✱

✱✱✱

Cell viability after 24 hrs (%)

Cell viability after 48 hrs (%)

0

20

40

60

80

0

20

40

60

80

0

20

40

60

80

0

20

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60

80
Figure 5.14 Loss of A20 expression sensitises activated IKK2 deficient T cells to RIPK1 dependent cell death

Lymph node cells were isolated from Cre -ve (WT), Ikk2ΔT<sup>CD4</sup>, Tnfaip3ΔT<sup>CD4</sup>, Tnfaip3.1kk2ΔT<sup>CD4</sup>, Tnfaip3.1kk2ΔT<sup>CD4</sup>RIPK1<sup>D138N</sup>, Tnfaip3.1kk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>−/−</sup> and Tnfaip3.1kk2ΔT<sup>CD4</sup>Tnfrsf1a<sup>−/−</sup>RIPK1<sup>D138N</sup> mice and labelled with CTV. Cells were stimulated with plate bound anti-CD3 and anti-CD28 for 48 hrs in the presence or absence of necrostatin-1. The cultures were analysed by flow cytometry.

(A) Density plots are of LIVE/DEAD dye vs CTV by total CD4<sup>+</sup> cells (top row) and CD8<sup>+</sup> cells (bottom row). Numbers indicate percentage of cells in each gate.

(B) Bar plots show cell viability (% live cells) of CD4<sup>+</sup> (first column) and CD8<sup>+</sup> (second column) T cells after culture for 24 hrs (top row) and 48 hrs (bottom row).

Data is pooled from 7 independent experiments. Error bars indicate SEM.
5.3 Discussion

A20 is well established as a negative regulator of NF-κB, but also functions to protect cells from TNF induced cell death (Lee et al., 2000). Here, we have characterised the role of A20 for T cell survival. We show that A20 is not an essential requirement of SP thymocytes or naïve T cells for protection against TNF induced cell death. A20 deficient SP thymocytes and T cells were completely resistant to TNF induced cell death in vitro, even at extremely high TNF concentrations. This corresponded with the phenotype of mice with T cell specific A20 deletion which display normal thymocyte development and unperturbed naïve T cell homeostasis, except for a small decrease in naïve CD8+ T cells. Our data is in line with previous studies examining mice with CD4Cre mediated A20 deletion (Fischer et al., 2017a; Just et al., 2016; Onizawa et al., 2015). The reason for the small decrease in naïve CD8+ T cells remains unclear but does not appear to be due to TNF induced cell death or reduced IL-7R expression. However, the finding that A20 is dispensable in T cells for protection against TNF induced cell death appears to contrast with other cell types. Deletion of A20 in MEFs and IECs sensitises them to TNF induced RIPK1 dependent cell death (Priem et al., 2019). This suggests that in absence of A20, other mechanisms are sufficient to restrict RIPK1 dependent cell death in T cells. Thus, the contribution of A20 for cell survival appears to vary between cellular contexts.

Our data suggests that although A20 is not critical for T cell survival, it plays a vital role in the regulation of RIPK1 in T cells. TNF triggers cell death of T cells when the activity of IKK2 alone or both IKK1 and IKK2 is blocked by acute pharmacological inhibition in vitro. But T cells can be completely rescued from TNF induced cell death when RIPK1 kinase activity is blocked with Nec-1, suggesting that cell death was RIPK1 dependent. This observation is consistent with data in MEFs showing that the IKK complex directly phosphorylates RIPK1 in complex I, to limit formation of complex IIb and subsequently prevent cell death (Dondelinger et al., 2015). Thus, in a similar fashion to SP thymocytes, survival of naïve T cells exposed to TNF
requires active repression of RIPK1 by the IKK complex (Webb et al., 2019). However, we found that in the absence of A20, the threshold of IKK inhibition required to unleash TNF triggered cell death was far lower. Death of A20 deficient T cells was also completely blocked with Nec-1, suggesting it was RIPK1 dependent. One interpretation of these results is that A20 deficiency in T cells necessitates greater IKK activity in order to prevent RIPK1 induced cell death. As such, A20 expression appears to fine tune the level of IKK activity that T cells require to control RIPK1 kinase activity.

Disrupting IKK activity genetically confirmed that A20 was required for optimal control of RIPK1 kinase by the IKK complex. IKK2 deficiency in T cells leads to impaired, but not absent, NF-κB activation mediated by IKK1 homodimers, which show weaker phosphorylation of IκBα (Schmidt-Supprian et al., 2003). Although not proven experimentally, we suspected that IKK1 homodimeric complexes also have weaker kinase activity towards RIPK1. In support of this, one gene copy of Ikbkb is sufficient to protect mature CD8+ SP thymocytes from TNF induced cell death whereas one gene copy of Chuk is not (Webb et al., 2019). Nonetheless, IKK2 deficient mice show normal thymocyte development and only a modest loss of mature T cells, thought to result from impaired IL-7R expression (Silva et al., 2014; Webb et al., 2019). Thus, in these mice, although regulation of RIPK1 by IKK may be sub-optimal, it is sufficient to protect T cells from cell death. In support of this view, we found that deletion of A20 in IKK2 deficient mice lead to profound peripheral T cell lymphopenia not observed in mice with single A20 or IKK2 deficiencies. Lymphopenia appeared to be due to a RIPK1 mediated death process, because kinase dead RIPK1 almost completely restored the peripheral T cell numbers to normal. This suggests that, in absence of A20, IKK1 homodimeric complexes are no longer sufficient to protect T cells from cell death. Therefore, repression of RIPK1 kinase activity in T cells with sub-optimal IKK activity is crucially dependent on A20 expression.

Normal SP thymocyte development is dependent upon IKK repression of RIPK1 dependent cell death (Webb et al., 2019). Ablation of either IKK1 or IKK2 has no effect on thymocyte survival whereas combined loss leads to
RIPK1 dependent cell death of SP thymocytes (Silva et al., 2014; Webb et al., 2016, 2019). Therefore, for thymocyte development, there is redundancy between IKK1 and IKK2 for control of RIPK1 kinase activity. In A20 IKK2 double deficient mice, CD4⁺ SP thymocyte development appeared normal. In contrast, there were fewer mature CD8⁺ SP thymocytes and blocking RIPK1 kinase activity restored their numbers, indicating susceptibility to RIPK1 dependent cell death. Thus, in absence of both A20 and IKK2, IKK1 homodimers alone remained sufficient for RIPK1 kinase control and survival of CD4⁺ SP and immature CD8⁺ SP thymocytes. In contrast, for normal survival of mature CD8⁺ SP thymocytes, repression of RIPK1 kinase activity by IKK1 homodimers is critically dependent on A20 expression.

Of the SP thymocyte subsets, mature CD8⁺ SP thymocytes have the greatest expression of RIPK1. This may explain why in mice with combined deletion of both A20 and IKK2, only the mature CD8⁺ SP thymocytes are sensitised to RIPK1 dependent cell death. In IKK1/2 deficient thymocytes, RIPK1 expression correlates with sensitivity to TNF induced cell death (Webb et al., 2019). Therefore, mature CD8⁺ SP thymocytes may require more stringent RIPK1 control mechanisms in place to protect against cell death. As such, in absence of IKK2, IKK1 homodimers may only just provide enough inhibition of RIPK1 to prevent complex Iib formation. Since we show that A20 also has a role in RIPK1 kinase control, additional deletion of A20 appears to tip the balance in favour of cell death. Interestingly, inhibition of RIPK1 kinase activity in vivo appears to provide a more complete rescue of mature CD8⁺ SP thymocytes in A20 IKK2 double deficient mice compared to IKK1/2 deficient mice (Webb et al., 2019). The observed RIPK1 independent death of mature CD8⁺ SP thymocytes with IKK1/2 deletion appears to be absent in mice deficient in both A20 and IKK2. Therefore, our data suggests that A20 and IKK2 are not required for control of this RIPK1 independent death pathway and suggests that IKK1 homodimers alone are sufficient.

TNF is the primary trigger of cell death in IKK1/2 deficient SP thymocytes (Webb et al., 2016). Here, we found that TNF also appeared to drive RIPK1 dependent death of mature CD8⁺ SP thymocytes lacking both A20 and IKK2.
Blocking TNF signalling either with anti-TNF antibody or through genetic ablation of TNFR1 both completely rescued the survival of mature CD8+ SP thymocytes. The extent to which survival was rescued mirrored that conferred by RIPK1 kinase dead and additional ablation RIPK1 kinase activity had no extra effect. Thus, it appears that in mature CD8+ SP thymocytes, TNF binding to TNFR1 is entirely responsible for inducing RIPK1 kinase activity and that both A20 and IKK2 are required for RIPK1 control downstream of TNFR1.

Our data suggests that TNF is also active in inducing RIPK1 dependent death of mature T cells in mice with combined A20 and IKK2 deficiency. Blockade of TNF signalling via treatment with anti-TNF antibody or genetic deletion of Tnfrsf1a could partially rescue naïve T cells in these mice. Acute blockade of TNF with antibody for 2 weeks gave an almost identical rescue of naïve T cells as knockout of TNFR1 throughout the T cell lifespan. This suggests that TNF triggers T cell death shortly after their export from the thymus. In contrast to TNF blockade, kinase dead RIPK1 rescued T cell numbers in A20 IKK2 double deficient mice to near normality. Ablation of both TNFR1 and RIPK1 kinase activity also gave a better rescue compared to ablation of TNFR1 alone. These data clearly indicate that A20 and IKK2 control RIPK1 kinase activity downstream of other receptors. Fas, TRAIL-R and DR3 are possible candidates since they all possess death domains and can recruit RIPK1 after ligand binding (Geserick et al., 2009; Holler et al., 2000; Lafont et al., 2017). But it remains unclear why receptors other than TNFR1 are triggering RIPK1 dependent cell death of naïve T cells in the periphery but not CD8+ SP thymocytes, since they also express other death receptors (Webb et al., 2019). Further work is needed to address whether this reflects an absence of other death ligands in the thymus or differing regulation of cell death pathways triggered by other receptors.

Canonical NF-κB induced IL-7R expression by new T cells is crucial for their normal peripheral homeostasis (Silva et al., 2014; Webb et al., 2019). Although A20 is a negative regulator of NF-κB and A20 deficient T cells show sustained NF-κB activity, this appeared to have no effect on IL-7R.
expression by naïve T cells (Fischer et al., 2017a; Giordano et al., 2014; Lee et al., 2000). A20 deficient naïve T cells showed normal IL-7R expression. In the setting of IKK2 deficiency, where impaired canonical NF-κB leads to reduced IL-7R expression, deletion of A20 did not increase the levels of IL-7R. This could suggest that A20 deletion does not enhance canonical NF-κB in T cells in the context of IKK2 deficiency. This confirms that A20 is important for normal naïve T cell homeostasis primarily by controlling RIPK1 kinase activity rather than by modulating IL-7R expression levels. We did observe that blocking RIPK1 kinase activity in mice with combined A20 and IKK2 deficiency restored naïve CD8\(^+\) T cells to numbers higher than in mice deficient in IKK2 alone. This occurred despite similar levels of IL-7R expression between the two strains. The reduced number of naïve CD8\(^+\) T cells in IKK2 deficient mice has previously been attributed to impaired IL-7R. However, this conclusion came from F5 TCR transgenic mice (Silva et al., 2014) and, as we showed previously, naïve F5 IKK2 deficient T cells are resistant to TNF induced cell death \textit{in vitro}. Here, our data in polyclonal IKK2 deficient mice suggests that mild sensitivity to RIPK1 dependent cell death also contributes to the phenotype. Thus, sensitivity of naïve IKK2 deficient T cells to TNF induced cell death appears to differ between F5 and polyclonal mice.

While it was clear from our data that A20 was important for RIPK1 kinase control in naïve T cells, we did not find evidence of a similar role in FoxP3\(^+\) Tregs and memory T cells. IKK2 deficient mice have significantly fewer FoxP3\(^+\) Tregs and memory T cells, in line with previous data (Schmidt-Supprian et al., 2003). In contrast, mice lacking A20 had an increased number of these populations, also consistent with earlier work (Fischer et al., 2017a; Onizawa et al., 2015). Canonical NF-κB is thought to play an important role for the generation of both FoxP3\(^+\) Tregs and memory T cells and, given the contrasting role of A20 and IKK2 for NF-κB regulation, appears to account for the different phenotypes (Isomura et al., 2009; Oh et al., 2017b; Ruan et al., 2009a; Webb et al., 2019; Zheng et al., 2003). However, additional deletion of A20 in IKK2 deficient mice did not compound the loss of these T cell populations and in addition, RIPK1 kinase dead
seemingly could not rescue these populations. The exception was CD4^+ T<sub>cm</sub> cells for which there was some evidence of RIPK1 dependent cell death in absence of A20 and IKK2. It is unclear why FoxP3^+ Tregs and memory T cells were apparently not sensitised to RIPK1 dependent cell death in absence of A20 and IKK2. There are multiple possible explanations for this. Perhaps these T cell populations do require IKK2 and A20 for normal control of RIPK1, however RIPK1 kinase dead may not be enough to rescue due to the impaired NF-κB activation. Alternatively, it could be that other mechanisms remained sufficient to limit RIPK1 kinase activity or that the populations in these mice were represented by some which had escaped Cre mediated deletion. Since, there wasn’t a reporter of Cre activity in this strain, we are unable to distinguish these scenarios.

Naïve F5 IKK2 deficient T cells are resistant to RIPK1 dependent cell death but become sensitised after activation. Sensitivity to RIPK1 dependent death by activated F5 IKK2 deficient T cells was associated with decreased expression of A20. A20 promotes the survival of naïve T cells by regulating RIPK1 kinase activity. Here, we demonstrate that A20 was also crucial for RIPK1 kinase control in activated T cells. IKK2 deficient T cells were initially resistant to RIPK1 dependent cell death early after activation, since there was no effect of Nec-1 on viability after 24 hrs. But by 48 hrs, there was a significant reduction in the viability of IKK2 deficient T cells which could be rescued with Nec-1, suggesting that T cells were undergoing RIPK1 dependent cell death in the absence of IKK2. This recapitulated what was observed after peptide stimulation of F5 IKK2 deficient T cells, thereby confirming that the potentially altered development of T cells in F5 mice had no bearing on the impact of IKK2 deficiency in activated T cells. Deletion of A20 in IKK2 deficient T cells appeared to render them susceptible to RIPK1 dependent cell death at the early time point since RIPK1 kinase dead could rescue cell viability. Thereby, this suggests that the ability of IKK1 homodimers to restrict RIPK1 dependent cell death depended on the expression of A20. We suspected that sensitisation of IKK2 deficient T cells to RIPK1 dependent death at the later time point was due to a loss in A20 expression. Crucially, ablation of A20 in IKK2 deficient T cells resulted in
similar susceptibility to RIPK1 dependent cell death as observed by IKK2 deficient T cells. Thus, this data indicates that the loss of A20 expression in activated IKK2 deficient T cells is responsible for sensitisation to RIPK1 dependent cell death. As such, the induction/maintenance of A20 expression in activated T cells is crucial for normal control of RIPK1 kinase activity by the IKK complex.

Analysing viability of A20 deficient T cells following activation revealed increased cell death that was not rescued by Nec-1. This suggests that A20 was also important in activated T cells to protect against RIPK1 independent cell death. This contrasts with previous work suggesting that A20 deficient T cells are sensitised to RIPK3 dependent necroptosis after activation (Onizawa et al., 2015). In this study, only activation of A20 deficient T cells in the presence of the caspase inhibitor Z-VAD was reported. Inhibition of caspase-8 is anticipated to trigger necroptosis and so it remains unclear whether A20 can induce necroptosis of activated T cells without blocking caspase-8 activity. Our data indicates that A20 deficiency does not induce necroptosis of activated T cells. Others suggest that A20 regulates autophagy in activated T cells (Matsuzawa et al., 2015), and this may account for the RIPK1 independent cell death we observed in activated A20 deficient T cells. Ultimately, further work is required to characterise how A20 is regulating this RIPK1 independent cell death pathway and how it is being triggered.

The mechanism by which A20 regulates RIPK1 kinase activity in T cells to promote survival is currently uncertain. Most studies have so far explored the role of A20 downstream of TNFR1. One possibility is that A20 may regulate the abundance of RIPK1 in complex I post-translationally. Early work using cell lines suggested that A20 controls ubiquitination of RIPK1 by utilising its OTU and ZnF4 domains (Wertz et al., 2004, 2015). In this scenario, the deubiquitinase activity of the OTU domain catalyses the removal of K63-linked ubiquitin chains from RIPK1. RIPK1 is then subsequently ubiquitinated by the ZnF4 domain of A20, which attaches K48-linked chains to target RIPK1 for proteasomal degradation. Thus, if A20 is deficient, perhaps more
RIPK1 can be incorporated into complex I which subsequently warrants optimal IKK activity to prevent complex IIb formation and cell death. More recent evidence suggests that instead of directly regulating RIPK1, A20 functions to stabilise complex I formed downstream of TNFR1 after TNF binding. A20 is thought to bind to M1-linked ubiquitin chains which decorate complex I using its ZnF7 domain (Wertz et al., 2015; Yamaguchi and Yamaguchi, 2015). In this way, it shields the M1-linked chains from removal by the deubiquitylase CYLD to prevent destabilisation of complex I and formation of complex IIb (Draber et al., 2015; Priem et al., 2019). Thus, faced with an increased propensity of RIPK1 to dissociate from complex I in absence of A20, T cells could become more reliant on IKK activity to ensure extensive RIPK1 phosphorylation and inhibition to prevent cell death. In addition, the IKK complex has been shown to phosphorylate CYLD and inhibit its deubiquitylase activity in Jurkat T cells (Reiley et al., 2005; Xu et al., 2020). As such, since IKK activity is impaired in absence of IKK2, CYLD activity may be enhanced and could further accelerate removal of M1-ubiquitin chains from complex I if A20 is deficient. Nonetheless, how A20 may protect T cells from RIPK1 dependent cell death downstream of other death receptors needs further investigation. LUBAC is currently the only known complex capable of synthesising M1-linked ubiquitin chains in vivo and the extent to which it is involved in signalling by other receptors is unclear, although a role for LUBAC in TRAIL-R signalling has been described (Lafont et al., 2017). Therefore, it could be that A20 employs different functions downstream of different receptors for normal RIPK1 control in T cells.
Chapter 6 - Extrinsic apoptotic pathways are regulated by the IKK complex in T cells

6.1 Introduction

Extrinsic apoptosis is induced by members of the TNFR superfamily that possess death domains, including TNFR1, Fas and TRAIL-R. After ligand binding, these receptors are capable of inducing the formation of cell death complexes which ultimately facilitate activation of caspase-8. Depending on the death receptor, these cell death complexes can form at the receptor or within the cytoplasm. Cell death is not the default outcome of TNFR1 signalling due to the existence of multiple cell death checkpoints. Disabling these checkpoints leads to formation of the cytoplasmic complex II. If NF-κB activation is impaired, apoptosis is induced by complex IIa, comprised of TRADD, FADD and caspase-8 (Micheau and Tschopp, 2003). But if upstream TNFR1 signalling components are inactivated, including cIAP1/2, LUBAC, TAK1 or IKK, complex II incorporates RIPK1 and potently induces apoptosis which is dependent on the kinase activity of RIPK1 (Dondelinger et al., 2013, 2015; Ikeda et al., 2011; Peltzer et al., 2014, 2018; Vanlangenakker et al., 2011). Unlike TNFR1, Fas and TRAIL-R can induce cell death via the formation of cell death complexes at the membrane, so called the death inducing signalling complex (DISC) (Lafont et al., 2018b; Lavrik and Krammer, 2012). The death domain of these receptors interacts with FADD which can then recruit pro-caspase-8 (Scott et al., 2009; Wang et al., 2010). If the level of c-FLIP incorporated into the DISC is low, pro-caspase-8 forms a dimer leading to cleavage of pro-caspase-8 to generate active caspase-8 (Lavrik and Krammer, 2012).

Although both Fas and TRAIL-R are potent inducers of cell death, they can also induce activation of NF-κB, but in a manner less efficiently than TNFR1. This is thought to occur through formation of a cytoplasmic “complex II” of which the core components are FADD, pro-caspase-8, RIPK1, cIAP1/2 and TRAF2 (Cullen et al., 2013; Henry and Martin, 2017; Jin and El-Deiry, 2006;
Lin et al., 2000; Varfolomeev et al., 2005). More recently, it has been shown that LUBAC is recruited to complex II downstream of TRAIL-R, where it catalyses the addition of linear M1-linked ubiquitin chains (Lafont et al., 2017). This subsequently permits recruitment and activation of the IKK complex. Whether this is the same mechanism by which Fas activates NF-κB remains uncertain. Nonetheless, it appears that both IKK and RIPK1 are important for complex II mediated NF-κB activation.

The IKK complex is known to directly repress RIPK1 kinase activity downstream of TNFR1 to prevent death of SP thymocytes (Dondelinger et al., 2015; Webb et al., 2019). Indeed, deletion of TNFR1 can almost completely restore normal SP thymocyte development in IKK1/2 deficient mice (Webb et al., 2016). While IKK and RIPK1 are downstream signalling components of death receptors such as Fas and TRAIL-R, a role for IKK to specifically restrain RIPK1 kinase activity has so far only been identified downstream of TNFR1. There are, however, several lines of evidence from my work suggesting that the IKK complex may indeed be repressing RIPK1 kinase activity downstream of other receptors. Firstly, inhibition of RIPK1 kinase provides a better rescue of activated F5 IKK2 deficient T cells from cell death than blockade of TNF signalling alone. Secondly, deletion of TNFR1 only partially rescues the peripheral lymphopenia in mice deficient in both A20 and IKK2. In contrast, kinase dead RIPK1 mediates a near complete rescue of peripheral naïve T cell numbers. Lastly, death of activated T cells lacking A20 and IKK2 is not rescued by ablation of TNFR1 but can be rescued with kinase dead RIPK1.

In this chapter, my aim was to understand if, in T cells, the IKK complex was required to restrain RIPK1 mediated cell death downstream of death receptors additional to TNFR1. To do this, we analysed T cells from mice with constitutive and inducible ablation of the IKK complex and compared the impact of Tnf and Casp8 deletion. We then explored which death receptors might trigger death of T cells in absence of the IKK complex.
6.2 Results

6.2.1 The IKK complex protects thymocytes and T cells from extrinsic apoptosis

We wanted to investigate whether the IKK complex controls cell death downstream of other death receptors, in addition to TNFR1. The activity of caspase-8 is essential for extrinsic apoptotic pathways (Varfolomeev et al., 1998). To more broadly investigate the extent to which the IKK complex protects T cells from extrinsic apoptosis pathways, regardless of the triggering receptor, we compared mice deficient in both IKK1 and IKK2 (IKK1/2ΔT\textsubscript{CD2}) to mice with additional Cre-mediated deletion of caspase-8 (IKK1/2Casp8ΔT\textsubscript{CD2}). We also analysed IKK1/2ΔT\textsubscript{CD2}Tnf\textsuperscript{f−/−} mice as a point of reference, since the rescue mediated by blocking TNF signalling has been previously defined (Webb et al., 2016). In these different strains, huCD2\textsuperscript{iCre} mediates gene deletion of IKK1 and IKK2 at the double negative 2 stage of thymocyte development, resulting in a complete block in IKK activity. SP thymocyte development in IKK1/2ΔT\textsubscript{CD2} mice was considerably disrupted since there was substantial reduction in the percentage of mature HSA\textsubscript{lo}CD62L\textsuperscript{hi}CD4\textsuperscript{+} and CD8\textsuperscript{+} SP thymocytes, as has been demonstrated previously (Fig. 6.1A) (Webb et al., 2016, 2019). Knockout of TNF appeared to restore normal CD4\textsuperscript{+} SP thymocyte development in IKK1/2ΔT\textsubscript{CD2} mice and significantly increased the percentage of mature HSA\textsubscript{lo}CD62L\textsuperscript{hi}CD8\textsuperscript{+} SP thymocytes, though this mature compartment did not reach levels of WT mice. In contrast, deletion of caspase-8 in IKK1/2ΔT\textsubscript{CD2} mice completely restored the percentage of CD4\textsuperscript{+} and CD8\textsuperscript{+} SP thymocyte subpopulations to WT levels.

In the periphery of IKK1/2ΔT\textsubscript{CD2} mice, there was a profound loss of naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, consistent with previous reports (Fig. 6.1B) (Webb et al., 2016, 2019). The shift in the CD4\textsuperscript{+} to CD8\textsuperscript{+} T cell ratio indicated a greater loss of naïve CD8\textsuperscript{+} T cells than naïve CD4\textsuperscript{+} T cells. Although there was clear evidence of an increased percentage of naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in IKK1/2ΔT\textsubscript{CD2}Tnf\textsuperscript{f−/−} mice, this remained far below levels observed in WT controls. In contrast to TNF ablation, deletion of caspase-8 resulted in a very
substantial increase in naïve CD4⁺ and CD8⁺ T cell frequencies and appeared to mediate a far stronger rescue of naïve T cells than TNF deficiency. Therefore, these data suggest that, in the thymus, TNF is the primary ligand responsible for the loss of CD4⁺ SP thymocytes in mice deficient in both IKK1 and IKK2. However, there were also indications that the IKK complex may be important for protecting mature CD8⁺ SP thymocytes and naïve T cells from extrinsic apoptosis that was not TNF driven since deletion of Casp8 permitted T cells in IKK1/2ΔTCD2 mice to accumulate to higher levels than TNF knockout.
A

Total

CD4+ SP thymocytes

CD8+ SP thymocytes

B

Live cells

CD4+ T cells

CD8+ T cells
Figure 6.1 The IKK complex protects thymocytes and T cells from extrinsic apoptosis

Flow cytometric analysis of Cre-ve (WT; n=4), IKK1/2ΔT^CD2 (Tnfr^-; n=2), IKK1/2ΔT^CD2 Tnfr^- (n=1), and IKK1/2Casp8ΔT^CD2 (n=4) mice. (A) Density plots are of CD4 vs CD8 by live thymocytes (top row) and HSA vs CD62L by gated CD4^+ SP thymocytes (middle row) and CD8^+ SP thymocytes (bottom row). Numbers indicate percentage of cells in each gate. (B) Density plots are of CD4 vs CD8 by live lymph node cells (top row) and CD25 vs CD44 by TCR^+CD4^+ cells (middle row) and TCR^+CD8^+ cells (bottom row). Numbers indicate percentage of cells in each gate.
6.2.2 Naïve T cells constitutively require the IKK complex for repression of extrinsic apoptosis

Our data strongly suggested that TNF ligand cannot fully account for triggering of extrinsic apoptosis pathways amongst new T cells entering the periphery from the thymus. We next assessed the role of the IKK complex in repression of extrinsic apoptosis specifically in fully mature naïve T cells. To do this, we analysed mice that permitted inducible ablation of IKK1 and IKK2 in CD4⁺ T cells (IKK1/2ΔTICD4). Floxed Ikk1 (Ikk1fxfx) and Ikk2 (Ikk2fxfx) alleles were deleted by a tamoxifen inducible CreERT under the control of the Cd4 locus (Cd4CreERT) (Śledzińska et al., 2013). In this way, the peripheral T cell compartment develops normally in these mice, avoiding the impact of IKK deficiency on thymocyte development. Tamoxifen was administered to mice for five consecutive days and mice were analysed on day 21. First, we asked if the IKK complex was required for naïve T cell survival. Tamoxifen treatment of IKK1/2ΔTiCD4 mice caused a skew in the CD4⁺ to CD8⁺ T cell ratio resulting from a profound reduction in the number of naïve CD4⁺ T cells compared to treated WT controls (Fig. 6.2A, B). The number of naïve CD8⁺ T cells was unchanged, indicating that deletion of IKK1/2 had not occurred in these cells.

Next, we asked whether the impaired survival of CD4⁺ naïve T cells in absence of IKK1/2 was due to a failure to regulate the extrinsic apoptotic pathway. To do this, we generated mice with inducible deletion of IKK1, IKK2 and caspase-8 (IKK1/2Casp8ΔTICD4). 21 days after the first tamoxifen injection, these mice had significantly more peripheral naïve CD4⁺ T cells than IKK1/2ΔTICD4 mice, but still fewer than in treated WT controls (Fig. 6.2A, B). This suggested that defective control of extrinsic apoptosis could, in part, account for the reduced naïve CD4⁺ T cell survival in mice following induced loss of IKK1/2. Next, we wanted to understand the extent to which TNF was responsible for triggering death of IKK1/2 deficient naïve CD4⁺ T cells. To investigate this, we crossed the IKK1/2ΔTICD4 strain with Tnf⁻/⁻ mice (IKK1/2ΔTICD4Tnf⁻/⁻). Following tamoxifen treatment, there was a substantial loss of naïve CD4⁺ T cells compared to treated Tnf⁻/⁻ controls, and similar to that observed in IKK1/2ΔTICD4 mice (Fig. 6.2A, B).
To best assess whether receptors other than TNFR1 were triggering death of IKK1/2 deficient naïve CD4^{+} T cells, we compared the impact of TNF and caspase-8 deletion in IKK1/2ΔT^{CD4} mice. Since there was a modest difference in total naïve T cell numbers between WT and Tnf^{-/-} mice, we first normalised the T cell numbers of IKK1/2ΔT^{CD4} strains to their respective controls. Ablation of caspase-8 in IKK1/2ΔT^{CD4} mice rescued peripheral naïve CD4^{+} T cell numbers from ~25% to ~60% of control numbers (Fig. 6.2C). However, absence of TNF had no effect on naïve CD4^{+} T cell numbers in IKK1/2ΔT^{CD4} mice.

Therefore, this data suggests that the IKK complex is crucial for naïve T cell survival and functions partly to repress extrinsic apoptosis induced by receptors other than, or in addition to, TNFR1.
Figure 6.2 Naïve T cells constitutively require the IKK complex for repression of extrinsic apoptosis

Cre-ve (WT, n=11), IKK1/2ΔT\textsuperscript{ICD4} (n=5), IKK1/2Casp8ΔT\textsuperscript{ICD4} (n=12), Tnf\textsuperscript{−/−} (n=6) and IKK1/2ΔT\textsuperscript{ICD4} Tnf\textsuperscript{−/−} (n=13) mice were treated I.P with tamoxifen for five consecutive days. 21 days after the first injection, mice were analysed by flow cytometry.

(A) Density plots are of CD4 vs CD8 by live lymph node T cells (top row) and CD25 vs CD44 by TCR\textsuperscript{+}CD4\textsuperscript{+} cells (middle row) and TCR\textsuperscript{+}CD8\textsuperscript{+} cells (bottom row). Numbers indicate percentage of cells in each gate.

(B) The total number of naïve (CD44\textsuperscript{lo}CD25\textsuperscript{−}) CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from the lymph nodes and spleens of the indicated strains.

(C) The total numbers of naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from the lymph nodes and spleens of the indicated strains plotted as a proportion of the controls. Ratio of numbers from IKK1/2ΔT\textsuperscript{ICD4} and IKK1/2Casp8ΔT\textsuperscript{ICD4} to WT controls and of IKK1/2ΔT\textsuperscript{ICD4}Tnf\textsuperscript{−/−} to Tnf\textsuperscript{−/−} controls.

Data is pooled from 7 independent experiments. Error bars indicate SD.
6.2.3 Naïve T cells depend on IKK signalling for normal IL-7R expression

It was clear that sensitivity to extrinsic apoptosis could only partly account for the impaired survival of IKK1/2 deficient naïve T cells. Absence of IKK1 and IKK2 in T cells also leads to a complete block in canonical NF-κB signalling. Previous work has shown that new T cells require canonical NF-κB signalling for peripheral survival through upregulation of IL-7R (Silva et al., 2014). However, inducible deletion of IKK2, thereby impairing but not completely ablating canonical NF-κB, does not affect IL-7R expression by naïve F5 T cells, nor their peripheral survival (Silva et al., 2014). Since this earlier study only imposed a partial block in IKK function by deleting Ikk2, we asked whether naïve T cells constitutively required the complete IKK complex for maintenance of IL-7R expression. To address this, we measured IL-7R on naïve T cells from IKK1/2ΔTiCD4 strains and normalised this to the respective controls. Tamoxifen treatment of IKK1/2ΔTiCD4 mice caused a ~40% decrease in IL-7R expression by naïve CD4+ T cells (Fig. 6.3). There were similar decreases in IL-7R on naïve CD4+ T cells from IKK1/2Casp8ΔTiCD4 and IKK1/2ΔTiCD4Tnf−/− mice compared to controls. As a control, we observed that naïve CD8+ T cells from all strains showed normal IL-7R expression after tamoxifen treatment, further indicating that IKK1/2 deletion was restricted to CD4+ T cells.

Together, these data suggest that naïve T cells require tonic IKK induced NF-κB signalling in order to maintain expression of IL-7R. The loss of IL-7R expression by IKK1/2 deficient naïve CD4+ T cells may also account for the impaired survival in vivo.
Figure 6.3 Naïve T cells depend on IKK signalling for normal IL-7R expression

Cre-ve (WT, n=11), IKK1/2ΔTICD4 (n=5), IKK1/2Casp8ΔTICD4 (n=12), Tnf−/− (n=6) and IKK1/2ΔTICD4 Tnf−/− (n=13) mice were treated I.P with tamoxifen for five consecutive days. 21 days after the first injection, mice were analysed by flow cytometry. Bar plots show the IL-7R MFI by live lymph node naïve CD4+ (left) and CD8+ (right) T cells of the indicated strains as a fraction of the IL-7R MFI by controls analysed at the same time. Ratio of IKK1/2ΔTICD4 and IKK1/2Casp8ΔTICD4 to WT controls and of IKK1/2ΔTICD4 Tnf−/− to Tnf−/− controls. Data is pooled from 7 independent experiments. Error bars indicate SD.
6.2.4 Blocking extrinsic apoptosis does not rescue IKK1/2 deficient regulatory or memory T cell populations

Next, we wanted to understand if constitutive IKK expression was required for normal homeostasis of other mature peripheral CD4+ T cell subpopulations. We first assessed the impact of IKK ablation on FoxP3+CD25+ Tregs. The IKK complex is important for the thymic generation of FoxP3+CD25+ Tregs but it remains unclear if mature peripheral FoxP3+CD25+ Tregs continue to require IKK signalling (Schmidt-Supprian et al., 2003; Webb et al., 2016). Tamoxifen treatment of IKK1/2ΔTiCD4 mice caused a ~50% reduction in the number of FoxP3+CD25+ Tregs after 21 days compared to treated controls (Fig. 6.4A, B). There was also a similar decrease in the number of FoxP3+CD25+ Tregs upon ablation of TNF or caspase-8 in IKK1/2ΔTiCD4 mice.

Similarly, it was uncertain whether CD4+ memory subsets constitutively required IKK signalling. 21 days after the first tamoxifen injection, there was a significant loss of both Tcm and Tem cells in IKK1/2ΔTiCD4, IKK1/2Casp8ΔTiCD4 and IKK1/2ΔTiCD4 Tnf−/− mice compared to controls (Fig. 6.4C, D). But it appeared that ablation of TNF or caspase-8 had no effect on both CD4+ memory T cell populations in IKK1/2ΔTiCD4 mice (Fig. 6.4E).

Together, this data suggests that the IKK complex is a continued requirement for normal homeostasis of peripheral FoxP3+CD25+ Tregs and CD4+ memory T cells. However, it remains unclear if the IKK complex functions to control extrinsic apoptosis in these T cell populations.
Figure 6.4 Blocking extrinsic apoptosis did not rescue IKK1/2 deficient regulatory or memory T cell populations

Cre -ve (WT, n=11), IKK1/2ΔT^{CD4} (n=5), IKK1/2Cas8ΔT^{CD4} (n=12), Tnf^{-/-} (n=6) and IKK1/2ΔT^{CD4} Tnf^{-/-} (n=13) mice were treated I.P with tamoxifen for five consecutive days. 21 days after the first injection, mice were analysed by flow cytometry.

(A) Density plots of FoxP3 vs CD25 by live lymph node TCR^{+}CD4^{+} cells. Numbers indicate percentage of cells in each gate.

(B) The total number of FoxP3^{+}CD25^{+} Tregs from the lymph nodes and spleens of the indicated strains (left) and as a proportion of controls (right). Ratio of numbers from IKK1/2ΔT^{CD4} and IKK1/2Cas8ΔT^{CD4} to WT controls and of IKK1/2ΔT^{CD4} Tnf^{-/-} to Tnf^{-/-} controls.

(C) Density plots are of CD62L vs CD44 by live lymph node TCR^{+}CD4^{+}CD25^{-} cells. Numbers indicate percentage of cells in each gate.

(D) The total number of CD4^{+} T_{cm} (CD44^{hi}CD62^{hi}) and T_{em} (CD44^{hi}CD62L^{lo}) cells from the lymph nodes and spleens of the indicated strains.

(E) The total number of CD4^{+} T_{cm} and T_{em} cells from the lymph nodes and spleens of the indicated strains plotted as a proportion of the controls. Ratio of numbers from IKK1/2ΔT^{CD4} and IKK1/2Cas8ΔT^{CD4} to WT controls and of IKK1/2ΔT^{CD4} Tnf^{-/-} to Tnf^{-/-} controls.

Data is pooled from 7 independent experiments. Error bars indicate SD.
6.2.5 Activated F5 T cells express multiple death receptors

Our cumulative data suggested that TNFR1 signalling could not solely account for triggering cell death of naïve T cells in absence of the IKK complex since ablation of caspase-8 gives a better rescue of IKK1/2 deficient T cells than knockout of TNF. Death receptors of the TNFR superfamily are capable of inducing apoptosis through activation of caspase-8. To identify potential candidate death receptors that trigger cell death when IKK activity is lost, we first investigated the expression of death receptors by T cells. Since we also found evidence of TNF independent but RIPK1 dependent cell death of activated F5 lkk2ΔTCD2 T cells, we asked if other death receptors were expressed that may be responsible for triggering cell death. Activated F5 CTRL T cells expressed Tnfrsf1a (TNFR1), Fas, Tnfrsf10b (TRAIL-R), Tnfrsf25 (DR3) and Tnfrsf21 (DR6) (Fig. 6.5). However, while Fas, Tnfrsf1a and Tnfrsf10b showed relatively high expression, nFPKM of Tnfrsf25 and Tnfrsf21 were much lower. Activated F5 lkk2ΔTCD2 T cells showed similar expression of death receptors, except for a small decrease in Tnfrsf10b. Therefore, T cells express several death receptors, which could have a role in triggering death in absence of IKK activity.
Figure 6.5 Activated F5 T cells express multiple death receptors

Analysis of RNA-seq data comparing activated F5 Ikk2ΔTCD2 and F5 CTRL T cells. Gene expression displayed as normalised fragments per kilobase exons per million fragments (nFPKM).
6.2.6 The IKK complex protects T cells from FasL induced cell death

Of the five death receptors investigated, Fas showed the highest expression in activated F5 T cells. Two forms of Fas ligand (FasL) are able to bind Fas receptors: membrane-bound FasL (mFasL) and soluble FasL (sFasL). mFasL and sFasL are thought to have competing and opposing functions since mFasL is a potent inducer of cell death, whereas there is evidence that sFasL triggers non-apoptotic signalling pathways, including NF-κB in cell lines (Ahn et al., 2001; O’ Reilly et al., 2009; Xiao et al., 2002), and may also act as a competitive inhibitor of mFasL (Hohlbau et al., 2000; Suda et al., 1997; Tanaka et al., 1998). Therefore, we asked whether the IKK complex could protect T cells from cell death downstream of Fas. First, we wanted to test if sFasL could induce death of T cells. To do this, we cultured T cells with increasing concentrations of sFasL for 24 hrs. Naïve CD4+ T cells were resistant to sFasL induced death while there was some death of naïve CD8+ T cells but only at the highest concentrations of sFasL used (Fig. 6.6A). Next, we asked if the IKK complex was protecting T cells from sFasL induced death. IKK2i was added to cultures to induce a partial block in IKK activity. In the presence of IKK2i, there was an increased percentage of dead naïve CD4+ and CD8+ T cells at the two highest concentrations of sFasL. Since partial inhibition of the IKK complex suggested a protective function following sFasL stimulation, we probed this further by inducing a complete block in IKK activity in T cells. To do this, we utilised mice with T cell specific deletion of IKK1 (Ikk1ΔTCD2). Loss of IKK1 has no effect on T cell development in this strain (Chen et al., 2015; Webb et al., 2019). Naïve T cells from Ikk1ΔTCD2 mice were almost completely resistant to sFasL induced death, showing only a slight increase in death at the highest sFasL concentration. However, in the presence of IKK2i, that in combination with genetic ablation of Ikk1, induced a complete block in IKK activity, naïve T cells were profoundly sensitised to sFasL induced cell death. Increased death of both naïve CD4+ and CD8+ T cells was apparent at lower concentrations of sFasL and almost all cells had died at the highest concentrations.
Since these results suggested that the IKK complex was important for protecting T cells from sFasL induced death, we next asked whether this was mediated by suppressive regulation of RIPK1 activity. The addition of Nec-1 to cultures to inhibit RIPK1 kinase activity almost completely rescued naïve T cells from sFasL induced death upon IKK blockade.

In the absence of IKK, sFasL induced death of T cells depended on RIPK1 kinase activity. However, the classical apoptotic pathway induced by mFasL does not require RIPK1 kinase activity (Geserick et al., 2009; Ting et al., 1996). Therefore, we asked whether the IKK complex was controlling an additional RIPK1 dependent cell death pathway downstream of Fas. To test this, we cultured T cells from Ikk1ΔTCD2 mice with increasing concentrations of aggregated FasL (aFasL) for 24 hrs. FLAG-tagged FasL is cross-linked at the cell membrane by anti-FLAG antibody (enhancer) to mimic the action of mFasL. This efficiently induced apoptosis of naïve CD4+ and CD8+ T cells and at the highest concentrations of aFasL, almost all cells had died (Fig. 6.6B). Nec-1 had no effect on cell death, confirming that this was independent of RIPK1 kinase activity. Adding IKK2i to the culture, thereby causing a complete block in IKK activity, profoundly sensitised naïve T cells to death at lower concentrations of aFasL. Inhibiting RIPK1 kinase activity with Nec-1 partially rescued naïve T cells from cell death but they showed a similar sensitivity to aFasL induced death as naïve T cells cultured without IKK2i.

Together, this data suggests that the IKK complex protects T cells from FasL induced cell death by controlling RIPK1 kinase activity. This FasL induced RIPK1 dependent cell death pathway appears additional to the classical RIPK1 kinase independent apoptotic pathway.
Figure 6.6 The IKK complex protects T cells from FasL induced cell death

(A) Lymph node cells were isolated from Ikk1ΔTCD2 and Cre -ve (WT) mice and cultured with different concentrations of FLAG-FasL (sFasL) for 24 hrs with or without PBS, 10 μM IKK2i and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.

(B) Lymph node cells were isolated from Ikk1ΔTCD2 mice and cultured with different concentrations of FLAG-FasL, and a fixed concentration of cross-linking enhancer, for 24 hrs with or without PBS, 10 μM IKK2i and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.

Data is pooled from 2 (A) independent experiments. Error bars indicate SEM.
Naïve T cells are resistant to TRAIL induced death in absence of IKK signalling

Analysis of death receptor expression by activated F5 CTRL T cells revealed that Tnfrsf10b (TRAIL-R) was highly expressed. TRAIL-R can activate NF-κB through recruitment of the IKK complex (Lafont et al., 2017; Lin et al., 2000). Consequently, we wanted to understand if the IKK complex may also be important in protecting T cells from TRAIL induced death. To address this, we cultured T cells from Ikk1ΔTCD2 mice with increasing concentrations of TRAIL. Naïve CD4+ and CD8+ T cells were apparently resistant to TRAIL induced death upon addition of IKK2i to completely block IKK activity (Fig. 6.7).
Figure 6.7 Naïve T cells are resistant to TRAIL induced death in absence of IKK signalling

Lymph node cells were isolated from \( lkk1\Delta T^{CD2} \) mice and cultured with different concentrations of TRAIL for 24 hrs with or without PBS, 10 µM IKK2i, and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.
6.2.8 Naïve CD8+ T cells require A20 to limit FasL induced cell death

Alongside the IKK complex, A20 also has a critical role in constraining RIPK1 kinase activity in T cells. Since Fas can trigger RIPK1 dependent cell death, we asked if A20 can protect T cells from FasL induced cell death. To test this, we first cultured T cells from Tnfaip3ΔTCD4 mice with increasing concentrations of sFasL. At the highest concentration of sFasL used, there was an increased percentage of dead naïve CD4+ and CD8+ T cells compared to WT controls (Fig. 6.8). It appeared that naïve CD8+ T cells were more sensitive to sFasL induced death than CD4+ T cells in absence of A20. Culturing cells with aFasL, to aggregate the Fas receptor, also induced death of naïve CD8+ T cells at lower concentrations compared to WT controls. Next, we wanted to understand if A20 protected T cells from FasL induced cell death by controlling RIPK1 kinase activity. However, blocking RIPK1 kinase activity with Nec-1 had no effect on cell death. Therefore, this suggests that A20 protects T cells, particularly CD8+ naïve T cells, from FasL induced cell death but via a RIPK1 independent mechanism.
Figure 6.8 Naïve CD8+ T cells require A20 to limit FasL induced cell death

Lymph node cells were isolated from Tnfaip3ΔTCD4 and Cre -ve (WT) mice and cultured with different concentrations of FLAG-FasL (sFasL) for 24 hrs with or without Nec-1 (top row). This was repeated in the presence of fixed concentrations of cross-linking enhancer (bottom row). Viability (% dead cells) of naïve T cells was determined by flow cytometry using a LIVE/DEAD dye.
6.2.9 A20 restricts FasL induced death of naïve T cells by controlling RIPK1 kinase activity

Disrupting IKK activity in naïve T cells had revealed that A20 was important for protection against RIPK1 dependent cell death in vivo, in absence of TNFR1. Since we had now identified a role for the IKK complex in controlling RIPK1 kinase activity downstream of Fas, we questioned whether A20 could also be required by T cells for optimal control of RIPK1 kinase activity by IKK downstream of Fas. To address this, we stimulated T cells from Tnfaip3ΔTCD4Tnfrsf1a−/− mice with sFasL in the presence of increasing concentrations of IKK inhibitor for 24 hrs. As A20 deficient naive CD8+ T cells were sensitive to RIPK1 independent death at the concentration of sFasL used, we first focused on naïve CD4+ T cells. At high concentrations of IKK2i, to partially block IKK activity, there was increased cell death of naïve CD4+ T cells from Tnfrsf1a−/− mice upon sFasL stimulation (Fig. 6.9A). But the addition of Nec-1 to cultures, to block RIPK1 kinase activity, rescued the T cells from cell death. In contrast, naïve CD4+ T cells from Tnfaip3ΔTCD4Tnfrsf1a−/− mice were sensitive to sFasL induced death at lower concentrations of IKK2i. At the highest concentrations of IKK2i used, sFasL had triggered death of almost all T cells. To understand if the increased sensitivity to sFasL induced death in A20 deficient T cells was due to a failure to control RIPK1, we added Nec-1 to cultures. Blocking RIPK1 kinase activity using Nec-1 almost completely rescued T cells from cell death.

To further validate that A20 was protecting T cells from sFasL induced RIPK1 dependent cell death, we stimulated T cells from Tnfaip3ΔTCD4Tnfrsf1a−/− mice with sFasL when IKK activity was completely blocked using IKK16. Since IKK16 induces death of almost all T cells at the highest concentration used, and to account for the sFasL induced RIPK1 independent death of A20 deficient T cells, we normalised cell death from 0 to 100%. At increasing concentrations of IKK16, sFasL stimulation led to increased cell death of naïve CD4+ and CD8+ T cells from Tnfrsf1a−/− mice (Fig. 6.9B). Nec-1 rescued the naïve T cells from death to the level of unstimulated controls. However, sFasL induced death of naïve T cells from Tnfaip3ΔTCD4Tnfrsf1a−/− mice at significantly lower IKK16 concentrations. The
addition of Nec-1 almost completely rescued the naïve T cells from sFasL induced cell death.

Since IKK inhibition revealed that A20 was also important for protecting naïve T cells from sFasL induced death, we wanted to assess the level of IKK activity required to protect A20 deficient T cells from sFasL triggered death. To do this, we calculated the LD50 concentrations of IKK16 in the presence and absence of sFasL. Compared to the unstimulated control, sFasL stimulation of naïve CD4+ T cells from *Tnfrsf1a*−/− mice resulted in a ~50% reduction in the LD50 of IKK16 (Fig. 6.9C). The IKK16 LD50 concentration for unstimulated naïve CD4+ T cells from *Tnfaip3ΔT*CD4* Tnfrsf1a*−/− mice was similar to sFasL treated cells from *Tnfrsf1a*−/− mice. However, stimulation of naïve CD4+ T cells from *Tnfaip3ΔT*CD4* Tnfrsf1a*−/− mice with sFasL resulted in a substantial drop in the IKK16 LD50 concentration. The addition of Nec-1 to sFasL stimulated cultures increased the LD50 of IKK16 to a similar concentration as for unstimulated controls. Altogether, for naïve CD4+ T cells from *Tnfrsf1a*−/− mice, sFasL stimulation led to a ~2 fold reduction in the IKK16 LD50, whereas there was a ~4 fold reduction for T cells from *Tnfaip3ΔT*CD4* Tnfrsf1a*−/− mice.

Compared to naïve CD4+ T cells, the IKK16 LD50 concentrations for unstimulated naïve CD8+ T cells from *Tnfrsf1a*−/− mice were slightly lower. But there was a similar ~2 fold reduction in the IKK16 LD50 concentration upon sFasL stimulation (Fig. 6.9D). However, for CD8+ naïve T cells from *Tnfaip3ΔT*CD4* Tnfrsf1a*−/− mice there was a ~3 fold decrease in the IKK16 LD50 concentration upon sFasL stimulation compared to unstimulated controls. It did appear however that the IKK16 LD50 was higher in the presence of Nec-1 compared to unstimulated cells.

Collectively, our data suggests that naïve CD4+ and CD8+ T cells needed greater IKK activity in absence of A20 to regulate RIPK1 kinase activity and protect from sFasL induced cell death. Therefore, this data suggests that A20 controls RIPK1 kinase activity downstream of Fas in T cells.
A

**Tnfrsf1a**

- PBS
- Nec-1
- sFasL
- sFasL + Nec-1

B

**Tnfrsf1a**

- Naive CD4+ T cells
- Naive CD8+ T cells

- PBS
- Nec-1
- sFasL
- sFasL + Nec-1

C

Naive CD4+ T cells

D

Naive CD8+ T cells
Figure 6.9 A20 restricts FasL induced death of naïve T cells by controlling RIPK1 kinase activity

(A) Lymph node cells were isolated from Tnfaip3ΔTCD4Tnfrsf1a⁻/⁻ and Tnfrsf1a⁻/⁻ mice and cultured with different concentrations of IKK2i for 24 hrs with or without PBS, 30 ng/ml FLAG-FasL (sFasL) and Nec-1. Viability (% dead cells) of naïve CD4⁺ T cells was determined by flow cytometry using a LIVE/DEAD dye.

(B) Lymph node cells were isolated from Tnfaip3ΔTCD4Tnfrsf1a⁻/⁻ and Tnfrsf1a⁻/⁻ mice and cultured with different concentrations of IKK16 for 24 hrs with or without PBS, 30 ng/ml FLAG-FasL (sFasL) and Nec-1. Viability (% dead cells) of naïve T cells was determined by flow cytometry, using a LIVE/DEAD dye, and normalised from 0 to 100%.

(C) IKK16 LD50 values for naïve CD4⁺ T cells from experiment shown in (B) (left). Ratio of IKK16 LD50 for PBS treated cultures vs IKK16 LD50 for sFasL and Nec-1 treated cultures (right).

(D) IKK16 LD50 values for naïve CD8⁺ T cells from experiment shown in (B) (left). Ratio of IKK16 LD50 for PBS treated cultures vs IKK16 LD50 for sFasL and Nec-1 treated cultures (right).

Error bars indicate 95% confidence intervals (C, D).
6.3 Discussion

The IKK complex is essential for repression of cell death downstream of TNFR1. Activation of NF-κB by the IKK complex induces the expression of pro-survival proteins. But IKK also phosphorylates and inhibits RIPK1 kinase to prevent complex II formation (Dondelinger et al., 2015, 2019). For SP thymocytes, NF-κB is dispensable for normal survival which instead relies upon IKK repression of TNF induced, RIPK1 dependent cell death (Webb et al., 2016, 2019). Here, we investigated whether the IKK complex also protects T cells from cell death induced by death receptors additional to TNFR1.

We show that the primary function of IKK for normal thymocyte development is the inhibition of TNF induced cell death, with limited involvement of other extrinsic apoptotic pathways. SP thymocyte development is blocked in absence of the IKK complex but ablation of TNF production is sufficient to restore normal survival of CD4⁺ SP thymocytes and partially rescues CD8⁺ SP thymocytes. This mirrors the phenotype of IKK1/2 deficient mice with deletion of Tnfrsf1a, suggesting that TNF appears to be inducing cell death principally via binding to TNFR1 (Webb et al., 2016). However, SP thymocyte development appears normal in IKK1/2 deficient mice with ablation of caspase-8. Since deletion of Casp8 gave a better rescue of IKK1/2 deficient CD8⁺ SP thymocytes than Tnf knockout, this suggests that death receptors additional to TNFR1 are triggering death in absence of the IKK complex.

RIPK1 kinase dead can completely restore CD4⁺ but not CD8⁺ SP thymocyte development in absence of IKK (Webb et al., 2019). Viewed in consideration with our data, this suggests that the regulation of extrinsic cell death pathways by IKK differs between CD4⁺ and CD8⁺ SP thymocytes. While IKK inhibition of RIPK1 downstream of TNFR1 is sufficient for normal CD4⁺ SP thymocyte survival in vivo, the survival of CD8⁺ SP thymocytes necessitates IKK control of both RIPK1 dependent and independent cell death mechanisms induced by multiple death receptors.
IKK repression of extrinsic apoptotic pathways separate to TNF induced death signalling appears to be crucial for survival of mature T cells. Constitutive deletion of IKK1/2 in thymocytes caused a profound decrease in peripheral T cells. Deletion of casp8 permitted T cells to accumulate to far higher levels in the periphery than ablation of TNF production, which in the case of CD4+ T cells occurred despite normal thymocyte development in both. Inducible ablation of IKK1/2 specifically in mature T cells caused a substantial loss of naïve T cells which could be rescued by deletion of Casp8 but not Tnf. This indicates that peripheral maintenance of mature T cells requires the IKK complex for repression of extrinsic apoptosis. In contrast to SP thymocytes, it appears that TNF has a less distinct role as a cell death trigger of mature T cells in absence of the IKK complex. Instead, there is likely to be redundancy between death receptors to induce death of mature T cells when IKK signalling is aberrant.

Sensitivity to extrinsic apoptosis could only partly account for the loss of mature T cells following inducible deletion of the IKK complex. IL-7R expression by T cells is important for normal peripheral homeostasis. Previous work has shown canonical NF-κB to be critical for IL-7R upregulation by T cells exiting the thymus (Silva et al., 2014; Webb et al., 2019). We show that inducible ablation of IKK1/2 in mature T cells, thereby causing a complete block in NF-κB activation, leads to a reduction in IL-7R expression. Thereby, tonic IKK signalling is required by mature T cells for normal expression of IL-7R. But an earlier study in which Ikk2 was inducibly deleted by R26RCreERT2 after tamoxifen administration, impairing NF-κB activation, did not observe reduced IL-7R expression by naïve T cells (Silva et al., 2014). This may be because T cells were analysed at a later point after tamoxifen injection than our study, thus potentially allowing more time for alternative mechanisms to restore normal IL-7R expression. Or it could indicate that IKK2 is dispensable for maintenance of IL-7R expression by naïve T cells. Thus, perhaps relatively less NF-κB activation is required for normal IL-7R expression by mature T cells compared to the initial upregulation of IL-7R by new T cells, which is defective in absence of IKK2 (Silva et al., 2014).
IKK expression by FoxP3+ Tregs and memory T cells also appeared to be important for peripheral maintenance. Inducible ablation of IKK1/2 caused a substantial drop in these T cell populations. However, neither deletion of Tnf or Casp8 gave any rescue, suggesting that other IKK functions were more important than repression of extrinsic apoptosis for maintenance FoxP3+ Tregs and memory T cells. Since canonical NF-κB is vital for generation of these T cell populations, it is likely that they continue to require NF-κB for normal maintenance (Isomura et al., 2009; Long et al., 2009b; Oh et al., 2017b; Ruan et al., 2009a; Webb et al., 2019; Zheng et al., 2003). In line with this view, inducible ablation of c-Rel or c-Rel/RelA specifically in mature FoxP3+ Tregs leads to a reduced percentage of Tregs in the spleen (Oh et al., 2017b). It is thought that c-Rel is required by mature Tregs for maintenance of FoxP3 expression (Oh et al., 2017b). Whether IKK controls extrinsic apoptosis in FoxP3+ Tregs and memory T cells cannot be determined from our data.

There are multiple death receptors which can trigger cell death by activating caspase-8. The mechanism by which the IKK complex regulates death downstream of TNFR1 is well characterised, but little is known about how IKK may be functioning downstream other death receptors. Here, we have revealed that the IKK complex regulates the Fas apoptotic pathway in T cells. T cells were largely resistant to soluble FasL (sFasL), which only weakly induced death of CD8+ T cells at high non-physiological concentrations. This is likely because sFasL fails to sufficiently aggregate the Fas receptor to facilitate formation of the DISC (Holler et al., 2003; Jang et al., 2003; Schneider et al., 1998). However, partial inhibition of the IKK complex was sufficient to sensitise T cells to sFasL induced cell death while complete IKK inactivation caused substantial T cell death. Our data suggests that the IKK complex actively protects T cells from FasL induced cell death. Whether FasL contributes to death of IKK1/2 deficient T cells in vivo remains to be determined. However, lpr/gld mice which have defective Fas signalling exhibit lymphoproliferation suggesting it does have a physiological role for T cells in vivo (Cohen and Eisenberg, 1991).
Our data suggests that IKK protects T cells from FasL induced cell death by regulating RIPK1. Blocking RIPK1 kinase activity could almost completely rescue T cells from sFasL induced cell death when IKK activity was inhibited. But, as is well known with other cell types, RIPK1 kinase activity is not required for the classical Fas apoptotic pathway in T cells (Geserick et al., 2009; Ting et al., 1996). Clustering the Fas receptor by cross linking FasL at the membrane robustly induces DISC formation (Holler et al., 2003; Huang et al., 1999; Jang et al., 2003; Schneider et al., 1998) and triggered death of T cells which was not rescued by blocking RIPK1 kinase activity. The RIPK1 dependent cell death triggered by sFasL in the absence of IKK activity appeared to be distinct to the classical Fas apoptotic pathway. At concentrations of sFasL which were seemingly insufficient to induce DISC formation, blockade of IKK caused RIPK1 dependent cell death. Additionally, clustering the Fas receptor induced death of T cells more potently than sFasL in the absence of IKK activity. But death could not be completely rescued when RIPK1 was inhibited. Therefore, this suggests that the RIPK1 dependent cell death pathway triggered by FasL and controlled by IKK is independent of the DISC.

The nature of this RIPK1 dependent cell death pathway downstream of Fas is currently unclear. Previous work using cell lines has only suggested a role for RIPK1 kinase activity during FasL induced caspase-8 independent necroptosis following depletion of cIAP1/2 or inhibition of caspase-8 or TRAF2 (Geserick et al., 2009; Holler et al., 2000; Karl et al., 2014). However primary naïve T cells are not sensitive to necroptosis because they do not express the effector protein MLKL (Webb et al., 2019). Thus, IKK is likely to be regulating a RIPK1 dependent apoptotic pathway. In addition to assembly of the DISC, FasL interaction with Fas also induces formation of a cytoplasmic complex II (Geserick et al., 2009; Lavrik et al., 2008). This consists of FADD, pro-caspase-8, RIPK1 and c-FLIP and is thought to be responsible for inducing NF-κB activation (Cullen et al., 2013). If and how the IKK complex interacts with complex II has not be demonstrated. However, a similar complex forms downstream of TRAIL-R and has been shown to recruit the IKK complex (Henry and Martin, 2017; Lafont et al., 2017;
Varfolomeev et al., 2005). This raises the possibility that the IKK complex phosphorylates and inhibits RIPK1 activity within this complex, in a similar fashion to that which occurs in complex I downstream of TNFR1 (Dondelinger et al., 2015, 2019). In absence of IKK activity, RIPK1 may permit further recruitment of FADD and caspase-8 to complex II and subsequently lead to apoptosis.

Despite a number of similarities between the Fas and TRAIL-R death pathways, we could not demonstrate a role for the IKK complex in regulating TRAIL induced cell death. T cells were completely resistant to TRAIL induced cell death with and without IKK inhibition. One reason for this may be because naïve T cells express very low levels of TRAIL-R (Mariani and Krammer, 1998; Zhang et al., 2003). Therefore, TRAIL is unlikely to be contributing to death of IKK1/2 deficient naïve T cells in vivo. The expression of TRAIL-R is upregulated during T cell activation, and as such, it may be more appropriate to explore whether IKK regulates TRAIL induced death in activated T cells.

We previously established that A20 fine tunes the regulation of RIPK1 by the IKK complex in T cells. But deletion of Tnfrsf1a only partially rescued peripheral T cells in mice deficient in both A20 and IKK2. Additional ablation of RIPK1 kinase activity further restored the peripheral T cell compartment. Here, we show that control of the FasL induced RIPK1 dependent cell death pathway by the IKK complex is perturbed in the absence of A20. Compared to WT T cells, the threshold of IKK inhibition required to sensitisise A20 deficient T cells to sFasL induced cell death was substantially reduced. Inhibition of RIPK1 completely rescued T cells from sFasL triggered cell death. Thus, T cells required A20 for optimal control of the FasL induced RIPK1 dependent cell death pathway by the IKK complex. Further work is required to establish if A20 is regulating RIPK1 downstream of Fas and TNFR1 by a similar mechanism.

In addition to regulating RIPK1 triggered cell death, we also found evidence that A20 protected T cells from FasL induced cell death by a distinct RIPK1
independent mechanism. A20 deficiency sensitised CD8+ T cells in particular, to both soluble and crosslinked FasL induced cell death. Importantly, blocking RIPK1 kinase activity did not rescue the CD8+ T cells from death, suggesting death triggered by Fas in the absence of A20 was RIPK1 independent. This suggests that A20 also regulates Fas triggered cell death pathways in a manner distinct from the IKK complex. It is possible such a mechanism is also responsible for the cell death we observed amongst activated A20 deficient T cells (chapter 3). Further work is required to understand the mechanism by which A20 controls FasL induced cell death. But earlier studies have suggested that A20 can post-translationally regulate caspase-8. Within the DISC formed by TRAIL-R, caspase-8 is polyubiquitinated by cullin-3 (Jin et al., 2009). This permits subsequent binding to p62 which acts to stabilise active caspase-8 in the cytosol and facilitate cell death. A20 is thought to deubiquitinate caspase-8 and thereby dampen caspase-8 activity (Jin et al., 2009). Whether such activity of A20 occurs in T cells downstream of Fas remains uncertain.
Chapter 7 - Final Discussion

The aim of this thesis was to understand the role of IKK signalling in T cells. It has long been established that the IKK complex is crucial for activation of NF-κB (Li et al., 1998; Mercurio et al., 1997; Zandi et al., 1998). But for some time, it was difficult to analyse the function of the IKK complex in T cells since knockout of any IKK component is sufficient to cause premature lethality (Hu et al., 1999; Li et al., 1999a, 1999b, 2000, 1999c; Rudolph et al., 2000; Takeda et al., 1999; Tanaka et al., 1999). This changed with the advent of conditional knockout strains. Tissue specific gene deletion in the T lineage has demonstrated important roles for IKK in the development, homeostasis, and function of T cells (Greve et al., 2007; Schmidt-Supprian et al., 2003, 2004c; Silva et al., 2014; Webb et al., 2016). Nonetheless, findings from these studies were interpreted in the context of NF-κB activation. It’s now clear that the IKK complex has many other functions in addition to activation of NF-κB. Indeed, IKK was identified as an important regulator of TNF induced cell death independently of NF-κB (Dondelinger et al., 2015). It is this function of IKK, not NF-κB activation, which is crucial for thymocyte development (Webb et al., 2019). In light of this, our goal was to understand if IKK repression of cell death was also important in peripheral T cells. To address this, we used a variety of constitutive and inducible mouse strains lacking IKK and other TNFRSF signalling components. Here, we have identified IKK and A20 as important regulators of cell death in T cells. We demonstrate that IKK and A20 function together to control both RIPK1 dependent and independent cell death pathways downstream of multiple death receptors in thymocytes, naïve T cells and activated T cells.

7.1 Repression of extrinsic apoptosis by IKK and A20 is essential for SP thymocyte development

The IKK complex is crucial for normal thymocyte development since IKK1/2 deficient mice exhibit a block at the mature SP stage (Webb et al., 2016, 2019). This is not due to impaired NF-κB activation since mice lacking all
three canonical NF-κB subunits show normal thymocyte development (Webb et al., 2019). Instead, IKK is thought to protect SP thymocytes from extrinsic apoptosis since blocking TNFR1 signalling rescues mature SP populations (Webb et al., 2016). Here, our data supports these findings. We show that deletion of caspase-8 in IKK1/2 deficient mice completely restores normal CD4+ and CD8+ thymocyte development. Thus, IKK is required by SP thymocytes solely for repression of extrinsic apoptosis. One mechanism by which IKK protects SP thymocytes from apoptosis is through phosphorylation and inhibition of RIPK1. Ablation of RIPK1 kinase activity in IKK1/2 deficient mice can almost completely rescue CD4+ SP populations development but only partially rescue CD8+ SP thymocyte development (Webb et al., 2019). Therefore, combined with our data, this confirms that IKK protects SP thymocytes from both RIPK1 dependent and independent extrinsic apoptosis.

The specific sensitivity of SP thymocytes to RIPK1 dependent cell death in absence of IKK is associated with RIPK1 expression. RIPK1 expression is developmentally regulated and mature SP thymocytes have the highest expression of RIPK1 of all thymocyte populations (Webb et al., 2019). RIPK1 expression correlates with RIPK1 activity and sensitivity to TNF induced cell death in IKK1/2 deficient SP thymocytes (Webb et al., 2019). We identified A20 as a regulator of RIPK1 dependent cell death in SP thymocytes. Although thymocyte development is normal in IKK2 deficient mice, additional deletion of A20 impaired CD8+ SP thymocyte development due to RIPK1 dependent cell death. One mechanism by which A20 is thought to control RIPK1 kinase activity is by shielding the network of M1-linked ubiquitin surrounding complex I from CYLD mediated deubiquitination, thereby stabilising complex I (Draber et al., 2015; Priem et al., 2019). We note that, in a similar fashion to RIPK1, the expression A20 and CYLD also appear to change throughout thymocyte development. The expression of Tnfaip3 is highest in DP thymocytes and decreases to reach its lowest point in mature SP thymocytes (Webb et al., 2019). On the other hand, the expression of Cyld is lowest in DP thymocytes and increases to reach its highest point in mature SP thymocytes (Webb et al., 2019). It is unclear how or why A20 and
CYLD expression is regulated during development. We speculate that this may not be related to fine tuning of canonical NF-κB activation since they both act as negative regulators. Instead, it could suggest fine tuning to the RIPK1 dependent cell death pathway. Thus, the loss of A20 and increase in CYLD and RIPK1 expression may serve to shift the balance of signalling away from survival and more towards death as thymocytes mature.

CD8\(^+\) SP thymocytes appear to require more stringent regulation of RIPK1 than CD4\(^+\) SP thymocytes. Deficiency in either IKK1 or IKK2 has no impact on CD4\(^+\) or CD8\(^+\) thymocyte development, probably due to redundancy between these kinase subunits (Chen et al., 2015; Schmidt-Supprian et al., 2003; Silva et al., 2014). However, mice with only one functional gene copy of IKK1, and not IKK2, have normal CD4\(^+\) SP thymocyte development, but a reduction in mature CD8\(^+\) SP thymocytes much like that of IKK1/2 deficient mice (Webb et al., 2019). Similarly, we show that deletion of A20 in IKK2 deficient mice leads to a lineage specific reduction in mature CD8\(^+\) but not CD4\(^+\) SP thymocytes, as a result of RIPK1 dependent cell death. One potential explanation for these observations could be the differing expression of RIPK1. Protein expression of RIPK1 is approximately 1.5 times greater in mature CD8\(^+\) versus CD4\(^+\) SP thymocytes (Webb et al., 2019).

### 7.2 TNF is the primary trigger of extrinsic apoptosis in IKK deficient SP thymocytes

Earlier work identified TNF as a crucial trigger of SP thymocyte death in absence of IKK. Deletion of TNFR1 can completely rescue CD4\(^+\) SP thymocyte development in IKK1/2 deficient mice but only partially rescue mature CD8\(^+\) SP thymocytes (Webb et al., 2016). We show that TNF knockout gives a similar phenotype, thus ruling out any role for TNFR2 induced death and demonstrating that TNF is acting solely by binding to TNFR1. However, since ablation of caspase-8 provides a more complete rescue of mature CD8\(^+\) SP thymocytes in IKK1/2 deficient mice than TNF or TNFR1 ablation, IKK also appears to be repressing cell death induced by
other death receptors (Webb et al., 2016). Nonetheless, deletion of TNFR1 completely rescues mature CD8+ SP thymocytes in mice deficient in both A20 and IKK2. While ablation of IKK1 and IKK2 results in both RIPK1 dependent and independent cell death of mature CD8+ SP thymocytes, combined A20 and IKK2 deficiency results more exclusively in TNF triggered RIPK1 dependent cell death (Webb et al., 2019). Taken together, these observations suggest that death receptors other than TNFR1 may be responsible for triggering the RIPK1 independent cell death pathway, that only appears to occur in the complete absence of IKK activity.

7.3 IKK and A20 control extrinsic apoptosis in mature T cells

The role of IKK has been less well defined in mature T cells compared to thymocytes and previously, IKK had only been considered in terms of NF-κB activation. IKK dependent NF-κB activation is crucial for normal homeostasis of new T cells by inducing expression of IL-7R (Silva et al., 2014). Here, we show that IKK is constitutively required by mature T cells for protection from extrinsic apoptosis. Inducible ablation of IKK1/2 in mature T cells caused a reduction in naïve T cells which could be rescued by deletion of Casp8.

One mechanism by which IKK restrains extrinsic apoptosis in mature T cells appears to be through direct repression of RIPK1 kinase activity. Partial or complete inhibition of IKK renders naïve T cells susceptible to TNF induced RIPK1 dependent cell death in vitro. But for optimal control of RIPK1 by IKK, we show that T cells require A20. In absence of A20, naïve T cells appeared to require greater IKK activity to protect from TNF induced RIPK1 dependent cell death in vitro. In A20 deficient mice, impairing IKK activity by genetic deletion of IKK2 lead to profound lymphopenia. But genetic inhibition of RIPK1 kinase activity could restore the peripheral T cell compartment, demonstrating that T cells were dying from RIPK1 dependent cell death. Since CD4+ naïve T cells, but not CD4+ SP thymocytes, were sensitised to RIPK1 dependent cell death in absence of A20 and IKK2, this suggests that more stringent regulation of RIPK1 is required once T cells exit the thymus.
However, compared to ablation of RIPK1 kinase activity, deletion of Casp8 appears to lead to far higher accumulation of naïve T cells in the periphery of mice deficient in IKK1/2 during thymocyte development (Webb et al., 2019). Thus, IKK also appears to control a RIPK1 independent cell death pathway in mature T cells.

7.4 TNFR1 and Fas cell death pathways are controlled by IKK and A20 in mature T cells

Although TNF is the primary trigger of extrinsic apoptosis in SP thymocytes, other death receptors appear to play a more prominent role in the periphery. Ablation of TNFR1 or antibody mediated blockade of TNF in mice deficient in both A20 and IKK2 can partially rescue the peripheral T cell lymphopenia, but not to the same extent as inhibition of RIPK1 kinase activity. Thus, TNFR1 is not the sole trigger of RIPK1 dependent cell death in peripheral T cells. Additionally, ablation of TNF production is insufficient to rescue peripheral T cells from death following inducible IKK1/2 deletion in mature T cells, whereas deletion of Casp8 does rescue, implicating a role for IKK in repression of extrinsic apoptosis triggered by receptors additional to TNFR1.

We show that FasL can induce RIPK1 dependent cell death of T cells following pharmacological IKK inhibition or combined loss of A20 and IKK2 activity in vitro. As such, FasL may be responsible for the observed TNFR1 independent extrinsic apoptosis in vivo. Furthermore, FasL can trigger RIPK1 independent cell death of T cells in absence of A20 in vitro. Thus, given that A20 deficient T cells are resistant to TNF in vitro, it is possible that FasL is responsible for the small but significant decrease of CD8+ naïve T cells in A20 deficient mice.

7.5 IKK signalling protects activated T cells from RIPK1 dependent cell death

TCR induced NF-κB activation is crucial during the very early stages of T cell activation by inducing the expression of myc and cell cycle genes amongst
others (Grumont et al., 2004). Thus, complete NF-κB blockade by inactivation of the CBM or IKK complexes leads to failure of T cells to blast transform and divide (Egawa et al., 2003; Hara et al., 2003; Newton and Dixit, 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003; Webb et al., 2019). But by impairing rather than completely ablating IKK activity, we have been able to separate its role as a repressor of cell death from its role in acute T cell activation. In absence of IKK2, NF-κB activation is impaired but not completely blocked and we have shown that this is sufficient for T cells to undergo normal priming, blast transformation and cell cycle progression. Instead, we show that activated IKK2 deficient T cells have insufficient IKK activity for control of RIPK1 kinase activity. In vitro experiments demonstrated that F5 TCR transgenic and polyclonal IKK2 deficient T cells undergo cell death following TCR stimulation. But the impaired IKK2 deficient T cell responses in vitro and in vivo could be rescued following pharmacological or genetic inhibition of RIPK1 kinase activity, demonstrating that cell death was RIPK1 dependent. Thus, our data indicates that repression of RIPK1 kinase activity by the IKK complex is crucial for survival of activated T cells and is consistent with IKK directly phosphorylating and inhibiting RIPK1 kinase (Dondelinger et al., 2015; Webb et al., 2019).

Collectively, as well as having an important role early during T cell activation, we now show that IKK has a role in the later stages, once T cell proliferation begins, for protection against extrinsic apoptosis.

7.6 IKK dependent induction of A20 during T cell activation promotes survival

The sensitivity of IKK2 deficient T cells to RIPK1 dependent cell death after activation contrasted with the predominant resistance of these cells to RIPK1 dependent cell death prior to activation. Naïve IKK2 deficient T cells from F5 mice were resistant to TNF induced cell death in vitro and polyclonal IKK2 deficient mice do not exhibit extensive RIPK1 dependent death of T cells. We hypothesised that the impaired NF-κB activation in absence of IKK2 was responsible for the altered regulation of RIPK1 after T cell activation.
Expression of the NF-κB gene target A20 was significantly reduced in activated IKK2 deficient T cells. Our data suggests that A20 promotes the survival of activated T cells via multiple mechanisms. Firstly, A20 controls a RIPK1 independent cell death pathway in activated T cells. A20 deficient T cells were sensitive to cell death following activation which could not be rescued by inhibiting RIPK1 kinase activity. However, the nature of this pathway and how it is triggered is unclear. Secondly, A20 was required for optimal regulation of RIPK1 by the IKK complex in naïve T cells. A20 deletion did not impact the sensitivity of activated IKK2 deficient T cells to cell death and, as such, this mechanism explained the increased sensitivity of IKK2 deficient T cells to RIPK1 dependent cell death following activation.

We position the IKK complex as a crucial regulator of extrinsic cell death pathways in activated T cells (Fig. 7.1). IKK directly phosphorylates and inhibits the activity of RIPK1 (Dondelinger et al., 2015; Webb et al., 2019). But in addition to this, IKK also drives NF-κB dependent A20 expression. In this way, A20 controls a RIPK1 independent cell death pathway but also acts to optimise repression of RIPK1 by the IKK complex. TCR signalling dynamically regulates the expression of A20 during T cell activation. TCR stimulation induces the degradation of A20 via Malt1 cleavage and by the proteasome (Coornaert et al., 2008; Düwel et al., 2009). A20 expression is restored in an NF-κB dependent manner (Coornaert et al., 2008). We posit that the TCR tunes sensitivity to extrinsic cell death pathways via A20. By inducing destruction of constitutive A20, activated T cells become rapidly reliant on de novo synthesised A20. Thus, following activation, T cells become more sensitive to extrinsic cell death if canonical NF-κB signalling is perturbed.

**7.7 IKK and A20 control cell death pathways downstream of multiple death receptors in activated T cells**

TNFR1 appears to be one trigger of RIPK1 dependent cell death in activated T cells when control of RIPK1 by IKK is disrupted. *In vitro* and *in vivo* F5
IKK2 deficient T cell responses can be partially rescued by ablation of TNFR1. However, deletion of Tnfrsf1a did not rescue death of activated T cells deficient in both A20 and IKK2. Since in these scenarios, ablation of RIPK1 kinase activity gave a more complete rescue of activated T cells than deletion of Tnfrsf1a, this suggests that other death receptors were triggering RIPK1 dependent cell death. Both Fas and TRAIL-R were expressed by activated F5 T cells at a relatively higher level compared to other death receptors. Since FasL can trigger RIPK1 dependent cell death in T cells following inactivation of IKK, perhaps FasL is also responsible for triggering death of activated IKK2 deficient T cells. FasL also triggers RIPK1 independent death of A20 deficient T cells and, as such, is likely to have a role following activation.
Normal IKK activity

Impaired IKK activity

Genes involved in T cell activation (e.g. myc)
In consideration of previous studies and the data generated in this thesis, we propose a mechanism for how IKK signalling functions during T cell activation. Following stimulation, the TCR induces activation of NF-κB via the IKK complex. Simultaneously, A20 is cleaved by Malt1 and degraded by the proteasome to ensure maximal NF-κB activation at the point of stimulation. In this way, NF-κB drives the expression of genes (such as myc) involved in acute activation, blast transformation and cell cycle entry. Activated T cells (and other cell types) produce TNF and sFasL which may function in an autocrine and/or paracrine fashion, potentially further driving NF-κB activation. Since Tnfaip3 is also an NF-κB gene target, A20 expression is restored. De novo synthesis of A20 acts to prevent sustained NF-κB, inhibits a RIPK1 independent cell death pathway and is required for optimal inhibition of RIPK1 kinase activity by the IKK complex downstream of TNFR1 and Fas, thereby preventing cell death.

In T cells with partial IKK activity, such as when IKK2 is deficient, TCR stimulation can induce sufficient NF-κB activation to drive the transcription of genes involved in early T cell activation. A20 is degraded by Malt1 and the proteasome however there is insufficient NF-κB activity to restore normal levels of A20. Triggering of TNFR1 induces complex I formation while ligation of Fas leads to complex II formation. Within these complexes, IKK phosphorylation and inhibition of RIPK1 kinase activity is impaired. Combined with the increased RIPK1 expression and activity, the lack of A20 compounds the already sub-optimal control of RIPK1 activity by IKK, thus leading to RIPK1 dependent cell death.
7.8 Future studies

There are still many unanswered questions regarding the triggers and regulation of extrinsic cell death pathways in T cells. Firstly, although we identified A20 as a regulator of RIPK1 in T cells, we don’t fully understand how it functions. Much of the work investigating how A20 represses TNF induced cell death has focused on other cell types. But A20 is regulated differently in T cells compared to most cell types, given that it is constitutively expressed to a high level. It is important to understand how A20 affects the ubiquitination of complex I in T cells and how this impacts on the phosphorylation of RIPK1 by the IKK complex. In addition, more work is required to understand how A20 represses cell death induced by other death receptors and whether it functions in the same way as described downstream of TNFR1.

We have identified roles of A20 and IKK in the repression of FasL induced cell death of T cells in vitro. However, these mechanisms have yet to be fully defined. Firstly, how does IKK repress RIPK1 kinase activity downstream of Fas? Is it through direct phosphorylation and inhibition? How does Fas trigger IKK activation? Whether inhibition of FasL induced cell death by A20 and IKK is important for thymocytes and mature T cells in vivo has yet to be directly investigated. To address this, mouse strains deficient in IKK1/2 or A20 and IKK2 could be crossed with either lpr/gld mice or mice with T cell specific deletion of Fas. Given the apparent redundancy between TNFRSF members for triggering cell death, perhaps additional ablation of TNF production or deletion of Tnfrsf1a would also help to confirm whether Fas is important in these scenarios. From our in vitro data, we could expect that deletion of Fas would rescue mature T cells populations in IKK1/2 deficient mice and mice lacking A20 and IKK2.

7.9 Clinical perspectives
Dysfunctional canonical NF-κB signalling has been implicated in a range of human diseases. Since NF-κB activation promotes cell survival, this pathway has been exploited by viruses and cancers which drive excessive NF-κB activity (Amaya et al., 2014; Kim et al., 2006; Yu et al., 2020). Thus, many have suggested targeting canonical NF-κB signalling pathways as a strategy to combat these diseases. However, in these scenarios, the role of canonical NF-κB signalling in T cells needs to be considered. We have shown that IKK plays a crucial role in T cells for repression of extrinsic cell death and activated T cells are particularly sensitive to even partial disruption of IKK. Therefore, targeting IKK in the context of these diseases is likely to negatively affect T cell homeostasis and effector responses and may be counterproductive. Instead, specifically targeting IKK2 might be beneficial during T cell mediated autoimmune diseases. In this way, it would have minimal impact on peripheral T cell homeostasis but would rapidly sensitise activated pathogenic T cells to RIPK1 dependent apoptosis. This would not lead to the release of DAMPs and therefore would not further potentiate inflammation. Nonetheless, given that IKK is important for regulatory T cell homeostasis, the benefits could be offset by a lack of immune regulation. Ultimately, the role of IKK in differing T cell populations needs to be closely evaluated before consideration of IKK as a therapeutic target.

A number of patients with primary immunodeficiency have been described with mutations in genes encoding canonical NF-κB signalling components. Patients with homozygous null mutations in IKBKB (encoding IKK2) exhibit severe combined immunodeficiency (SCID) (Cuvelier et al., 2019; Mousallem et al., 2014; Nielsen et al., 2014; Pannicke et al., 2013). Although these mutations cause a complete absence of IKK2 protein, unlike in mouse, it does not lead to embryonic lethality (Li et al., 1999b, 1999c; Tanaka et al., 1999). These patients have normal numbers of peripheral T cells, but presented with lower number of regulatory T cells and reduced memory T cells, despite recurrent infections (Pannicke et al., 2013). T cells showed impaired proliferative responses to a range of TCR stimuli (Mousallem et al., 2014; Nielsen et al., 2014; Pannicke et al., 2013). Thus, the impact of IKK2 absence in human T cells resembles that observed in mouse T cells.
(Schmidt-Supprian et al., 2003; Silva et al., 2014). Since we show that IKK2 is crucial during T cell activation for repression of RIPK1 dependent cell death in mouse, it is likely that the same is true in patients with IKBKB mutations. Although these patients ultimately require a haematopoietic stem cell transplant, perhaps in some circumstances they would benefit from treatment with RIPK1 kinase inhibitors, such as during infection or vaccination, to boost T cell responses.

Loss of function mutations in TNFAIP3 have been described which cause A20 haploinsufficiency (Zhou et al., 2016). These patients show a substantial decrease in A20 protein and present with early onset autoinflammatory disease. Some patients with A20 haploinsufficiency also exhibit autoimmune lymphoproliferative syndrome (ALPS), resembling those with mutations causing a dysfunctional Fas pathway (Kadowaki et al., 2018; Takagi et al., 2017). These patients are characterised by a normal number of naïve and memory CD8+ T cells populations. Stimulation of T cells in vitro demonstrated increased sensitivity to cell death. Thus, the T cell phenotype of patients with A20 haploinsufficiency resembles mice with T cell specific deletion of A20. However, they also exhibit expansion of double negative T cells (Takagi et al., 2017). sFasL was present at high levels in patient blood (Takagi et al., 2017). Since our data shows that T cells from A20 deficient mice were sensitised to sFasL induced RIPK1 independent cell death, this indicates that patient T cell abnormalities are likely to be T cell intrinsic.

Numerous studies have identified single nucleotide polymorphisms (SNPs) within the TNFAIP3 gene locus that have been linked to T cell mediated autoimmune diseases such as type I diabetes, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (Catrysse et al., 2014; Malynn and Ma, 2019). Many of the SNPs identified are present within regulatory regions upstream of coding regions and thus may affect A20 expression. Whether the SNPs are affecting A20 expression and function in T cells and if this is important for disease susceptibility is yet unclear. Nonetheless, it is important to understand the role of A20 in T cells and we show that A20 is an important regulator of extrinsic cell death pathways in T cells.
In conclusion, intervention of NF-κB and extrinsic cell death pathways offer the potential to allow augmentation of desirable T cell responses, by enhancing NF-κB and blocking cell death, while sensitising cells to TNFRSF induced death offers potential for short-term targeting intervention therapy to ablate undesirable T cell responses. However, detailed knowledge of complex TNFRSF pathways is necessary to be able to design specific and highly targeted therapeutic interventions.
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