The Role of DOCK8 in the Maintenance of $CD8^+$ T cell Memory and Invariant NKT cells



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Abstract

The use of genome wide ENU mutagenesis screening has uncovered vast numbers of novel genes involved in the control of the immune system. This thesis describes the characterization of a novel mutant, Captain Morgan (*CPM*), originally identified in an immunization screen designed to evaluate both the initial antibody response to antigen and the ability to sustain antibody production. Mapping of this mutant lead to the identification of a single base pair mutation in a novel guanine nucleotide exchange factor, dedicator of cytokinesis 8 (DOCK8). The mutation was found to result in altered gene splicing of the DOCK8 protein leading to the truncation of the protein and loss of catalytic function.

The importance of understanding the role of DOCK8 in host immunity has been recently underlined by the discovery that cohorts of patients suffering from autosomal recessive forms of hyper-IgE syndrome have loss-of-function or deletions in this novel guanine nucleotide exchange factor. Disease in these patients is characterised by recurrent viral and bacterial infections mainly of the skin and lungs, with reduced levels of peripheral $CD4^+$ and $CD8^+$ T cells in the blood of patients. Patients also have high levels of IgE and eosinophilia in the blood and are highly atopic with increased prevalence of allergic diseases including asthma.

Loss of DOCK8 function results in a number of phenotypes in *CPM* mice, which may help understand the immunodeficiency syndrome experienced by DOCK8 deficient patients. *CPM* mice, like DOCK8 deficient patients, are lymphopenic with losses of both $CD4^+$ and $CD8^+$ T cells in the blood and secondary lymphoid organs. Challenge of *CPM* mice with modified vaccina virus (MVA) and influenza strain X31

demonstrated normal primary anti-viral responses. However, similar to the loss of germinal centre B cells previously described in these mice, memory T cell responses were diminished, which may explain the susceptibility of DOCK8 deficient patients to recurrent infections. In addition to the loss of peripheral T cells, rare populations of lymphocytes such as invariant natural killer T cells (iNKT) were also reduced in the liver and thymus. Due to their roles in bacterial and viral responses and cancer immunosurveillance it is expected that loss of these cells will contribute to disease severity.

Together these findings illustrate the importance of the ENU mutagenesis model for generating new mutants, which can enhance our understanding of mammalian genes and create disease models of human disease. Further characterization of DOCK8 deficiency and the molecular mechanisms of DOCK8 function will have important implications for disease diagnosis and ongoing treatment for patients.

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List of Abbreviations

ACC	Aplasia cutis congenital
AD	Autosomal dominant
AIDS	Acquired immunodeficiency syndrome
AR	Autosomal recessive
BCR	B cell receptor
B.Pertussis	Bordetella Pertussis
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
C.elegans	Caenorhabditis elegans
CFSE	Carboxyfluorescein Succinimidyl Ester
CGG	Chicken gamma globulin
CPM	Captain Morgan
cSMAC	central Supramolecular Activation Clusters
DH-PH	Dbl–pleckstrin homology domain
DHR	DOCK homology region
DN	Double negative
DNA	Deoxyribonucleic acid
DOCK	Dedicator of Cytokinesis
DP	Double positive
EDTA	Ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G (0,1,2)	Generation (0,1,2)
GAP	GTPase-activating proteins
GC	Germinal centre
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HAU	Hemagglutination units
HIES	Hyper IgE syndrome
HIV	Human immunodeficiency virus
HSA	Heat stable antigen
HSCT	Haemopoetic stem cell transplant
ICAM	Intercellular adhesion molecule
I.D.	Intradermal
IEL	Intraepithelial lymphocytes

IFN	Interferon
Ig	Immunoglobulin
iNKT	Invariant natural killer T cell
I.P.	Intraperitoneal
I.V.	Intravenous
LFA-1	Lymphocyte function-associated antigen 1
MACS	Magnetic assisted cell sorting
MCP-1	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MVA	Modified Vaccina Ankara
MZ	Marginal zone
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIDs	Primary immunodeficiency diseases
pLNs	Peripheral lymph nodes
PLZF	Promyelocytic leukaemia zinc finger
PMA	Phorbol myristate acetate
Pri	Primuris
pSMAC	peripheral Supramolecular Activation Clusters
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma T
RTEs	Recent thymic emigrants
SAP	SLAM associated protein
SDF-1	Stromal cell-derived factor 1
SI	Small intestine
SLAM	Signalling lymphocyte activation molecule
SP	Single positive
TB	Tuberculosis
TCR	T cell receptor
TLR	Toll like receptor
TNF-α	Tumor necrosis factor α
Treg	T regulatory
TTLD	Terminal transverse limb defects
VCAM	Vascular cell adhesion protein 1
VLA-4	Very Late Antigen 4
WASp	Wiskott-aldrich sydrome protein
WGS	Whole genome sequencing
WT	Wildtype
XLP	X-linked lymphoproliferative disease
αGalCer	Alpha-galactosylceramide

Chapter 1

Introduction

1.1 The Importance of Understanding Immunological Memory

One of the most important goals in the field of immunology is the development of new, effective vaccines to decrease the burden of infectious diseases. At present, three diseases (AIDS, TB and Malaria) account for approximately half of deaths per year globally. These diseases as a whole account for roughly 5 million deaths per year and underline the desperate need for new vaccines. Great successes have been achieved in the past with the successful implementation of vaccines against yellow fever and smallpox, which have played crucial roles in the control of these infectious diseases [1]. However, attempts thus far to formulate new vaccines have been unsuccessful illustrating that there are still great caveats in our understanding of how immunological memory is generated. Therefore, further research to improve our understanding of the mechanisms underlying the generation and maintenance of immunological memory are crucial to the success of the vaccination approach.

The study of immunological memory in humans is made difficult by several factors including: accessibility to biological material from humans is restricted (most routinely involving blood samples) and as the induction of memory takes place in the secondary lymphoid organs, which we cannot gain access to, the relevance of these samples are questionable; differences in exposure to infectious agents can alter measurements taken; and the human population contains a vast array in genetic susceptibility to pathogens which makes the interpretation of data unclear. Therefore, the use of animal models has been key to advances in this field. The use of mouse

models as infectious models is hugely advantageous for research, as animals have defined genetic backgrounds and can be housed in pathogen free environments to ensure previous exposures to pathogens will not affect the outcome of research models. We now understand that factors including the dosage and nature of infectious agent, the induction of inflammatory factors such as TNF-α and the route of vaccination all have important implications for the outcome of vaccination through the use of such models [2-4].

Understanding both the cellular and molecular mechanisms of immunological memory is of key importance to improving vaccine design. Many of our discoveries in the vaccination field have been through knockout models of key genes hypothesized to take part in the regulation and formation of memory responses. However, a disadvantage of this approach is that the generation of knockout models is difficult and some prior knowledge to the function or importance of the gene itself is required. Because of this, other approaches have been sought to identify new genes involved in host immunity. One strategy to identify new genes involved in host immunity and to generate new models of human disease is the use of the mutagen N-ethyl-N-nitrosourea (ENU).

1.2 ENU Strategy and Identification of Captain Morgan (*CPM*)

1.2.1 ENU Strategy

N-ethyl-N-nitrosourea (ENU) is a highly potent mutagen used to induce single base pair mutations in mice with the goal of identifying novel genes by forward genetics [5]. This approach is based on "phenotype driven" analysis of pedigrees generated from ENU treated founder mice to allow the identification of novel genes involved in an area of interest. Original successes of this "phenotype driven" approach were in naturally occurring mutants in genes such as Foxn1, FoxP3 and Fas which gave rise to well established mouse strains known as "nude", "scurfy" and "lpr" mice [6]. These mice have easily recognisable disease phenotypes, which are associated with human diseases including severe combined immunodeficiency (SCID), autoimmune polyendocrinopathy, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) and autoimmune lymphoproliferative syndrome respectively. The use of these animal models has led to the dissection of the molecular mechanisms underlying these diseases, which has now advanced the diagnosis and treatment of patients. However, naturally occurring variants are rare and therefore methods to accelerate the natural rate of mutation in hosts have been sought.

The mutagen ENU is estimated to induce 1 single base pair change per 1MB of genomic DNA most commonly inducing A/T to T/A transversions and A/T to G/C transitions [6-9]. However, recent work by our research group, with the use of whole genome sequencing (WGS), more accurately measures the rate of mutation at 1/700,000 base pairs (Bull et al, unpublished). Mutations generated in male founder mice (G0) are propagated to allow screening for dominant mutations (expected in roughly 2% of G1 mice). Recessive mutations are bred to homozygosity by breeding

strategies including backcrossing of G2 mice to G1 mice or inter-crossing with G2 mice (Fig1). There are several advantages to ENU mutagenesis compared to alternative methods of inducing mutation (radiation and other chemical mutagens) and targeted gene knockout models. The nature of mutation mimics that of natural human variation introducing changes in genes including: loss or gain of function mutations, altered gene splicing and truncation of genes, making these models more biologically relevant. Point mutations are also less likely to create embryonic lethality in offspring compared with gene knockout methods or radiation treatment, which can cause large deletions or inversions involving multiple genes. However, the main advantage of the ENU strategy is identifying novel genes due to the unbiased nature of the experimental approach. Investigation is driven by phenotypes observed with no prior knowledge of the genes altered thus allowing for new genes and alternative pathways to be investigated. This approach can be extremely costly and labour intensive due to the nature of breeding to maintain phenotypes, and thus genotypes, of interest. Another downfall due to the random nature of mutation is this screening process may result in the discovery of classically described genes, which may have already been extensively researched. With advances in whole genome sequencing and the characterization of large numbers of already existing ENU mutants for the frequency of mutation it may soon be possible to omit the difficult and risky breeding strategy required to maintain phenotypes and increase the rate of the discovery of new genes.

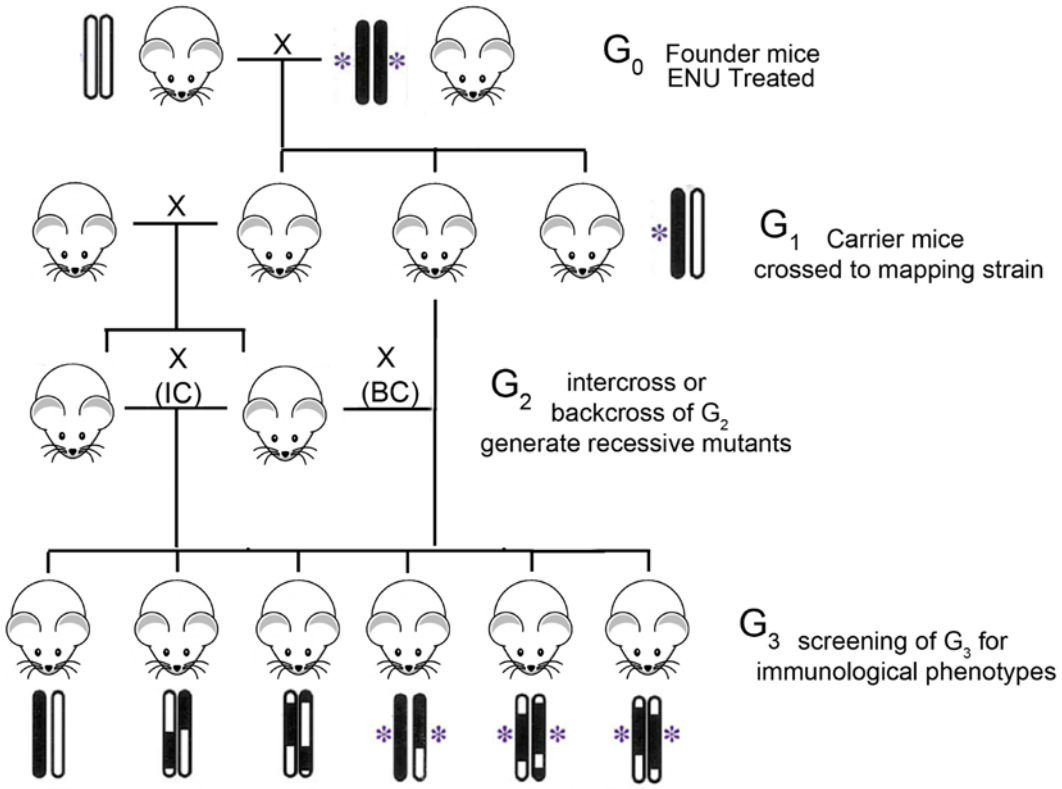


Figure1: The ENU pedigree structure for the discovery of novel genes. N-ethyl-N-nitrosourea (ENU) treated mice, G0, containing point mutations are bred to C57BL/6 mice giving rise to G1 offspring. For identification of mutations, out-crossing of G1 mice to mapping strains gives rise to a second generation (G2), which are then inter-crossed (IC) or backcrossed (BC) to produce G3 animals. G3 animals are screened and animals of interest genotyped to identify causative mutations.

1.2.2 Identification of novel ENU mutant – Captain Morgan (*CPM*)

To discover new genes involved in the induction of humoral immunity and memory B cells, our research group in collaboration with Chris Goodnow from the Australian National University (Canberra, Australia) developed an immunization screen to identify ENU mutants defective in the initiation and polarization of early antibody responses and maintaining long-term humoral immunity. ENU mutants were

immunized with a mixture of killed bordetella pertussis (B.pertussis) bacteria, and chicken-γ-globulin (CGG) protein and bled 14 days post immunization [10]. IgG2c (Th1 response) and IgG1 (Th2 response) were measured to B.pertussis and CGG respectively to establish successful initiation of responses and appropriate polarization [10]. The majority of antibody produced at this stage is derived from the clonal expansion of extra-follicular B cells occurring after interaction with T cells [11]. 28 days post immunization mice were bleed a second time to examine IgG levels, which are derived from mature germinal centre responses. This immunization screen originally identified two ENU mutants propagated from independent pedigrees with reduced antibody titres 28 days post immunization but with normal primary B cell responses [10]. These strains were later named "captain morgan" (*CPM*) and "primurus" (*pri*). Mapping of both strains resulted in the identification of two different mutations within the novel guanine nucleotide exchange factor, dedicator of cytokinesis 8 (DOCK8) [10, 12, 13]. *CPM* contained a T-C substitution in intron 20, which leads to the disruption of the exon 20 splice donor sequence whereas primurus contained a T-C substitution in exon 43 which is part of the DOCK Homology Region 2 (DHR2) catalytic domain (Fig2A). The mutation found in *CPM* cells resulted in altered gene splicing of the DOCK8 protein with differing lengths of DOCK8 observed after amplification of mRNA encoding exons 18-21 (Fig2B). This altered gene splicing results in frameshifts and the introduction of premature stop codons, which leads to the loss of the DOCK8 protein as demonstrated by the lack of detection in western blots (Fig2C).

The mutation found in *pri* results in an alteration in codon 1827 changing a serine residue to a proline residue [10]. This conserved residue lies within a α-Helix

structure, which is integral to the binding of the DHR2 domain to target molecules and therefore the disruption of this secondary structure is expected to disrupt DOCK8 function.

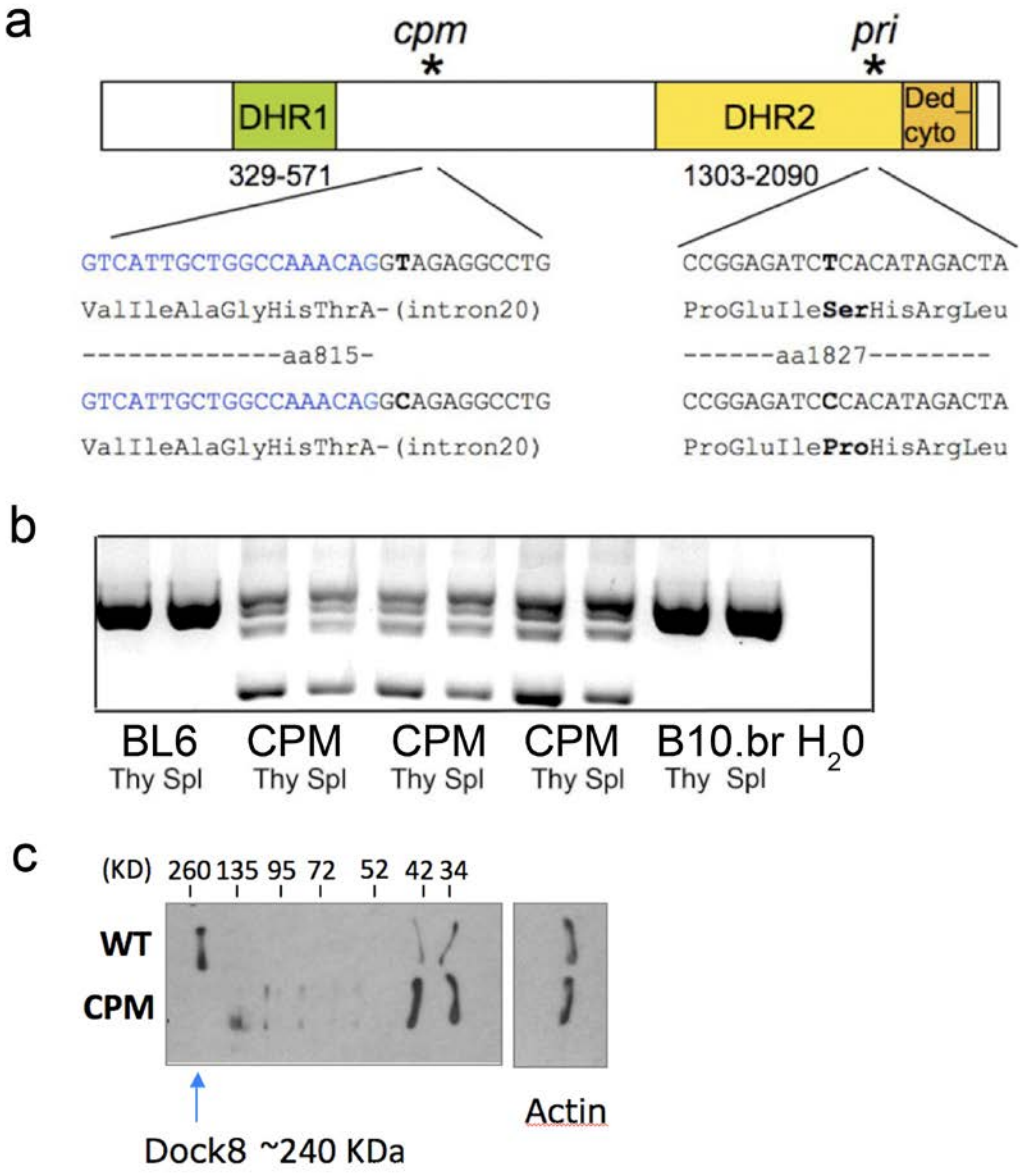


Figure 2: DOCK8 domain structure and location of ENU mutations. a) The DOCK8 protein contains two evolutionary conserved domains, the DHR1 (light green) and DHR2 (yellow) domains that are integral to its function (the predicted GTP-binding region is depicted in orange). Mutations in *CPM* and *pri* are shown with an asterisk (*) which were both characterized as T-C substitutions [10]. b) Amplification of

DOCK8 exons 18-21 from cDNA generated from WT (BL6 and B10) or Captain Morgan (*CPM*) lymphocytes derived from the thymus and spleen. Multiple bands are only observed in *CPM* cells. c) Analysis of DOCK8 protein expression by western blotting demonstrates loss of expression in *CPM* cells.

The relevance of this model of DOCK8 deficiency in animals has been reinforced by the discovery of patients with mutations/deletions in the DOCK8 genes. These patients suffer from a primary immunodeficiency disease with increased susceptibility to bacterial and viral pathogens. However, the role of DOCK8 in haemopoetic cells is still to be investigated.

1.2.3 Phenotypes observed in *CPM* mice

The deficiencies in humoral immunity in *CPM* and *pri* mice indicated DOCK8 had a crucial role in the adaptive immune system. Therefore, detailed analyses have been carried out in both ENU strains. The two mutants show no significant differences in B cell development with no obvious reductions of immature B cells in the bone marrow [10, 12, 13]. In the periphery, no differences were seen in the numbers of follicular B cells in organs such as the spleen. However, a severe reduction in marginal zone (MZ) B cells is observed in the absence of DOCK8 function [10]. This phenotype is cell intrinsic based on mixed chimera experiments and has been observed in both ENU mutants. Importantly, the discovery of two ENU mutants with mutations in the same gene made it possible to generate compound heterozygotes to confirm that causative mutations were indeed in the DOCK8 gene. Compound heterozygote $CPM^{+/-}$ $pri^{+/-}$ display similar phenotypes to homozygote *CPM* and *pri* strains with loss of antibody responses following immunization and loss of MZ B cells. These data act as strong

evidence that the causative mutations in these strains are in the novel guanine nucleotide exchange factor, DOCK8. After further investigation of the failure of *CPM* mice to maintain antibody responses, an intrinsic defect in germinal centre (GC) B cell survival was established. Therefore, as well as the loss of MZ B cells, GC B cells are also defective in the absence of DOCK8 [10]. However, the role of DOCK8 in maintaining these cell populations is still to be elucidated.

1.2.4 Summary

ENU mutagenesis offers a unique approach to identifying novel genes involved in immune regulation and to generating new mouse models of mendelian disease. The approach has several advantages compared to gene knockout models including: 1) the ability to identify new genes without any prior knowledge to gene function; 2) mutations are more physiologically relevant as mutations induced by ENU mimic natural variation in the human population; 3) many gene knockout models can be embryonically lethal due to the removal of the entire gene; and 4) the ability to design an immunological screen to identify genes involved in an area of interest to allow research to be focused. The development of one such screen to identified mutants with normal primary immune responses to immunization but lacking in long-term antibody responses has led to the identification of a new gene involved in humoral immunity – the guanine nucleotide exchange factor DOCK8. The discovery of the mechanisms underlying DOCK8 function in maintaining humoral immunity could provide insights into improving future vaccination designs and also enable therapeutic targeting in patients suffering from autoimmunity.

1.3 Autosomal Recessive Hyper IgE Syndrome and DOCK8

1.3.1 Primary Immunodeficiency and DOCK8

As mentioned briefly above, the importance of understanding the role of DOCK8 in host immunity is underlined by the discovery of a rare primary immunodeficiency syndrome found in patients with loss-of-function or deletions in this novel guanine nucleotide exchange factor. Disease in these patients is characterised by recurrent viral and bacterial infections affecting the skin and lungs, including herpes simplex and zoster, molluscum contagiosum, adenovirus, respiratory syncitial virus, pneumocystis and staphylococcal, streptococcal and haemophilus influenza infections [14-17]. Patients are also highly atopic, with elevated levels of IgE and hypereosinophilia. As a result of this, patients are prone to extensive food and environmental allergies including anaphylaxis and asthma. Three of 11 patients in one study also died at a young age from cancer, including metastatic squamous carcinoma [15].

The majority of patients recruited in these studies are classified as sufferers of hyper-IgE syndromes (HIES). HIES are rare primary immunodeficiency diseases characterized in patients by recurrent viral and bacterial infections with elevated levels of serum IgE (>10 times normal levels) [14, 18, 19]. However, the genetic etiologies of HIES are diverse with some yet to be identified. Both autosomal dominant and recessive etiologies have been described. Autosomal dominant forms of HIES (AD-HIES) present with the above recurrent infections, hyper IgE but also with addictional manifestations including skeletal and dental abnormalities. The causative mutation in patients with AD-HIES is in the signal transducer and activator of transcription 3 (STAT3) protein (60%-70% of patients investigated) [14]. Autosomal

recessive forms of HIES (AR-HIES) are also characterized by recurrent infection and hyper IgE (including exaggerated levels of eosinophils in the blood) but do not present with any skeletal or dental abnormalities. In these patients, the genetic factors underlying disease are largely unknown. Thus, an understanding of the genetic etiologies underlying autosomal recessive forms of HIES may aid in both diagnosis and treatment of patients.

Genetic analyses of AR-HIES patients have attempted to identify causative mutations in a range of genes. Notably, mutation in the tyrosine kinase, TYK2, has been described in one patient suffering from AR-HIES [20]. However, mutations in TYK2 have not been present in other analyses of AR-HIES patients [14, 21]. Work carried out by Zhang et al using comparative genomic hybridization arrays led to the discovery of DOCK8 as a major cause of AR-HIES. Genetic analysis showed mutations/deletions in the DOCK8 gene resulting in loss of DOCK8 expression in haemopoetic cells from all 11 patients enrolled in this study [17]. This was the first study to link loss of DOCK8 function/expression to AR-HIES. This work was later supported by Engelhardt et al. Through the use of homozygosity mapping, Engelhardt et al were able to identify gene loci associated with AR-HIES and identified mutation in DOCK8 as the cause of AR-HIES. In this study, 21 out of 27 patients recruited were found to have deletions or truncations of the DOCK8 protein [14].

The importance of diagnosis and intervention in AR-HIES is crucial as disease manifestation occurs at a very young age and has a poor prognosis. The successful treatment of one patient with autosomal recessive DOCK8 deficiency by allogeneic haematopoietic stem cell transplantation (HSCT) has been reported [22]. Post HSCT,

this patient's IgE and eosinophilia levels and T cells had returned to normal levels within 6 months and 12 months respectively.

Prior to these studies, two unrelated patients had been described by Griggs et al to have deficiencies in DOCK8 [23]. These patients were diagnosed with mental retardation and developmental disabilities. Griggs et al showed that both patients had truncated forms of a DOCK8 isoform. Notably, DOCK6 and DOCK7 are expressed at high levels in the brain and nervous tissue and have been demonstrated to have key roles in axon formation and neuronal polarization. Therefore, loss of DOCK8 may be expected to result in disruption of neurological cells. However, no immunological phenotypes were described. As both patients carried single copy deletions in the DOCK8 gene along with disruption to other unknown genes, this may explain why these patients did not present with the classical HIES phenotype.

1.3.2 Mechanisms Underlying Hyper IgE Syndromes

The molecular mechanisms of hyper-IgE diseases in patients lacking DOCK8 expression or function are unclear. More work has focused on the autosomal dominant representation of HIES and how mutation in STAT3 results in susceptibility to recurrent infections and hyper-IgE. Importantly, several groups have demonstrated that STAT3 plays a critical role in TH17 differentiation in both humans and in mouse models and have speculated that this loss in the immune repertoire will predispose patients to recurrent staphylococcal infections of the skin and lung [24-28]. STAT3 alongside the master regulator of TH17 development, RORγt, are key transcriptional factors that regulate the IL-17 gene. Conditional knockout studies using CD4-Cre and $STAT3^{flox}$ transgenic mice have demonstrated the dependence of TH17 differentiation

on STAT3, showing loss of RORγt and IL17 expression in its absence [29]. However, no such role for DOCK8 in TH17 differentiation has been described. It is also difficult to interpret these findings in patients due to differences in lymphopenic state and the influence of persistent infection.

Treatment of HIES patients has also focused on the control of the atopic phenotype including atopic dermatitis and asthma. Some success has been shown in patients treated with therapies such as Omalizumab, an anti-IgE monoclonal treatment (abstracts, Sanchez-Ramon et al). However, greater understanding of the mechanisms underlying hyper-IgE secretion is sought. Loss of regulation by regulatory T cells can result in allergic diseases [30-33]. Therefore, work has been carried out to examine T cell differentiation and regulatory T cells in the STAT3 deficient patients. STAT3 deficient patients have normal levels of T regulatory cells (Treg) in the blood and T cells undergo normal T cell differentiation into Th1 and Th2 subsets under appropriate polarizing conditions. Importantly, Treg function is also intact ex vivo indicating that the hyper-IgE syndrome in STAT3 patients is not due to a loss of immune regulation by T cells [34]. However, STAT3 deficiency did have a strong influence on cells of the innate immune system. Monocyte-derived dendritic cells are unresponsive to IL-10 resulting in a loss of dendritic cells of a tolerogenic phenotype. The loss of tolerogenic dendritic cells may have consequences for the induction and maintenance of inducible T regulatory (iTreg) populations important in controlling and regulating immune responses [34]. Thus, we can speculate that loss of IL-10 responsiveness in dendritic cells and therefore loss of iTreg populations may help explain the clinical manifestations we see in HIES patients.

Differences in the presentation of patients suffering from autosomal dominant and recessive forms of HIES support the idea that DOCK8 and STAT3 take part in different signalling pathways and that different molecular pathways are afflicted by their absence. However, there is some evidence that DOCK8 may function upstream of STAT3. Consistent with our ENU mutant, work by Jabara et al has shown that DOCK8 deficient patients lack serological memory with deficiencies in protective antibody titres after being given protective vaccination at a young age [35]. Of particular interest, 2 of the 5 patients assessed for protective antibody titres showed early antibody responses after immunization, but failed to maintain protective antibodies thereafter [35]. This is in contrast to healthy controls that maintain protective antibody titres for years post vaccination. This is similar to our *CPM* strain which can mount extra-follicular primary antibody responses but cannot sustain GC B cells, which are responsible for the formation of high affinity antibodies and memory B cell development, resulting in levels of secondary antibody responses being lost [10]. Flow cytometry analysis of DOCK8 deficient patients also showed a loss of circulating CD27+ memory B cells in the blood of patients seeming to confirm a loss of serological memory [35]. Specifically, DOCK8 deficient B cells failed to respond to the TLR9 ligand, CpG, in *in vitro* culture assays with no proliferation or secretion of IgM and IgG antibodies. In contrast, B cells responded normally to other stimuli including anti-CD40 and IL-4. Of note, when stimulated with anti-CD40 and IL-4, no differences in the secretion of IgE were observed indicating no intrinsic bias of DOCK8 deficient B cells to IgE secretion [35].

Analysis of signalling pathways and effector molecules downstream of TLR9 in DOCK8 deficient cells revealed a loss of CpG-driven phosphorylation of STAT3

which has been previously shown to be important in B cell proliferation and differentiation. Therefore loss of DOCK8 signalling, STAT3 phosphorylation and effector function may help to explain deficiencies in B cell memory observed in both autosomal dominant and recessive forms of HIES [35]. However, an intrinsic defect in B cell immunology does not appear to be apparent in relation to the hyper-IgE phenotype. Moreover, effects of recurrent infections make it difficult to interpret results in patients.

1.3.3 Summary

Understanding of genetic etiologies of HIES is key to the accurate diagnosis and development of new therapies for the treatment of patients. 60% - 70% of autosomal dominant forms of HIES are found to have mutations in the protein STAT3, a transcription factor important in TH17 differentiation and B cell activation and differentiation. Loss of TH17 differentiation and B cell unresponsiveness to TLR9 activation may help to explain the susceptibility of patients to recurrent stapylcoccal infections in the lung and skin. In the absence of STAT3, monocyte-derived dendritic cells also lose responsiveness to tolergenic factors such as IL-10 resulting in immune dysregulation. This may be a factor underlying the atopic phenotype seen in patients.

The genetic factors responsible for the autosomal recessive form of HIES are largely unknown. Two candidate genes have been uncovered, TYK2 and DOCK8. TYK2 has only been confirmed in one patient thus far whereas DOCK8 has now become a much more prominent candidate. Loss of DOCK8 in mouse models, reported in our lab, results in loss of B cell memory as well as T cell immunodeficiency. This is similar to the phenotype seen in patients. However, no hyper IgE phenotype has been observed

in either *CPM* or *pri* animal strains and therefore the role of DOCK8 in atopy is unknown.

1.4 Guanine Nucleotide Exchange Factors – DOCK family

1.4.1 Introduction to DOCK family proteins

The Ras superfamily of small GTPase proteins form a large family of proteins involved in a wide range of cellular processes including cell division, migration, phagocytosis and cytoskeletal regulation. Mutations in these proteins have a strong association with human cancers and have become a strong focus of research to improve our understanding of cellular biology and to produce drug targets for the treatment of cancer. To date, over 150 members of this family have been identified in humans based on sequence homology. These proteins cycle between a guanine diphosphate (GDP)-bound inactive form to an active guanine triphosphate (GTP)-bound state in which these proteins can interact and induce cell signalling and effector molecules. Two families of proteins tightly regulate the activity of these GTPase proteins: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Rho GTPase specific GEFs can be divided into two main subfamilies, the classical Dbl–pleckstrin homology domain (DH-PH)-containing family and the DOCK180-related proteins containing the DOCK Homology Region (DHR) motif [36, 37]. The latter family consist of eleven mammalian members that have been implicated in a wide variety of critically important biological processes, including chemotaxis, phagocytosis, myoblast fusion and neuronal cell growth and differentiation [38-41].

The DOCK family of proteins comprises a number of GEFs that are critical activators of Rho/Rac/Cdc42 small GTPase proteins in worms, flies and mammals. Divided into 4 subfamilies (Fig3), they have little sequence homology outside their evolutionary

conserved DHR1 and DHR2 domains. The DHR1 domain in DOCK proteins binds PI3,4,5P3 lipids generated by PI3 kinase activity and promotes localized membrane binding and activation, while the DHR2 domain binds to Rho-family GTPases-proteins (Rac, Cdc42) and provides catalytic GEF activity to stimulate these proteins [37].

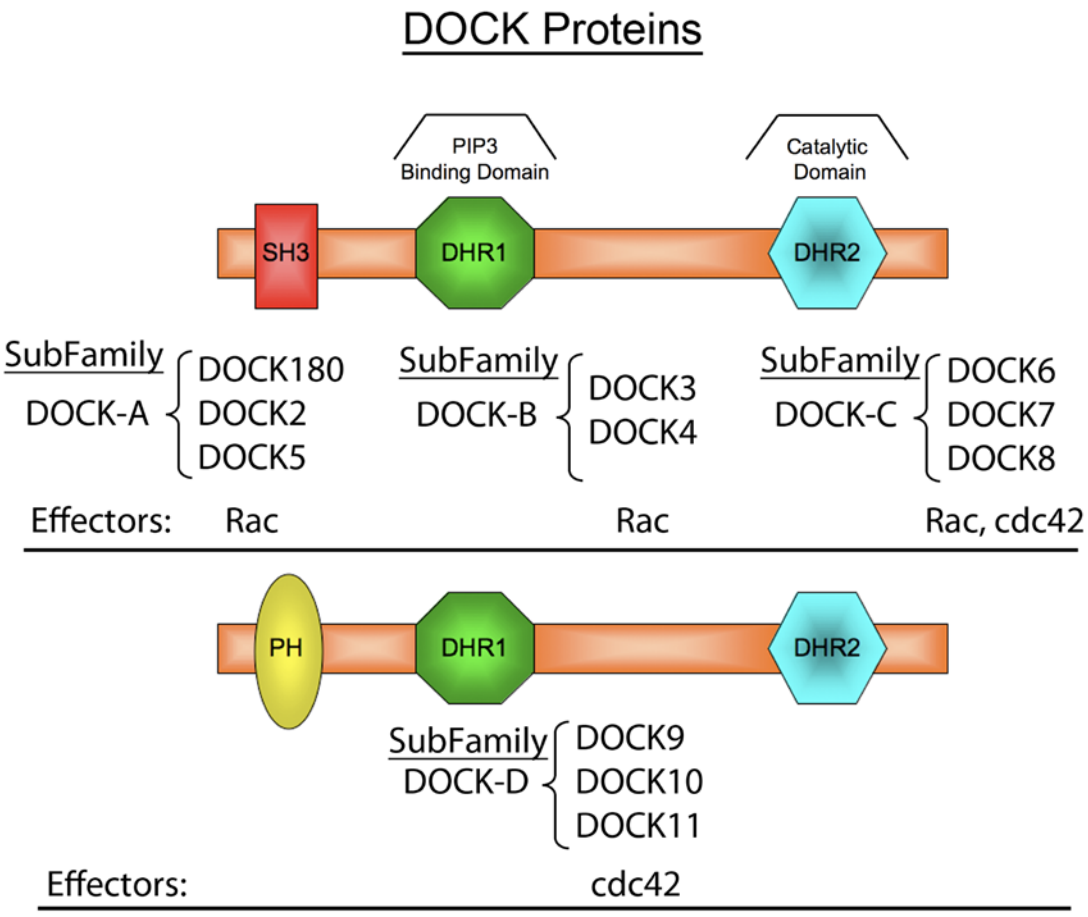


Figure 3: The DOCK family proteins and their effectors. The DOCK family proteins share little sequence homology apart from two evolutionary conserved domains. The DHR1 domain contains a phospholipid (including PIP3) binding domain whereas the DHR2 domain is important for interaction with effector molecules, such as Rac and cdc42. DOCK family proteins can be further subdivided based on sequence homology in the DHR1/2 domains and on the presence of a SH3 domain (found in DOCK-A, B

and C subfamily) and a pH domain (DOCK-D subfamily). Different subfamilies have been demonstrated to specifically interact with different downstream effectors.

1.4.2 DOCK180 and DOCK2 proteins

To date, the two best-characterized DOCK family proteins are the prototypical DOCK1 protein - DOCK180 and DOCK2. DOCK180 was cloned in 1996 during a search for proteins interacting with the proto-oncogene Crk [42]. It was later found that DOCK180 played a crucial role in Rac activation during a number of biological processes such as cell migration, phagocytosis and cell death [43-45]. Genetic screening in Caenorhabditis elegans (C.elegans) and Drosphila melanogaster lead to the discovery of DOCK180 orthologues in these species. Work carried out on the C.elegan DOCK180 ortholog Ced-5, has lead to a greater understanding of the molecular mechanisms controlling DOCK protein activity and the downstream activation of Rac. Importantly, Ced-5 was shown to regulate Ced-10 (ortholog of mammalian Rac) activity in C.elegans in conjunction with other proteins including Ced-12 (ortholog of mammalian protein ELMO) [46, 47]. Evidence now suggests that interaction between DOCK180, inactive Rac and ELMO confers catalytic activity on DOCK180 to allow effective Rac activation [46, 48]. ELMO alone does not appear to have an enzymatic activity of its own and acts simply as a scaffolding protein to facilitate this process. It may also promote the targeting of DOCK180 to the plasma membrane in response to a number of stimuli.

Despite the widespread expression of DOCK180 and its importance in cell movement, peripheral blood leukocytes show little or no expression. However, $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and neutrophils express high levels of the related

protein DOCK2 and the creation of DOCK2 knockout mice has demonstrated a critical role for DOCK2 in regulating the immune system [49-51]. DOCK2 knockout mice show severe lymphopenia with losses of $CD4^+$ and $CD8^+$ T cells and B cells in secondary lymphoid organs including the spleen and peripheral lymph nodes (PLNs). This phenotype was shown to be due to defective cell migration in response to a wide range of homeostatic chemokines including CCL19, CCL21, stroma-derived factor (SDF)-1 and CXCL13 [49]. Calcium influx, protein kinase B and ERK1/2 activation are normal in DOCK2 knockout mice compared to heterozygote littermates. However, phosphorylation of Rac1 was absent after stimulation of chemokine receptors demonstrating a crucial link between these receptors, DOCK2 and cell migration [49].

As well as deficiencies in adaptive immunity, innate responses are also limited by the absence of DOCK2. Although chemotactic responses of monocytes to chemokines SDF-1 and monocyte chemotactic protein (MCP)-1 are normal in DOCK2 deficient mice, neutrophil migration and activation by N-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol myristate acetate (PMA) are diminished, again due to lack of Rac1 activation and failure to induce polarized accumulation of F-actin [52].

1.4.3 DOCK C subfamily of proteins

The GEF DOCK8 is part of the DOCK-C subfamily of DOCK proteins, along with proteins DOCK6 and DOCK7, based on sequence homology and similarities in domain structures [41] (Fig3). DOCK6 and DOCK7 are expressed at high levels in the brain and other neuron tissues and have been shown to play a critical role in

dendrite formation, axonal growth and neurite survival [53, 54]. Recessive mutations in DOCK6 in humans are associated with Adams-Olivier Syndrome, a neurological disorder defined by the combination of aplasia cutis congenita (ACC) and terminal transverse limb defects (TTLD) [55]. In relation to this, it may be significant that two cases of mental retardation and developmental disabilities have been documented in patients found to carry DOCK8 mutations [23]. However, gene expression data shows that DOCK8, unlike DOCK6 and DOCK7, is expressed mainly in haemopoetic cell types including B and T cell subpopulations and other innate cell types including granulocytes, macrophages and dendritic cells. However, the role of DOCK8 in maintaining immune function and homeostasis is still to be investigated.

DOCK8 was first isolated in a yeast two-hybrid system screening for Cdc42-interacting proteins [56]. As well as binding to cdc42, it was also shown that Rac1 could also be targeted by DOCK8 [56]. This finding is supported by the result that DOCK6 and DOCK7 and have been demonstrated to interact with cdc42 and Rac1 respectively. However, a stable interaction between DOCK8 and Cdc42 or Rac1 could not be established using pull-down experiments and immunoprecipitations. As the yeast two-hybrid system is more sensitive and is able to detect transient transactions between proteins, this raises the possibility of a chaperone molecule being required to stabilize DOCK8 interactions with target molecules. This theory has been demonstrated to be true with reference to DOCK180 and ELMO to enhance Rac activation [46]. However, to date there is no clear candidate to carry out this role for DOCK8.

1.4.4 Mechanisms of DOCK protein catalytic function

The structural mechanisms underlying activation of GTPases by the exchange of GDP for GTP by DOCK family proteins has been difficult to elucidate. However, Yang et al through the use of crystal structures of the DOCK9 DHR2 domain in complexes with nucleotide free Cdc42, GDP-bound and GTP-bound Cdc42 have been able to reveal the structural basis of DOCK activation of GTPases [57]. As experimental evidence suggests that the main downstream target of DOCK8 is the GTPase, Cdc42, this work provides insight into how DOCK8 may function.

Initially for GEFs to activate their target molecule (such as Rac or Cdc42) they must lower the affinity of GTPases for GDP before the addition of GTP. This process of lowering affinity for GDP occurs during the interaction of the DOCK DHR2 domain with the target molecule, such as Cdc42. The work done by Yang et al firstly shows that, binding of Cdc42 to the DOCK9 DHR2 domain exposes the nucleotide binding site of GDP so it can be removed. The binding of Cdc42 also causes exclusion of Mg^{2+} from the complex by a Val residue found at position 1951 of the DHR2 domain. As Mg^{2+} is important in increasing affinity of nucleotides for GTPases [58], the exclusion of Mg^{2+} makes GDP more susceptible to removal. The importance of the Val 1951 residue is evident by the fact that it is evolutionarily conserved in all DOCK homologues from plants to humans [59]. The mutagenesis of this residue also severely disrupts DOCK catalytic function. This mechanism of Mg^{2+} exclusion is fundamentally different to other classically described GEFs as normally a conserved residue of the GTPase (e.g. Alanine residue 59 in Ras proteins) is responsible for interfering with Mg^{2+} binding as opposed to residues within the GEF itself [60, 61]. The second stage of activation of a GTPase is the substitution of GDP for GTP. Once

GDP affinity is sufficiently lowered, GDP can be displaced by GTP resulting in the activation of the GTPase. The nucleotide free form of cdc42 binds tightly to the DOCK DHR2 domain and therefore the re-attachment of nucleotide is required for the discharge of the GTPase. The binding of GTP induces conformational changes affecting both Cdc42 and the DHR2 domain. This reorganization of the complex results in the disengagement of the GTPase and increases its affinity for GTP. GDP itself does not induce equivalent conformational changes compared to GTP and therefore is out competed [57]. As mentioned previously, DOCK proteins appear to require chaperone molecules such as ELMO to stabilize such reactions.

1.4.5 Recruitment and activation of DOCK proteins

A key question concerning the role of DOCK proteins within the immune system is what upstream signals recruit and activate DOCK proteins. Based on the study of DOCK2 knockout mice, DOCK proteins may have roles downstream of chemokine receptors in regulating cell movement [50]. In support of this, DOCK8 has also been shown to be activated downstream of chemokine receptors in response to sphingosine-1-phosphate (S1P) and CCL21 in single positive thymocytes [62]. However, DOCK proteins have been shown to be activated most notably after engagement of integrins. DOCK180 is recruited in response to integrin activation to induce phagocytosis, cell adhesion and cytoskeletal rearrangements [38, 43, 44, 63, 64]. To date, this has not been formally demonstrated for DOCK8, but DOCK proteins have also been associated with inside out signaling of integrins. In the absence of DOCK2, B cells suffer from severe defects in integrin activation by CXCL13 despite comparable levels of integrin expression [49]. Failure to activate integrins has also been observed downstream of both the T cell receptor (TCR) and

the B cell receptor (BCR) in the absence of DOCK8 [10, 13]. As these defects were observed during the formation of the immune synapse, loss of DOCK proteins is expected to have consequences for the activation and differentiation of immune cells and in active immune responses.

Upon interaction of B cells with antigen, B cells undergo spreading and cytoskeleton rearrangements which allow the clustering of antigen into a central signalling structure known as the central supramolecular activation clusters (cSMAC). During the formation of this structure, other accessory molecules such as integrins and tyrosine phosphatase become segregated. Integrins such as LFA-1 form a ring around the cSMAC known as the peripheral supramolecular activation clusters (pSMAC) and stabilize the structure by interaction with ICAM-1 expressed on antigen presenting cells [65, 66].

Analysis of the activation of DOCK8 deficient B cells from our *CPM* strain using *in vitro* assays demonstrated that in the absence of DOCK8 B cell activation was intact [10]. *CPM* B cells underwent equivalent cellular division after stimulation with anti-IgM when compared to WT B cells and also up regulated activation markers including CD25 and CD86. However, when *CPM* B cells were lowered onto lipid bilayers containing fluorescently tagged molecules associated with B cell synapse formation, differences in integrin localization were observed. *CPM* B cells cluster antigen normally into the cSMAC but fail to segregate the integrin LFA-1 into the pSMAC [10]. This deficiency may have important consequences for the selection and activation of *CPM* B cells *in vivo* and may help to explain the loss of GC B cells in this model. Therefore, DOCK family proteins can have roles not only in the activation

of integrins such as LFA-1 but also their localization within structures such as the immune synapse [10, 13].

1.4.6 Summary

The DOCK family represents a unique subfamily of GEFs capable of regulating chemotaxis, phagocytosis, cell adhesion and cell survival. The family is comprised of 11 members which are expressed primarily in the nervous system and in haemopoetic cells. These proteins contain two evolutionary conserved domains, the DHR1 and DHR2 domains that are responsible for DOCK protein function. The DHR1 domain is important in the localization of these proteins to the cell membrane by interaction with phospholipids whereas the DHR2 contains the catalytic activity of the protein and has been demonstrated to be important in the regulation and activation of GTPases including Cdc42 and Rac1 proteins. Dysregulation of these proteins is predicted to affect a number of cellular processes most notably affecting cytoskeletal regulation. The importance of these proteins is demonstrated by phenotypes observed when their expression or function is disrupted. DOCK2 knockout mice have a severe loss of both T and B cells due to abolished chemokine responses due to a failure to activate Rac1. Other cell types including neutrophils are also disrupted by the absence of DOCK2. Our DOCK8 deficient strain, *CPM*, does not display such severe phenotypes. However, defects in GC B cells after immunization and in MZ B cells suggest that DOCK8 may play a more specialized role in the immune system.

1.5 Maintenance of Naïve and Memory T cell Survival

1.5.1 Factors regulating the naïve T cell pool

Guanine nucleotide exchange factors and the actin cytoskeleton have been shown to have an important role in maintaining normal T cell homeostasis. This has been shown in a number of knockout models where the removal of key GEFs, GTPases and cytoskeletal regulators has lead to abnormal T cell development, diminished T cell motility and migration and lymphopenia [41, 50, 67]. However, the mechanisms underlying these losses in T cell function are still unclear and are under investigation.

The maintenance of the naïve T cell pool is dependant on two main factors: (1), the release of new, mature naïve T cells from the thymus throughout life and (2), the maintenance of already existing naïve T cells in the peripheral lymphoid organs. $CD4^+$ and $CD8^+$ T cells are generated in the thymus after undergoing stringent selection processes to establish a pool of mature T cells with a diverse array of TCR specificities to foreign antigens while eliminating potentially harmful self reactive cells. Immature $CD4^+CD8^+$ double positive (DP) T cells possessing TCR specificities with weak affinity binding for self-peptide MHC complexes are selected in the thymus during 'positive selection' whereas T cells with high affinity binding, which are therefore potentially self reactive and harmful, are subsequently deleted during 'negative selection'. This model predicts the level of signal generated during these initial interactions between TCR and peptide-MHC complexes is extremely important in the selection process. It is also important to note at this stage that T cells that produce TCR specificities that do not recognize self-peptide MHC complexes also undergo apoptosis due to a lack of TCR stimulation. This is also true of naïve T cells in the peripheral tissues. Mature, naïve T cells in the periphery are kept alive by tonic

signaling through the TCR, demonstrated by work showing the ablation of the TCR results in the shortening of the lifespan of naïve T cells [68]. Other survival factors, most notably cytokines such as IL7 are also important [69, 70]. Therefore, signaling via the TCR is crucial for both the selection and the survival of naïve T cells throughout their lifespan.

1.5.2 Effects of cytoskeletal proteins and GEFs on thymic development

Based on the importance of TCR signaling in both positive and negative selection of T cells, much work has been done to uncover the relative importance of the different TCR signaling molecules during T cell development. Deficiencies in the Vav family of Rho guanine nucleotide exchange factors have shown these GEFs are crucial in both T and B cell development [71, 72]. Vav1-null mice show an early block in the transition from double negative (DN) to double positive (DP) T cells with a further block observed in the positive and negative selection of single positive (SP) $CD4^+$ and $CD8^+$ T cells [72]. These observations are consistent with Vav1 being involved in TCR signaling. Mice deficient in Vav2 or Vav3 undergo relatively normal T cell development. However, the use of combined knockouts of the three GEFs shows a hierarchy in the importance of these proteins. The role of Vav3 in T cell development only becomes apparent in Vav1 and Vav3 knockout mice with more severe blocks in DN to DP and DP to SP transitions [72]. In the absence of all 3 GEFs, T cell development is almost completely blocked. Thus, Vav proteins can compensate for the loss of other proteins, as illustrated by the fact that when expression of either Vav2 or Vav3 is lost, T cell development can still occur through Vav1 [72]. DOCK2 knockout mice have also been shown to have defects in TCR activation, due to failures in the regulation of synapse formation [51]. Analysis of T cell development in

the absence of DOCK2 showed similar blocks in DN to DP and DP to SP transitions [51]. Based on this, DOCK8 may play a role in the development of mature T cells. However, as seen with different Vav proteins, redundancy is apparent within this protein family and therefore DOCK proteins may provide similar compensatory effects.

Interestingly, critical roles of DOCK proteins have also been demonstrated in the colonization of the thymus by T-lymphoid progenitor cells generated in the fetal liver [73]. Work carried out by Fukui et al demonstrated the impaired migration of lymphoid progenitors to fetal thymus lobes when both DOCK180 and DOCK2 expression was lost. This phenotype was not seen when either DOCK180 or DOCK2 were expressed on their own therefore demonstrating that DOCK proteins can indeed have compensatory roles, similar to that seen in Vav knockout studies [73].

1.5.3 Tonic TCR signaling and T cell lymphopenia

As mentioned previously, tonic TCR signaling in the periphery is also a crucial factor in maintaining T cell lifespan. The mechanisms as to how tonic TCR signaling influences T cell survival are poorly understood. However, one possible mechanism is by regulating responsiveness to cytokines such as IL7. This has been demonstrated by showing naïve CD8 T cells become unresponsive to cytokines such as IL7 when transferred into MHC-I deficient hosts [74, 75]. Studies involving deletions or alterations in TCR signaling cascades show reduced T cell lifespan involves proteins including Vav proteins and the cytoskeletal protein wiskott-aldrich syndrome protein (WASp) [72, 76].

As a consequence of this, large proportions of peripheral T cells in such lymphopenic mice are $CD44^{hi}$ memory-like T cells. The most commonly used explanation for this phenomenon is increased homeostatic proliferation due to the increased availability of cytokines including IL7 and IL15 [74]. However, as TCR signaling may be important in regulating responsiveness to homeostatic cytokines, other mechanisms should also be considered. One such mechanism may be the loss of contact with regulatory cells which control/inhibit the proliferation of naïve T cells. It has been demonstrated that T regulatory populations have a crucial role in maintaining peripheral T cell tolerance by mechanisms controlling the proliferation of T cells *in vitro* and *in vivo* [77]. Therefore, loss of regulatory populations in these mice may be responsible for the expansion of $CD44^{hi}$ populations. Due to loss of cytoskeletal regulation, cells may also have intrinsic defects in their ability to interact and form contacts with regulatory elements. For example, interaction with dendritic cells has been shown to induce tolerance in CD8 T cells by ligands including PD-1 and CTLA-4 [78].

1.5.4 Role of GEFs in active immune responses

GEFs have also been demonstrated to have crucial roles in the initiation of both T cell and B cell responses. As seen in our *CPM* mutant, B cells lacking DOCK8 are able to mount primary responses to antigen but fail to maintain responses and generate long-term memory [10]. Other GEF knockout models have also shown similar defects in active immunity including defective CD8 T cell responses, impaired T cell help and diminished NK cell activation [79-81].

Through the use of immunological tools such as tetramers to detect antigen specific T cells and TCR transgenic mice, it is now possible to track immune responses and

study the factors that regulate memory induction. The adaptive immune response occurs in the draining lymph nodes of the site of infection and other secondary lymphoid organs such as the spleen. It is initiated by the migration of dendritic cells that have encountered antigen at the site of infection and migrated to the spleen and lymph nodes although other cells have been reported to induce primary T cell responses [82, 83]. The generation of antigen specific T cells in response to pathogen can be divided into 3 distinct stages. Upon encounter with antigen, an expansion phase occurs where a program of activation and differentiation is induced in antigen specific T cells to allow the formation of an effector T cell population, capable of cytokine production and cytolytic activity. This effector population is responsible for the clearance of antigen. After antigen is successfully cleared from the system, the antigen specific pool of effector cells undergo a phase of contraction where >95% of the effector T cell population die from apoptosis. However, a small proportion of antigen specific cells undergo a final phase of differentiation into a long-lived, memory population, which provides protection against further infection [2-4].

1.5.5 Factors regulating memory T cell differentiation and survival

Loss of memory T cells, in both animal models and in humans, has serious implications resulting in recurrent viral and bacterial infections and reduced life span. We now understand a wide range of factors are important in the induction and maintenance of memory cells. Generation of memory is dependent on the level of TCR stimulation at the time of antigen recognition as well as co-stimulation from activatory molecules including CD28, CD40, ICOS and OX40 [84]. Loss of responsiveness to pro-inflammatory cytokines including IL12 and IFNγ does not affect the primary expansion of antigen specific T cells but results in diminished

memory T cell formation [85]. Importantly, evidence suggests that the maintenance and control of memory T cell pool is dependant on homeostatic cytokines including IL-15 and IL-7 [2-4, 70]. This can be seen in IL-15 transgenic mice where a selective expansion of $CD8^+$ memory T cells is observed in response to higher IL-15 levels [86, 87]. Reduced $CD8^+$ memory T cell populations are also present in IL-15 and IL15Rα knockout mice [88, 89].

There is now increasing evidence that memory T cells also rely on other factors including cell-cell adhesive molecules and cytoskeletal proteins. Adoptive transfer of OT-1 effector T cells into intercellular adhesion molecule (ICAM)-1 knockout mice show reduced survival of memory T cells inferring the requirement of the integrin LFA-1 in the maintenance of memory cells [90]. However, whether this is due to the induction of survival factors downstream of LFA-1 engagement or due to the specialized localisation of memory cells to homeostatic niches is unclear. Indeed, $CD4^+$ and $CD8^+$ memory cells have been demonstrated to preferentially home to the bone marrow where cells are believed to undergo homeostatic proliferation [91, 92]. Memory $CD4^+$ T cells associate primarily with VCAM-1 expressing stromal cells, which are a prominent source of IL-7, through the integrin α4β1 [93]. Experiments interfering with α4β1/VCAM-1 binding or conditional knockout studies have demonstrated the importance of cell-cell adhesion mechanism for homing to the bone marrow [94-96].

In relation to this work, loss of DOCK8 has previously been shown to result in diminished GC B cells consequently resulting in a loss of B cell memory and

secondary antibody responses. However, at present no role of DOCK proteins have been described in primary or secondary T cell responses to antigen.

1.5.6 Summary

Maintenance of naïve T cell repertoire is important in protecting individuals from pathogen challenge. This is clear in patients with primary immunodeficiency diseases with loss of peripheral T cells. These patients normally present with susceptibility to a wide range of bacterial and viral infections due to defects in mounting immune responses. However, the selection and maintenance of this pool of naive T cells is tightly controlled by a number of factors. The development of the naïve T cell repertoire occurs within the thymus where T cells undergo selection based on their reactivity to self-peptide MHC complexes. The alteration of TCR signalling components such as GEFs including Vav proteins and DOCK2 results in blocks in T cell development. Deficiency in DOCK8 might also be expected to have effects on the selection of naïve T cells in the thymus.

Defects in TCR signalling have been demonstrated to be important in the survival of naïve T cells due to the requirement of tonic TCR signalling in relaying survival factors. In addition to this, survival factors including homeostatic cytokines such IL7 ad IL15, cell-to-cell adhesion molecules and cytoskeletal proteins are all important in cell survival. Increasing our understanding of all these factors and the mechanisms that relay these survival signals is crucial to our understanding of control of the naïve T cell repertoire.

Susceptibility of some immunocompromised patients to infection may be due primarily to an inability to mount normal effector and memory T cell responses. Through the use of animal models and experimental tools such as tetramers, the antigen specific T cell response has been measured through the course of infection and new factors involved in this response have been uncovered. Specifically, defects in TCR sensitivity due to alterations in signalling components such as GEFs have been linked to defects in responsiveness to antigen challenge and deficiencies in both effector and memory T cell populations. However, our understanding of memory cell induction and maintenance are still incomplete and different approaches are underway to identify new factors to help improve our knowledge of memory cells and ultimately, improve the design of future vaccines to induce strong protective responses in humans.

1.6 Invariant NKT development and Homeostasis

1.6.1 Importance of Natural killer T cells

Natural Killer T cells (NKT cells) are a unique population of immunoregulatory T lymphocytes that support strong immunity to cancers and microorganisms, including viruses and bacteria [97-101]. Invariant NKT cells (iNKT) form the main branch of these cells and express a semi-invariant TCR composed of a Vα14-Jα18 rearrangement which preferentially associates with the Vβ8, Vβ7, or Vβ2 gene segments. Unlike conventional T cells, NKT cells recognise glycolipids presented in the groove of an MHCI-like molecule CD1d [100, 102]. The importance of CD1d in the selection and maintenance of iNKT cells is now well documented. However, the identity of the endogenous ligand responsible for iNKT function is still highly debatable. The discovery of a naturally occurring ligand for this specific TCR rearrangement, alpha-galactosylceramide (αGalCer), has allowed the development, homeostasis and function of these cells to be well characterized using αGalCer-loaded tetramers [103].

1.6.2 Factors regulating invariant NKT cell development

iNKT cells, like conventional T cells, are dependant on the thymus for their development as both *nude* and thymectomized mice have no detectable iNKT cell population [104]. However, the selection and development of this cell type is unique and different to that of conventional T cells. iNKT cells precursors are found at an extremely low frequency in the thymus (1 in $1x10^6$ thymocytes) expressing high levels of heat stable antigen (HSA) and proliferate extensively after positive selection [105, 106]. In contrast to conventional αβ T cell development, which occurs by interaction of double positive $CD4^+CD8^+$ T cells with cortical epithelial cells

expressing MHC molecules, iNKT cells undergo positive selection by interaction with DP T cells expressing CD1d (based on knockout and chimeric models restricting the expression of CD1d to haemopoetic or epithelial cells) [105-108]. As we now understand, selection depends on additional signals to iNKT cells through SLAM receptors. SLAM receptors are expressed on DP T cells and signal via molecules including Src kinase Fyn and signalling lymphocytic activation molecule (SLAM) associated protein (SAP) [109]. X-linked lymphoproliferative syndrome (XLP) is caused by mutations in SAP and these patients also show deficiencies in iNKT cells [110]. In support of the role of SLAM receptors in positive selection of iNKT cells, SAP knockout mice show a complete block in early iNKT cell development [110].

After positive selection by DP T cells expressing CD1d and signalling by the accessory SLAM receptors, development occurs in a linear fashion with stages identified by CD44 and NK1.1 expression (along with other markers including DX5, CD4, Tbet and GATA3). Cells progress from low expression of both CD44 and NK1.1 ($CD44^{-}NK1.1^{-}$) to $CD44^{+}NK1.1^{-}$ cells before a final maturation step to become $CD44^{+}NK1.1^{+}$ (Fig4) [104, 111]. The expression of NK1.1 is the second distinct feature of iNKT cells following the expression of the Vα14-Jα18 TCR. This final stage of mature iNKT cells is a long-lived, resting population of cells found within the thymus with an as yet unknown function. Adoptive transfer experiments of thymic iNKT cells into autoimmune models such NOD mice shows the effective suppression of diabetes in these mice [112]. However, as mature iNKT cells are retained within the thymus, it is difficult to understand how they can mediate such immunoregulatory effects. In addition, no differences in thymic or T cell development

are observed in the absence of thymic iNKT cells. Therefore, the role of these cells is still under investigation.

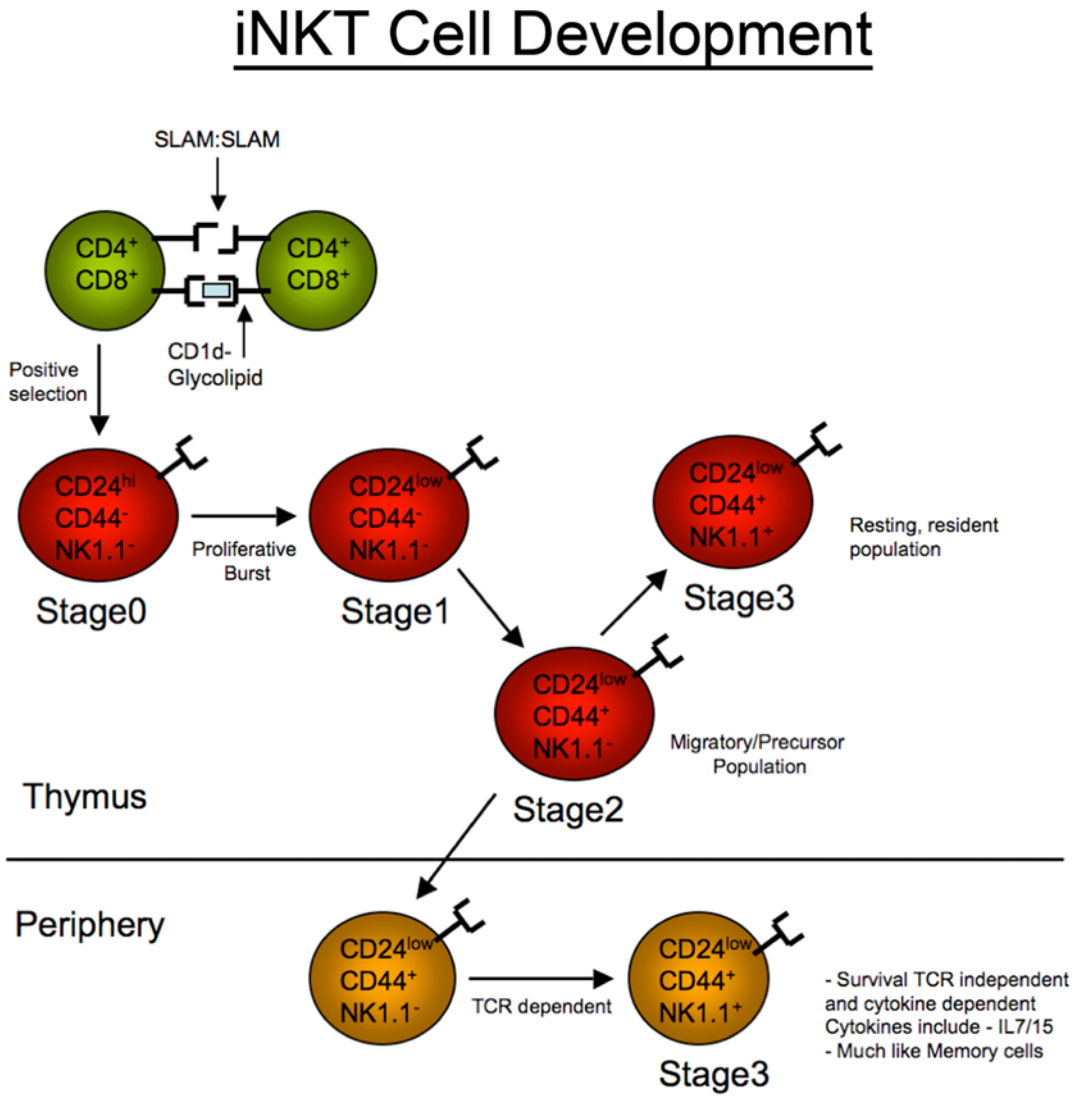


Figure 4: The development and maturation of iNKT cells. iNKT cell development is initiated by interaction of DP T cells bearing the Vα14-Jα18 TCR rearrangement with other CD1d expressing DP T cells. This interaction is also dependent on homotypic interactions mediated by SLAM receptors. Positive selection of cells induces a program of differentiation resulting in cells modulating cell markers including HSA^{hi}, CD44 and NK1.1. Stage2 cells possess the ability to egress from the thymus and undergo further development in the periphery upon interaction of CD1d. However, Stage2 cells may also remain within the thymus and undergo further

maturation in Stage3 iNKT cells. This population is a resting, resident population within the thymus.

1.6.3 Factors regulating peripheral iNKT cell function and survival

Peripheral iNKT populations are derived from the $CD44^{+}NK1.1^{-}$ population in the thymus that has been shown to egress into the blood under the influence of sphingosine-1-phosphate (S1P) [113, 114]. These cells will preferentially localize in the liver and spleen where they will up regulate NK1.1 after further interaction with CD1d molecules [115].

Although the development of iNKT cells is now well documented the factors involved in the survival and maturation of iNKT cells in the periphery are complex and uncertain. Through the use of CD1d knockout models and adoptive transfers the semi-invariant TCR and its ongoing interaction with CD1d molecules has been shown to have differing roles in each process. Maturation of $CD44^{+}NK1.1^{-}$ cells in the periphery is reliant on CD1d interaction as demonstrated by the specific failure of adoptively transferred $CD44^{+}NK1.1^{-}$ iNKT cells to up regulate NK1.1 in CD1d knockout hosts [115]. However, the survival of these cells was unimpaired for up to 6 weeks post transfer showing that iNKT survival is TCR independent. It is now accepted that factors including cytokines, cellular niches and cell-to-cell interactions via molecules such as integrins and other adhesion molecules are required [116-119].

iNKT cells do not rely on tonic TCR signalling to transduce survival signals to maintain iNKT cells. In this respect, iNKT cells are similar to memory-like populations which are dependent on cytokines such as IL7 and IL15 [120]. It has been

demonstrated that this is indeed true for iNKT cells with IL15 playing a key role in their survival, proliferation and maturation. IL15 knockout mice show reduced iNKT populations in the thymus, liver and spleen with cells showing reduced levels of survival factors such as Bcl2 [120, 121]. The addition of exogenous IL15 to iNKT cells in *in vitro* cultures has been demonstrated to increase levels of such survival factors. *In vivo*, dendritic cells are capable of trans-presentation of IL15 and are important regulators of iNKT cell turnover and activation [122]. In the absence of IL15 sensitivity, iNKT cells become unresponsive to activation. This defect can be rescued by exposing cells to IL15 prior to stimulation supporting a key role for IL15 in iNKT cell homeostasis [122].

The importance of chemokines and adhesion molecules has also been a key area of interest in understanding iNKT homeostasis and migratory behaviour. Interestingly, following adoptive transfer, splenic and liver iNKT cells show the same ability to home to both liver and spleen showing plasticity in iNKT cell migration [116]. This was an unexpected result as iNKT cells from different secondary lymphoid organs have been shown to have differential responses to a number of chemokines [113]. For example, CXCR6 has been shown to have a crucial role in the accumulation of iNKT cells within the liver. CXCR6 knockout mice show a loss in liver iNKT cells with altered homing capacity to the bone marrow [123]. Work also carried out by Thomas et al demonstrated that iNKT cells become retained in peripheral lymphoid organs and do not re-circulate [116]. This retention of iNKT cells in organs such as the liver is dependent on the expression of the signature transcription factor of the NKT cell lineage, promyelocytic leukaemia zinc finger (PLZF). Ectopic expression of this transcription factor in $CD4^+$ T cells results in altered $CD4^+$ T cell development with

cells taking on more NKT cell traits including the high expression of the integrin LFA-1 [116]. LFA-1 knockout mice show specific losses of liver iNKT cells while thymic and splenic iNKT cell populations remain intact [124]. The importance of LFA-1 in the retention of liver iNKT cells is demonstrated by the injection of LFA-1/ICAM-1 interfering antibodies into WT mice. Disruption of LFA-1/ICAM-1 complexes on iNKT cells results in the recirculation of cells into the blood. This effect is specific to LFA-1 as injection of neutralizing antibodies to other integrins including very late antigen (VLA)-4 has no effect on retention of liver iNKT cells [116].

Proteins involved in cytoskeletal rearrangements have also been implicated in the development and survival of iNKT cells. DOCK2 knockout mice show losses of all iNKT cell populations including thymic, splenic and liver cells [125, 126]. This defect is linked to the early development of iNKT cells in the thymus with a severe block in the positive selection of iNKT cell precursors. As mentioned before, this selection is based on signalling via the TCR by interaction with CD1d molecules and SLAM receptors. The block observed in DOCK2 knockouts is similar to that of SAP knockout mice. However, phenotypes observed in TCR activation in SAP deficient T cells are not seen in DOCK2 knockout T cells therefore DOCK2 may be important in signalling pathways independent of SAP. This raises the possibility that DOCK2 plays a role in signalling downstream of the TCR. DOCK2 knockout T cells do show defects in immunological synapse formation and have blocks in development of T cells when crossed to a TCR transgenic background [51]. Therefore, a role for DOCK2 in immune synapse formation and regulating TCR signalling during processes such as positive selection of iNKT cells looks likely. The cytoskeletal

protein, Wiskott-Aldrich syndrome protein (WASp) has also been implicated in iNKT cell development. Peripheral iNKT cell populations in the liver and spleen are lost with defects in iNKT maturation to $NK1.1^{+}$ state [127, 128]. Blocks in iNKT cell development are observed although this is found in the transition from $CD44^{+}NK1.1^{-}$ to $CD44^{+}NK1.1^{+}$ indicating a role of WASp in iNKT maturation. In addition to this, loss of WASp expression was associated with altered integrin expression with decreased LFA-1 staining [127, 128]. This has important implications for iNKT cell activation and homing in these mice.

1.6.4 Summary

iNKT cells have been demonstrated to regulate a wide range of immunological cascades including responses to bacterial and viral infections, autoimmune diseases and cancer immunosurveillance. Therefore, much work has been undertaken to understand the factors that regulate the activation and development of these cells in order to exploit them for therapeutic purposes. iNKT cells undergo a unique developmental pathway within the thymus in parallel to normal T cell development but development is dependent on expression of glycolipids in the context of CD1d to allow the positive selection of T cells expressing a restricted repertoire of TCR recombinations. After selection, iNKT cells undergo further maturation including the up regulation of the NK cell marker – NK1.1. This maturation step can take place in lymphoid organs such as the liver or spleen. However, iNKT maturation can also take place in the thymus, giving rise to a large population of resting, resident cells of unknown function. Several extrinsic and intrinsic factors have now been shown to regulate these cells including cytokines, integrins, chemokine receptors and

cytoskeletal proteins such as WASp. However, understanding of iNKT cell homeostasis and activation and their role in normal physiology is incomplete.

1.7 Objective of this thesis

The aim of this thesis was to investigate and understand the mechanisms underlying the immunodeficiency disease described in patients with mutations in the novel guanine nucleotide exchange factor – DOCK8. Patients deficient in this GEF have been described who suffer from a severe primary immunodeficiency disease characterized by recurrent bacterial and viral infections, atopy with high IgE levels and eosinophilia and susceptibility to cancer. Studying samples taken from these patients *ex vivo* can give insight to the status of the immune system in the absence of DOCK8. However, it is difficult to interpret findings due to ongoing infections, heterogeneity in genetics, differences in environmental factors and possible differences in ongoing treatment. Work is also limited due to access to samples and assays that can be carried out. Therefore, to achieve the above goals I carried out a detailed analysis of a DOCK8 deficient mouse strain, Captain Morgan (*CPM*). Through the use of ENU mutagenesis, our lab identified this mutant during a screening process for ENU mutants with deficiencies in humoral immunity.

Leading up to this project, analysis of B cell homeostasis and function in these mice uncovered the novel role of DOCK8 in humoral immunity. The development of *CPM* B cells was normal in the bone marrow and the secondary lymphoid organs such as the spleen. However, selective losses of MZ B cells and B1 cells were seen in the spleen and peritoneal cavity respectively. The most profound effect of DOCK8 deficiency was in the differentiation and survival of GC B cells. Loss of DOCK8 results in an intrinsic loss of GC B cells leading to a loss of high affinity antibody responses and memory B cells.

However, recurrent viral infections in patients indicates a possible defect in T cell homeostasis and activation and therefore I have carried out a detailed analysis of T cell development, T cell homeostasis in the peripheral lymphoid organs (including spleen, lymph nodes and blood) and T cell turnover and survival. To explore these pathways, I used a variety of *in vivo* and *in vitro* assays including the use of bone marrow chimeras to evaluate cell intrinsic versus extrinsic effects. These experiments confirm a loss of naïve T cells in *CPM* mice, which mirrors that seen in DOCK8 deficient patients. My results show this is due to a cell intrinsic defect in the survival of these cells.

Importantly, and in relation to the B cell phenotypes already documented in *CPM*, I investigated the ability of *CPM* T cells to undergo activation and differentiation in response to viral challenge. Inability of DOCK8 deficient cells to undergo activation and control viral infection may explain the phenotypes seen in patients. These experiments show that *CPM* mice mount normal primary responses to viral challenge, but fail to maintain memory T cells. This phenotype is similar to that of the B cell immunization defect we see in the absence of DOCK8, with initial levels of antibody produced being normal, but lost over time.

Finally, the susceptibility of patients to other immunological related phenotypes such as an increased prevalence of tumours raised the question of other cell types being regulated by DOCK8. In the remaining part of my thesis, I show that invariant natural killer T cells are decreased in *CPM* mice, with losses observed in all lymphoid organs. Unlike related knockout strains such as DOCK2 knockout mice, development of iNKT cells in *CPM* mice was normal. However, differences in the ability of iNKT

cells to undergo full maturation were observed in the thymus, spleen and liver. Defects in the spleen were rescued in mixed bone marrow settings, suggesting these defects were due to extrinsic factors such as antigen presentation. However, losses of thymic and liver iNKT were principally cell intrinsic and will contribute to the susceptibility of patients to recurrent infections and cancer.

1.8 References

1. Poland, G. and A. Barrett, The old and the new: successful vaccines of the 20th century and approaches to making vaccines for the important diseases of the 21st century. Curr Opin Immunol, 2009. 21(3): p. 305-7.
2. Sprent, J. and C.D. Surh, Generation and maintenance of memory T cells. Curr Opin Immunol, 2001. 13(2): p. 248-54.
3. Seder, R.A. and R. Ahmed, Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol, 2003. 4(9): p. 835-42.
4. Kaech, S.M., E.J. Wherry, and R. Ahmed, Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol, 2002. 2(4): p. 251-62.
5. Papathanasiou, P. and C.C. Goodnow, Connecting mammalian genome with phenome by ENU mouse mutagenesis: gene combinations specifying the immune system. Annu Rev Genet, 2005. 39: p. 241-62.
6. Vinuesa, C.G. and C.C. Goodnow, Illuminating autoimmune regulators through controlled variation of the mouse genome sequence. Immunity, 2004. 20(6): p. 669-79.
7. Cook, M.C., C.G. Vinuesa, and C.C. Goodnow, ENU-mutagenesis: insight into immune function and pathology. Curr Opin Immunol, 2006. 18(5): p. 627-33.
8. Nelms, K.A. and C.C. Goodnow, Genome-wide ENU mutagenesis to reveal immune regulators. Immunity, 2001. 15(3): p. 409-18.
9. Hoebe, K. and B. Beutler, Unraveling innate immunity using large scale N-ethyl-N-nitrosourea mutagenesis. Tissue Antigens, 2005. 65(5): p. 395-401.

10. Randall, K.L., et al., Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol, 2009. 10(12): p. 1283-91.
11. MacLennan, I.C., et al., Extrafollicular antibody responses. Immunol Rev, 2003. 194: p. 8-18.
12. Lambe, T., et al., DOCK8 is essential for T-cell survival and the maintenance of CD8+ T-cell memory. Eur J Immunol, 2011. 41(12): p. 3423-35.
13. Randall, K.L., et al., DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. J Exp Med, 2011. 208(11): p. 2305-20.
14. Engelhardt, K.R., et al., Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. J Allergy Clin Immunol, 2009. 124(6): p. 1289-302 e4.
15. Su, H.C., Dedicator of cytokinesis 8 (DOCK8) deficiency. Curr Opin Allergy Clin Immunol, 2010. 10(6): p. 515-20.
16. Su, H.C., H. Jing, and Q. Zhang, DOCK8 deficiency. Ann N Y Acad Sci, 2011. 1246: p. 26-33.
17. Zhang, Q., et al., Combined immunodeficiency associated with DOCK8 mutations. N Engl J Med, 2009. 361(21): p. 2046-55.
18. Freeman, A.F. and S.M. Holland, Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. Pediatr Res, 2009. 65(5 Pt 2): p. 32R-37R.
19. Grimbacher, B., S.M. Holland, and J.M. Puck, Hyper-IgE syndromes. Immunol Rev, 2005. 203: p. 244-50.

20. Minegishi, Y., et al., Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. Immunity, 2006. 25(5): p. 745-55.

21. Woellner, C., et al., The hyper IgE syndrome and mutations in TYK2. Immunity, 2007. 26(5): p. 535; author reply 536.

22. Barlogis, V., et al., Successful allogeneic hematopoietic stem cell transplantation for DOCK8 deficiency. J Allergy Clin Immunol, 2011. 128(2): p. 420-22 e2.

23. Griggs, B.L., et al., Dedicator of cytokinesis 8 is disrupted in two patients with mental retardation and developmental disabilities. Genomics, 2008. 91(2): p. 195-202.

24. Yang, X.O., et al., STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem, 2007. 282(13): p. 9358-63.

25. Milner, J.D., et al., Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature, 2008. 452(7188): p. 773-6.

26. Milner, J.D., N.G. Sandler, and D.C. Douek, Th17 cells, Job's syndrome and HIV: opportunities for bacterial and fungal infections. Curr Opin HIV AIDS, 2010. 5(2): p. 179-83.

27. Ghoreschi, K., et al., T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. Trends Immunol, 2011. 32(9): p. 395-401.

28. Bettelli, E., T. Korn, and V.K. Kuchroo, Th17: the third member of the effector T cell trilogy. Curr Opin Immunol, 2007. 19(6): p. 652-7.

29. Mathur, A.N., et al., Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol, 2007. 178(8): p. 4901-7.

30. Wildin, R.S., S. Smyk-Pearson, and A.H. Filipovich, Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet, 2002. 39(8): p. 537-45.

31. Umetsu, D.T. and R.H. DeKruyff, The regulation of allergy and asthma. Immunol Rev, 2006. 212: p. 238-55.

32. Lin, W., et al., Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. J Allergy Clin Immunol, 2005. 116(5): p. 1106-15.

33. Altin, J.A., et al., Decreased T-cell receptor signaling through CARD11 differentially compromises forkhead box protein 3-positive regulatory versus T(H)2 effector cells to cause allergy. J Allergy Clin Immunol, 2011. 127(5): p. 1277-85 e5.

34. Saito, M., et al., Defective IL-10 signaling in hyper-IgE syndrome results in impaired generation of tolerogenic dendritic cells and induced regulatory T cells. J Exp Med, 2011. 208(2): p. 235-49.

35. Jabara, H.H., et al., DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation. Nat Immunol, 2012. 13(6): p. 612-20.

36. Cote, J.F. and K. Vuori, Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. J Cell Sci, 2002. 115(Pt 24): p. 4901-13.

37. Cote, J.F. and K. Vuori, GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. Trends Cell Biol, 2007. 17(8): p. 383-93.

38. Albert, M.L., J.I. Kim, and R.B. Birge, alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. Nat Cell Biol, 2000. 2(12): p. 899-905.

39. Hall, A. and G. Lalli, Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring Harb Perspect Biol, 2010. 2(2): p. a001818.

40. Moore, C.A., et al., A role for the Myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion. Development, 2007. 134(17): p. 3145-53.

41. Rossman, K.L., C.J. Der, and J. Sondek, GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol, 2005. 6(2): p. 167-80.

42. Hasegawa, H., et al., DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. Mol Cell Biol, 1996. 16(4): p. 1770-6.

43. Kiyokawa, E., et al., Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev, 1998. 12(21): p. 3331-6.

44. He, X., et al., Rac1 is essential for basement membrane-dependent epiblast survival. Mol Cell Biol, 2010. 30(14): p. 3569-81.

45. Sanematsu, F., et al., DOCK180 is a Rac activator that regulates cardiovascular development by acting downstream of CXCR4. Circ Res, 2010. 107(9): p. 1102-5.

46. Gumienny, T.L., et al., CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell, 2001. 107(1): p. 27-41.

47. Wu, Y.C. and H.R. Horvitz, C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. Nature, 1998. 392(6675): p. 501-4.

48. Brugnera, E., et al., Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. Nat Cell Biol, 2002. 4(8): p. 574-82.

49. Fukui, Y., et al., Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature, 2001. 412(6849): p. 826-31.

50. Reif, K. and J. Cyster, The CDM protein DOCK2 in lymphocyte migration. Trends Cell Biol, 2002. 12(8): p. 368-73.

51. Sanui, T., et al., DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-theta and LFA-1, in T cells. Immunity, 2003. 19(1): p. 119-29.

52. Kunisaki, Y., et al., DOCK2 is a Rac activator that regulates motility and polarity during neutrophil chemotaxis. J Cell Biol, 2006. 174(5): p. 647-52.

53. Miyamoto, Y., et al., Dock6, a Dock-C subfamily guanine nucleotide exchanger, has the dual specificity for Rac1 and Cdc42 and regulates neurite outgrowth. Exp Cell Res, 2007. 313(4): p. 791-804.

54. Watabe-Uchida, M., et al., The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18. Neuron, 2006. 51(6): p. 727-39.

55. Shaheen, R., et al., Recessive mutations in DOCK6, encoding the guanidine nucleotide exchange factor DOCK6, lead to abnormal actin cytoskeleton organization and Adams-Oliver syndrome. Am J Hum Genet, 2011 89(2): p. 328-33.

56. Ruusala, A. and P. Aspenstrom, Isolation and characterisation of DOCK8, a member of the DOCK180-related regulators of cell morphology. FEBS Lett, 2004. 572(1-3): p. 159-66.

57. Yang, J., et al., Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor. Science, 2009. 325(5946): p. 1398-402.

58. Vetter, I.R. and A. Wittinghofer, The guanine nucleotide-binding switch in three dimensions. Science, 2001. 294(5545): p. 1299-304.

59. Meller, N., et al., Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. Nat Cell Biol, 2002. 4(9): p. 639-47.

60. Worthylake, D.K., K.L. Rossman, and J. Sondek, Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature, 2000. 408(6813): p. 682-8.

61. Boriack-Sjodin, P.A., et al., The structural basis of the activation of Ras by Sos. Nature, 1998. 394(6691): p. 337-43.

62. Mou, F., et al., The Mst1 and Mst2 kinases control activation of rho family GTPases and thymic egress of mature thymocytes. J Exp Med, 2012. 209(4): p. 741-59.

63. Hsu, T.Y. and Y.C. Wu, Engulfment of apoptotic cells in C. elegans is mediated by integrin alpha/SRC signaling. Curr Biol, 2010. 20(6): p. 477-86.

64. Katoh, H. and M. Negishi, RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature, 2003. 424(6947): p. 461-4.

65. Krummel, M.F. and M.M. Davis, Dynamics of the immunological synapse: finding, establishing and solidifying a connection. Curr Opin Immunol, 2002. 14(1): p. 66-74.

66. Dustin, M.L., Cell adhesion molecules and actin cytoskeleton at immune synapses and kinapses. Curr Opin Cell Biol, 2007. 19(5): p. 529-33.

67. Meller, N., S. Merlot, and C. Guda, CZH proteins: a new family of Rho-GEFs. J Cell Sci, 2005. 118(Pt 21): p. 4937-46.

68. Polic, B., et al., How alpha beta T cells deal with induced TCR alpha ablation. Proc Natl Acad Sci U S A, 2001. 98(15): p. 8744-9.

69. Tan, J.T., et al., IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A, 2001. 98(15): p. 8732-7.

70. Schluns, K.S., et al., Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol, 2000. 1(5): p. 426-32.

71. Tedford, K., et al., Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat Immunol, 2001. 2(6): p. 548-55.

72. Fujikawa, K., et al., Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. J Exp Med, 2003. 198(10): p. 1595-608.

73. Lei, Y., et al., Role of DOCK2 and DOCK180 in fetal thymus colonization. Eur J Immunol, 2009. 39(10): p. 2695-702.

74. Sprent, J. and C.D. Surh, Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nat Immunol, 2011. 12(6): p. 478-84.

75. Cho, J.H., et al., T cell receptor-dependent regulation of lipid rafts controls naive CD8+ T cell homeostasis. Immunity, 2010. 32(2): p. 214-26.

76. Cotta-de-Almeida, V., et al., Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. Proc Natl Acad Sci U S A, 2007. 104(39): p. 15424-9.

77. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol, 2003. 4(4): p. 330-6.

78. Probst, H.C., et al., Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol, 2005. 6(3): p. 280-6.

79. Gulbranson-Judge, A., et al., Defective immunoglobulin class switching in Vav-deficient mice is attributable to compromised T cell help. Eur J Immunol, 1999. 29(2): p. 477-87.

80. Penninger, J.M., et al., The oncogene product Vav is a crucial regulator of primary cytotoxic T cell responses but has no apparent role in CD28-mediated co-stimulation. Eur J Immunol, 1999. 29(5): p. 1709-18.

81. Galandrini, R., et al., Role for the Rac1 exchange factor Vav in the signaling pathways leading to NK cell cytotoxicity. J Immunol, 1999. 162(6): p. 3148-52.

82. Itano, A.A. and M.K. Jenkins, Antigen presentation to naive CD4 T cells in the lymph node. Nat Immunol, 2003. 4(8): p. 733-9.

83. Ingulli, E., et al., In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J Exp Med, 1997. 185(12): p. 2133-41.

84. Duttagupta, P.A., A.C. Boesteanu, and P.D. Katsikis, Costimulation signals for memory CD8+ T cells during viral infections. Crit Rev Immunol, 2009. 29(6): p. 469-86.

85. Keppler, S.J., et al., Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8(+) T cells. Eur J Immunol, 2009. 39(7): p. 1774-83.

86. Nishimura, H., et al., Differential roles of interleukin 15 mRNA isoforms generated by alternative splicing in immune responses in vivo. J Exp Med, 2000. 191(1): p. 157-70.

87. Marks-Konczalik, J., et al., IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. Proc Natl Acad Sci U S A, 2000. 97(21): p. 11445-50.

88. Lodolce, J.P., et al., IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity, 1998. 9(5): p. 669-76.

89. Kennedy, M.K., et al., Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med, 2000. 191(5): p. 771-80.

90. Ye, Z., et al., LFA-1 defect-induced effector/memory CD8+ T cell apoptosis is mediated via Bcl-2/Caspase pathways and associated with downregulation of CD27 and IL-15R. Mol Immunol, 2010. 47(14): p. 2411-21.

91. Mazo, I.B., et al., Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. Immunity, 2005. 22(2): p. 259-70.

92. Di Rosa, F. and A. Santoni, Memory T-cell competition for bone marrow seeding. Immunology, 2003. 108(3): p. 296-304.

93. Tokoyoda, K., et al., Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. Immunity, 2009. 30(5): p. 721-30.

94. Koni, P.A., et al., Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. J Exp Med, 2001. 193(6): p. 741-54.

95. Papayannopoulou, T., et al., Molecular pathways in bone marrow homing: dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. Blood, 2001. 98(8): p. 2403-11.

96. DeNucci, C.C., et al., Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. J Immunol, 2010. 184(5): p. 2458-67.

97. Berzins, S.P., M.J. Smyth, and A.G. Baxter, Presumed guilty: natural killer T cell defects and human disease. Nat Rev Immunol, 2011. 11(2): p. 131-42.

98. Crowe, N.Y., et al., Differential antitumor immunity mediated by NKT cell subsets in vivo. J Exp Med, 2005. 202(9): p. 1279-88.

99. Diana, J. and A. Lehuen, NKT cells: friend or foe during viral infections? Eur J Immunol, 2009. 39(12): p. 3283-91.

100. Godfrey, D.I., S. Stankovic, and A.G. Baxter, Raising the NKT cell family. Nat Immunol, 2010. 11(3): p. 197-206.

101. Tupin, E., Y. Kinjo, and M. Kronenberg, The unique role of natural killer T cells in the response to microorganisms. Nat Rev Microbiol, 2007. 5(6): p. 405-17.

102. Kinjo, Y. and M. Kronenberg, V alpha14 i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. J Clin Immunol, 2005. 25(6): p. 522-33.

103. Stronge, V.S., et al., A closer look at CD1d molecules: new horizons in studying NKT cells. Trends Immunol, 2007. 28(10): p. 455-62.

104. Das, R., D.B. Sant'Angelo, and K.E. Nichols, Transcriptional control of invariant NKT cell development. Immunol Rev, 2010. 238(1): p. 195-215.

105. Godfrey, D.I. and S.P. Berzins, Control points in NKT-cell development. Nat Rev Immunol, 2007. 7(7): p. 505-18.

106. Benlagha, K., et al., Characterization of the early stages of thymic NKT cell development. J Exp Med, 2005. 202(4): p. 485-92.

107. Griewank, K., et al., Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity, 2007. 27(5): p. 751-62.

108. Egawa, T., et al., Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity, 2005. 22(6): p. 705-16.

109. Nunez-Cruz, S., et al., Differential requirement for the SAP-Fyn interaction during NK T cell development and function. J Immunol, 2008. 181(4): p. 2311-20.

110. Pasquier, B., et al., Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med, 2005. 201(5): p. 695-701.

111. Berzins, S.P., et al., Long-term retention of mature NK1.1+ NKT cells in the thymus. J Immunol, 2006. 176(7): p. 4059-65.

112. Hammond, K.J., et al., alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. J Exp Med, 1998. 187(7): p. 1047-56.

113. Johnston, B., et al., Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. J Immunol, 2003. 171(6): p. 2960-9.

114. Allende, M.L., et al., S1P1 receptor expression regulates emergence of NKT cells in peripheral tissues. FASEB J, 2008. 22(1): p. 307-15.

115. McNab, F.W., et al., The influence of CD1d in postselection NKT cell maturation and homeostasis. J Immunol, 2005. 175(6): p. 3762-8.

116. Thomas, S.Y., et al., PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. J Exp Med, 2011. 208(6): p. 1179-88.

117. Matsuda, J.L., et al., Homeostasis of V alpha 14i NKT cells. Nat Immunol, 2002. 3(10): p. 966-74.

118. Geissmann, F., et al., Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol, 2005. 3(4): p. e113.

119. Coquet, J.M., et al., IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J Immunol, 2007. 178(5): p. 2827-34.

120. Gordy, L.E., et al., IL-15 Regulates Homeostasis and Terminal Maturation of NKT Cells. J Immunol, 2011. 187(12): p. 6335-45.

121. Townsend, M.J., et al., T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity, 2004. 20(4): p. 477-94.

122. Castillo, E.F., et al., Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation. Blood, 2010. 116(14): p. 2494-503.

123. Germanov, E., et al., Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. J Immunol, 2008. 181(1): p. 81-91.

124. Ohteki, T., et al., Cutting edge: LFA-1 is required for liver NK1.1+TCR alpha beta+ cell development: evidence that liver NK1.1+TCR alpha beta+ cells originate from multiple pathways. J Immunol, 1999. 162(7): p. 3753-6.

125. Kunisaki, Y., et al., DOCK2 is required in T cell precursors for development of Valpha14 NK T cells. J Immunol, 2006. 176(8): p. 4640-5.

126. Emoto, M., et al., Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. J Immunol, 1999. 162(9): p. 5094-8.

127. Locci, M., et al., The Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function. J Exp Med, 2009. 206(4): p. 735-42.

128. Astrakhan, A., H.D. Ochs, and D.J. Rawlings, Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. J Immunol, 2009. 182(12): p. 7370-80.

Chapter 2

Materials and Methods

2.1 Animal work

2.1.1 Animal facilities and procedures

Mice were kept in pathogen free conditions. All experiments performed at the University of Oxford were approved by the Oxford University Ethical Review Committee and were performed under UK Home Office project licence 30/2455 and personal licence 30/6284.

2.1.2 Mouse ENU mutagenesis

Male 8-15 week old C57BL/6 mice were injected 3 times at an interval of 1 per week with 100mg/kg of N-ethyl-N-nitrosourea (ENU) (Sigma) in 10% ethanol in citrate buffer, pH 5.0. After 8 weeks, ENU treated males (G0) were mated with C57BL/6 females to generate G1 progeny carrying ENU induced mutations (G1). G1 carrier mice were then mated with G0 founder strains to produce offspring, which were then inter-crossed to produce G3 progeny, which are expected to carry homozygous recessive mutations. At this stage animals were screened for immunological phenotypes of interest. Mutagenesis and initial screening were performed at the John Curtin School of Medical Research at the Australian National University (Canberra, Australia) and in accordance UCSF Institutional Animal Care and User Committee approved protocols.

2.1.3 ENU mutant captain morgan and mouse transgenics

The mouse strain, Captain Morgan (*CPM*), was derived from the ENU mutagenesis program based at the John Curtin School of Medical Research (Canberra, Australia). *CPM* mice were inter-crossed to 129 F1 mice before the use of strain specific micro-satellite markers to map the *CPM* mutation to chromosome 19. Sequencing showed that *CPM* mice contained a T-C substitution in the intron 20, leading to the disruption of the exon 20 splice donor sequence of the guanine nucleotide exchange factor, DOCK8, which results in altered gene splicing consequently inducing frameshifts and premature stop codons in this protein.

CPM mice are on a C57BL/6 background and individual mice were screened for the *CPM* mutation to confirm homozygote mutants before use in experiments. *CPM* mice were also crossed to transgenic mouse strains including UBI-GFP/BL6 mice (ordered from Jackson Laboratories) to allow tracking of DOCK8 deficient cells during adoptive transfer experiments. B6SJL CD45 mice were purchased from Biomedical Services, John Radcliffe Hospital in Oxford. These mice carry a different CD45 allotype compared to C57BL/6 mice and were therefore used as hosts for adoptive transfer experiments and the generation of bone marrow chimeras. CD103 knockout (B6.129S2(C)-Itgaetm1Cmp/J) mice were purchased from Jackson Laboratory for phenotyping purposes.

2.1.4 DNA extraction (ear punch protocol)

At the time of weaning, all mouse strains were given ear punches to identify individual mice and allow screening of mice for the *CPM* mutation or other transgenes such as GFP. DNA was extracted from ear punches by incubating the

tissue with 1mg/ml proteinase K (Sigma) in ear punch buffer (50 mM Tris HCl pH 8.0, 10 mM EDTA, 1% SDS, 2 mM NaCl) at 55°C for 40 minutes, vortexing the samples once after 20 minutes. DNA samples were then diluted 1/10 with PCR grade water and heated to 100°C for 5 minutes to inactive the proteinase K. DNA was then stored at -20°C before PCR screens were carried out.

2.1.5 Genotyping and screening of mice by PCR

As described, animals with mutation in the guanine nucleotide exchange factor DOCK8 were generated by ENU mutagenesis. Mice that were homozygous or heterozygous for the mutated DOCK8 gene were screened by PCR with 0.2 μM of each primer (wildtype screen: DOCK8 and DOCK8 WT; mutant screen: DOCK8 and DOCK8 MUT), 0.31 μM of each dNTP, 2 mM $MgCl_2$ and 60 U/μL *Taq* DNA polymerase in ammonium PCR buffer. The PCR was run for 5 mins at 95°C; 34 cycles of 10 seconds at 95°C, 30 seconds at 68°C and 45 seconds at 72°C.

DOCK8	5’ CTGAAACTCAGCATCATCTGCCTCAAC 3’
DOCK8 WT	5’ CTCCCTACCCACAGGCCTCTA 3’
DOCK8 MUT	5’ CTCCCTACCCACAGGCCTCTG 3’

Where appropriate, mice were also screened for the presence of the transgene, GFP. This screen was carried out with 0.2 μM of each primer, 0.31 μM of each dNTP, 2 mM $MgCl_2$ and 60 U/μL *Taq* DNA polymerase in ammonium PCR buffer. The PCR was run for 5 mins at 95°C; 29 cycles of 10 seconds at 95°C, 30 seconds at 64°C and 30 seconds at 72°C.

GFP (F) 5’ ACG TAA ACG GCC ACA AGT TC 3’

GFP (R) 5’ AAG TCG TGC TGC TTC ATG TG 3’

2.1.6 Generation of bone marrow chimeras

To produce bone marrow chimeras, B6SJL CD45 mice were irradiated with 2 fractions of 4.5 Grays spaced by 3 hours, reconstituted by intravenous injection with 5-10×10^6 of donor bone marrow cells and left for 8–10 weeks before analysis. For the first 3 weeks post irradiation, mouse drinking water was supplemented with the antibiotic Baytril to prevent infection by gut microflora. Donor bone marrow cells comprised of either *CPM* or C57BL/6 bone marrow to produce “Straight Chimeras” or 20:80 mixtures of B6SJL CD45 (CD45.1^+) bone marrow and either *CPM* or C57BL/6 (CD45.2^+) to generate “Mixed Chimeras”.

2.1.7 Preparation of primary lymphocytes

Mouse lymphocytes were isolated from bone marrow, thymus, lymph nodes and spleen. Briefly, spleens, thymuses and lymph nodes were forced through a nylon mesh, pelleted and re-suspended in RPMI 1640 medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum (FCS, Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin, 100 U/ml penicillin and 20 μM Hepes pH 7.4 (Gibco). Bone marrow was taken from one tibia and one femur per mouse and homogenized by forcing cells through a 23-gauge needle. White blood cells were then enumerated by staining cells with white blood cell counting fluid (1.5% acetic acid, 0.5% methyl violet in water) and counted on a haematocytometer.

2.1.8 Liver lymphocyte preparation

Lymphocytes from mouse livers were isolated in order to study and analyze liver invariant NKT cells. Briefly, after perfusion of livers by injection of 2ml PBS via the portal vein, livers were homogenized, passed through a nylon mesh, pelleted and re-suspended in 40% Percoll (100% Percoll made up as follows: 9ml Percoll, 0.5ml FCS, 0.5ml RPMI 10x stock, 50ul biocarbonate and diluted to appropriate concentrations by addition of PBS) and layered onto an equivalent volume of 80% Percoll. Cells were then spun at 800g for 25 mins with the brake left off. Lymphocytes were recovered from the interphase and washed with media containing 10% FCS twice before red blood cell lysis.

2.1.9 Intraepithelial lymphocyte preparation

Intraepithelial lymphocytes (IELs) were prepared and isolated from the small intestine (SI) of animals. Gut contents and peyers patches were removed before the intestine was opened longitudinally. The gut wall was washed using ice cold PBS before being cut into small pieces, ~ 1-2cm^2 in size. Tissue was added to 50ml Falcons containing 25ml of media containing 10% FCS and 0.5mM EDTA and incubated at 37°C for 45mins. Supernatants were then collected, filtered through a nylon mesh, pelleted, re-suspended in 40% Percoll (as done during “Liver Lymphocyte Preparation”) and overlaid onto 70% Percoll. After being spun at 800g for 20mins with the brake off, lymphocytes were recovered from the interphase and washed with RPMI containing 10% FCS before cell staining.

2.2 Cellular Based Immunology Assays

2.2.1 Flow cytometry staining

$5x10^5$ cells were plated in 96 U-bottom plates before staining with fluorescently-conjugated antibodies against cellular markers diluted in staining media (HANKS buffer supplemented with 2% FCS, 2% NaAzide and Hepes). Antibodies were from B.D. Pharmingen or eBioscience® unless otherwise stated. FITC-conjugated antibodies were against: CD24, BrdU, CD8α, CD62L, CD18, CD103 and CD44. Phycoerythrin-(PE) conjugated antibodies were against: CD5, CD3, CD69, CD62L, CD45.1, CD25, TcR, IL4, IFNγ, CD11α, Bcl-2 and CD4. PE-Cy7 conjugated antibodies were against B220, CD69, CD8α, CD4 and NK1.1. PerCp-Cy5.5 conjugated antibodies were against CD8α and B220. Allophycocyanin-(APC) conjugated antibodies were against: CD44 and CD4. APC-Cy7 conjugated antibodies were against CD4 and CD45.1. Pacific Blue (PB) conjugated antibodies were against B220 and CD45.1. QDot650 and QDot605 conjugated antibodies were against CD4 and CD44. Near-Infrared and Aqua Invitrogen Live/dead cell kits were used to identify dead cells.

CD1d-αGalCer loaded tetramers were kindly provided by Vincenzo Cerundolo for the purposes of researching the role of DOCK8 in iNKT cells. Briefly, CD1d heavy chain containing a BirA tag and β_2M were synthesized using a prokaryotic expression system (pET, Novagen). Both proteins were expressed in Escherichia coli strain BL21 and isolated from inclusion bodies. Oxidative refolding chromatography was then used for *in vitro* refolding of CD1d-β_2M complexes in the presence of αGalCer. This refolding mixture was then concentrated, incubated overnight at room temperature with a BirA ligase enzyme in the presence of biotin for labelling purposes, before

purification of CD1d-αGalCer-biotin complexes by size-exclusion chromatography using a HiLoad 26/60 Superdex-75 (Amersham Pharmacia). CD1d-αGalCer loaded tetramers were then conjugated to strepavidin-allophycocyanin-(APC) to allow detection of tetramers by flow cytometry.

All FACS samples were collected on FACSCanto™ or LSRII machines (B.D.) and analysed with FlowJo (Tree Star, Inc.) or FACSDiva (B.D.).

2.2.2 Intracellular flow cytometry staining

The BD Cytofix/Cytoperm staining kit was used for staining of intracellular, cytoplasmic markers. Firstly, cells were stained as described in "Flow Cytometry Staining". After cell surface staining, cells were fixed with 100ul of Fixation solution for 10mins on ice before carrying out multiple washes with permeabilization solution (diluted 1/10 in distilled water). Cells were then stained with appropriate antibodies for intracellular targets diluted in permeabilization buffer on ice for 20mins. Once intracellular staining was complete, cells were washed twice with permeabilization buffer and twice with staining media before collecting samples on FACSCanto™ or LSRII machines (B.D.).

2.2.3 Intracellular staining for transcription factors

Staining for transcriptional factors, Tbet and GATA3 was carried out using the Foxp3 Staining Buffer Set from eBioscience. Cells were stained as described previously for surface markers, before fixation using Fixation/Permeabilization Concentrate diluted 1/4 in Fixation/Permeabilization diluent (all buffers provided in kit). Cells were fixed for 2 hours on ice before washing with permeabilization buffer and staining for

transcription factors Tbet and GATA3 diluted appropriately in permeabilization buffer. Cells were then washed and samples collected on FACSCanto^{TM} or LSRII machines (B.D.).

2.2.4 Cell sorting experiments

To isolate pure populations of cells (>99% purity) cells were stained with the flow cytometry protocols described above and run on a Beckman Coulter Legacy MoFlo MLS High Speed Cell Sorter at the flow cytometry core facility within the Jenner Institute, Oxford.

2.2.5 Magnetic-Activated Cell Sorting (MACS)

To carry out enrichment of rare lymphocyte populations such as iNKT cells, magnetic-activated cell sorting was carried out. All cell enrichments were carried out with 'negative selection' protocols to prevent the inadvertent alteration of cellular behaviour. Lymphocytes were stained with combinations of biotinylated antibodies including anti-B220, CD8α, Ter119, CD11c and Ly-6G (20mins on ice) before washing and addition of 15ul of strepavidin microbeads for 10mins on ice (Miltenyi Biotech). Samples were then run on LS columns (Miltenyi Biotech) and enriched cells were collected in the flow through and analyzed by flow cytometry to confirm depletion was successful.

2.2.6 *In vitro* chemotaxis assays

Chemotaxis assays were performed to assess chemotactic responses of primary lymphocytes. To carry out *in vitro* chemotaxis assays, Costar® Transwell® Permeable Supports were used in 12 well tissue culture plates. In principle, cells are

separated from chemoattractants by a permeable cell membrane. Cells from *CPM*, wildtype (WT) and B6SJL CD45 transgenics were isolated from primary and secondary lymphoid organs and prepared as normal. *CPM* or WT cells were mixed at 1:1 ratios and placed on the upper layer of transwell chambers. Separated by the permeable cell membrane, different chemoattractants including sphingo-1-phosphate (S1P) (used at 1000nM, 100nM and 10nM), CCL19 (1μg/ml), CCL21 (1μg/ml), CXCL12 (0.3μg/ml) and CCL25 (3.33μg/ml) were added to the lower chambers of the plate. Cells were incubated for 3 hours at 37°C before analysis of migratory cells by flow cytometry. Migration was measured as a percentage of input cells and *CPM* or WT cells were compared directly to CD45.1 WT cells.

2.2.7 T cell activation

To assess T cell activation, splenocytes were plated in 96 well plates coated with anti-CD3 antibody at 10μg/ml (145-2C11) and 10μg/ml anti-CD28 antibody (37.51) in PBS. After excess antibody was washed off coated plates, cells were plated at $5x10^5$ cells/well and incubated at 37°C overnight in RPMI 1640 containing 10% FCS before analysis of activation markers including CD25 and CD69 by flow cytometry.

2.2.8 T cell proliferation assayed by CFSE labelling

To assess proliferation, WT and *CPM* lymphocytes were labelled with Carboxyfluorescein Succinimidyl Ester (CFSE) and cultured for 3 days. To label cells with CFSE, cells were re-suspended at $2x10^6$ cells/ml and labelled for 8 min at room temperature with an equivalent volume of CFSE (final 2μM). The reaction was quenched by the addition of FCS and washed 3 times with RPMI-1640 containing

10% FCS. $2x10^5$ CFSE labelled cells were cultured on anti-CD3 and CD28 coated plates for 3 days at 37°C and analyzed by flow cytometry.

2.2.9 Identification of T helper subsets by intracellular cytokine staining (ICS)

To assess biases in T cell activation to different cytokine producing populations, lymphocytes from the spleen and lymph nodes were stimulated for 6 hours before intracellular cytokine staining was carried out. Firstly, cells were plated in 96-F bottom plates coated with 10μg/ml anti-CD3 antibody (145-2C11) at $2x10^5$ cells/well and incubated at 37°C. After 2 hours, GolgiPlug^{TM} was added to all cells for the final 4 hours of the stimulation. After 6 hours of stimulation, cells were stained for cytokines including IFNγ, IL4 and IL17. Cells were stained for surface markers, fixed and permeabilized using the BD Cytofix/Cytoperm staining kit.

2.2.10 $CD4^+$ T cell differentiation cultures

To assess differentiation of $CD4^+$ T cells, WT and *CPM* lymphocytes were CFSE-labeled as described and stimulated for 5 days with plate bound anti-CD3 (10μg/ml). Cultures were supplemented with IL2 (10μg/ml) (neutral conditions); IL2 (10ng/ml), IL12 (10ng/ml) and anti-IL4 (10μg/ml) (TH1 conditions); or IL2 (10ng/ml), IL4 (10ng/ml) and anti-IL12 (10ug/ml) (TH2 conditions). All cytokines and neutralizing antibodies were purchased from Peprotech. After 5 days of culture, to measure T cell differentiation into appropriate T helper cell subsets, cells were stained for transcriptional factors Tbet and GATA3. Analysis was carried out on live, $CD4^+$, $CFSE^{low}$ cells.

2.2.11 Adoptive transfers of primary lymphocytes

Cells were enriched from WT GFP, *CPM* GFP and WT mice by negative selection using the magnetic assisted cell sorting (MACS) enrichment protocol already described. Enriched cells were then mixed 1:1 with *CPM* GFP or WT GFP cells and transferred into B6SJL CD45 transgenic mice by intravenous injection (i.v.). Recipients were sacrificed at designated times and cell populations were analyzed for adoptively transferred cells.

2.2.12 Analysis of cell turnover by bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU) (Sigma/ B.D.) was given to animals in their drinking water at 0.25mg/ml and supplemented with 1% glucose (Sigma), for up to 15 days. Recipients were sacrificed at designated time points. Cells were then stained using BD Pharmingen™ BrdU Flow Kit. Briefly, cells were stained with cell surface markers before fixation and permeabilization. To expose incorporated BrdU, cells were then treated with 100ul of 300μg/ml DNAse for 1 hour at 37°C. Cells were then stained with FITC-conjugated anti-BrdU 3D4 antibody (B.D. Pharmingen) before collection on FACSCanto™ or LSRII machines (B.D.)

2.2.13 *In vivo* activation iNKT cell populations

To activate iNKT cells *in vivo*, *CPM* and WT mice were intravenously injected (i.v.) with 1ug of the glycolipid, α-galactosylceramide (αGalCer) or PBS and left for 1hour before the harvesting of organs. After isolating lymphocytes from livers and preparation of splenocytes, cells were cultured for a further 4 hours in GolgiPlug (BD Biosciences) before carrying out intracellular cytokine staining as described previously.

2.2.14 Cytokine cultures

Liver and thymic iNKT cells from *CPM* and WT mice were cultured for 3 days in different cytokine milieu (IL2, IL7, IL15, IL21) and stained with Live/dead kit (Invitrogen) to test for cell viability. $1x10^6$ cells were plated in 96-F bottom plates for these purposes. All cytokines were purchased from Peprotech and used at 20ng/ml.

2.2.15 RNA extraction

Due to the nature of the RNA extraction experiments carried out, the RNAqueous®-Micro Kit from Ambion was used as it is specialized in RNA extraction from limited numbers of cells. In brief, RNA was isolated from flow cytometry sorted populations from WT and *CPM* mice. Cells were pelleted, lyzed, and passed through silica-based filter that selectively binds RNA. After washes were carried out with specialized buffers (provided with kit), RNA was eluted and quantified by spectral density.

2.2.16 Microarray experiments

Commercially available high-density oligonucleotide, MouseWG-6_V2 chips from Illumina, (Illumina Inc, San Diego, California, USA) were used for whole-genome gene expression analysis. In brief, 500ng of total RNA was reverse transcribed to synthesize first and second strand complementary DNA (cDNA), followed by *in vitro* transcription to synthesize biotin-labelled complementary RNA (cRNA) using TotalPrep-96 RNA amplification kit from Ambion accordingly (Ambion Inc, Austin, TX). A total of 1500ng of biotin-labelled cRNA from each sample was used in the hybridization process at 58C for 18 h. The hybridized BeadChip was washed and labelled with streptavidin-Cy3 according to the manufacture's protocols.

2.3 Active Immunity

2.3.1 Anaesthesia

To carry out immunization experiments, animals were first anaesthetised to ensure the safe delivery of viruses. Mice were anaesthetized using a mixture of 75mg/kg Ketaset^{TM} (ketamine hydrochloride) plus 1mg/kg Domitor^{TM} (medetomidine hydrochloride) diluted in endotoxin-free water and administered by intraperitoneal injection (i.p.). After procedures were carried out on anaesthetized mice, mice were given a 100ul i.p. injection of a 1:10 solution of Antisedan^{TM} (atipamezole hydrochloride) diluted in endotoxin-free water. Animals were monitored for up to 30 minutes after Antisedan injection to ensure recovery from the anaesthetic was successful.

2.3.2 Modified vaccina virus and influenza challenge

To investigate T cell responses to viral challenge, mice were immunized with both attenuated and virulent viral strains and viral responses were tracked in the blood and secondary lymphoid organs of challenged animals. As described above, mice were anaesthetised prior to immunization. Mice were immunised intradermally (I.D.) with Modified Vaccinia Ankara (MVA) expressing green fluorescent protein at a dose of $1x10^6$ PFU/mouse ($2x10^7$ PFU/ml) or infected intranasally with 25µl of Influenza X-31 (A/Aichi/68; H3N2) at a dose of 512 HAU/ml. For mice infected with live influenza X-31 strain, mice were weighed daily as an indicator of health up to 10 days post infection. Animals reaching 80% of original body weight were humanely culled. Mice were sacrificed at designated time points to assess the level of immune response produced.

2.3.3 Viral peptides

Detection of anti-viral responses in challenged animals was carried out by *in vitro* stimulation of lymphocytes with viral peptides combined with intracellular cytokine staining. All peptides used in cellular assays were commercially synthesised by Protein Peptide Research or Sigma Aldrich, UK and dissolved in DMSO at a concentration of 20mg/ml and stored at -80°C. Working stocks were made by further dilution to 1mg/ml in PBS and stored at -20°C. In all cultures, peptides were used at a final concentration of 1-2ng/ml.

2.3.4 *In vitro* identification of antigen specific cells

To identify antigen-specific cells, lymphocytes isolated from individual mice were stimulated *in vitro* using peptides derived from MVA virus including the B8R epitope (sequence TSYFKESV) or a 1µg/well of pooled 20mer peptides spanning conserved internal nucleoprotein and matrix 1 protein epitopes of the influenza virus. Antibodies against LAMP-1 were also added to cells at the time of stimulation to stain antigen specific cells. After 2 hours of stimulation at 37°C, GolgiPlug^{TM} (BD Biosciences) was added to all samples and cells were cultured for a further 4 hours. After 6 hours of stimulation in total, cells were stained for IFNγ, TNF-α and IL-2 using the BD Cytofix/Cytoperm staining kit as described previously.

2.3.5 Statistical Data Analysis

Statistical comparisons were performed with Prism 4.0 Software (GraphPad Inc.), using ANOVA or two-tailed paired t-tests for comparisons.

Chapter 3

The Novel Role of DOCK8 in the Maintenance and Survival of Naïve T cells

3.1 The use of *CPM* to examine the role of DOCK8 in T cell homeostasis

Sufferers of immunodeficiency diseases normally present with a wide range of recurrent bacterial and viral infections with low peripheral T cell numbers. However, the genetic aetiologies of such diseases are complex and still to be elucidated. The identification of the genetic factors involved in some primary immunodeficiencies, such as Wiskott-Aldrich Syndrome (WAS, deficiency in Wiskott-Aldrich protein (WASp)) and X-linked lymphoproliferative disease (XLD, deficiency in signalling lymphocytic activation molecule-associated protein (SAP)) has improved our knowledge of these immunodeficiencies and advanced the diagnosis and treatment of these patients. Therefore, understanding the genetic factors underlying further diseases will also help to improve diagnosis for these patients and contribute to the development of novel therapies.

Through the genetic screening of patients suffering from AR-HIES, the guanine nucleotide exchange factor, DOCK8, was identified as an important disease factor in these patients [1]. In parallel to this, the discovery of an ENU mutant, Captain Morgan (*CPM*), has allowed for the role of DOCK8 within the immune system to be further characterized [2]. These original findings established that loss of DOCK8 resulted in the loss of antibody responses, which was later confirmed in DOCK8 deficient patients [3]. However, based on the phenotypes seen in AR-HIES patients, I concluded that it was likely that DOCK8 would also play a crucial role in maintaining the naïve T cell repertoire and would be important in active immune responses.

To understand the precise role of DOCK8 in T cell immunity, I have carried out a detailed analysis of the naïve T cell compartment in *CPM* mice. Processes including the positive and negative selection of T cells in the thymus, chemotactic responses of peripheral T cells and T cell turnover and survival were all examined.

3.2 T-cell lymphopenia in DOCK8 deficient humans

In collaboration with Helen Su from the NIH (Bethesda, USA), blood samples were collected from a cohort of patients suffering from DOCK8 deficiency. Flow cytometry staining was carried out on these samples and numbers of naïve CD45RA and activated effector/memory CD45RO $CD4^+$ and $CD8^+$ T cells were calculated and compared to healthy donors. This work revealed a reduction in both naïve and effector/memory T cells, although the latter populations appeared less affected and even normal in some patients (Fig5). It is difficult to interpret the significance of these results as patients are constantly exposed to a wide range of infectious agents and are undergoing different levels of treatment based on the severity of their disease.

To overcome the some of the difficulties of studying human cells *ex vivo*, I began a detailed analysis of our recently described ENU mutant, *CPM*, which carries homozygous null alleles of DOCK8. The *CPM* splice site mutation truncates the native protein, removing the DHR2 GEF domain inducing a functional DOCK8 knockout. Firstly, to investigate the effects of DOCK8 deficiency on T cells, I enumerated $CD4^+$ and $CD8^+$ T cells in the secondary lymph organs of DOCK8-deficient *CPM* mice. In this work, I am grateful for the day to day supervision of Teresa Lambe, a postdoctoral scientist in the lab, who helped me with experiments and contributed to the T cell data.

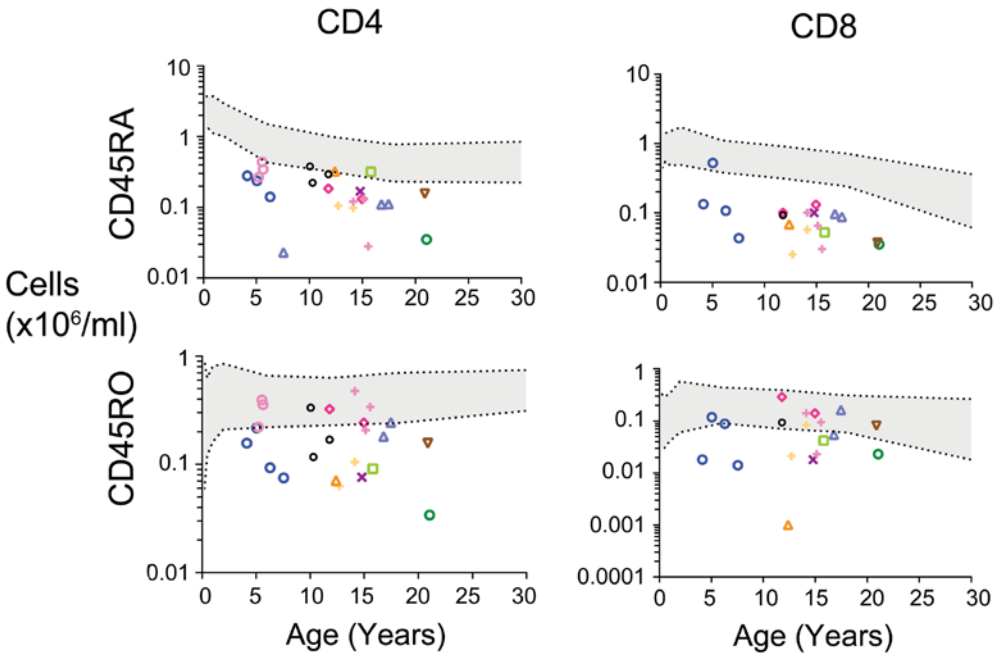


Figure 5: T cell lymphopenia observed in human DOCK8 deficiency. A) Blood samples were taken from DOCK8 deficient patients and healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from samples and staining for CD4, CD8, CD45RA and CD45RO) was carried out to enumerate levels of T cells. Unique symbols represent individual patients over time. Grey area defines healthy range showing the normal 10-90 percentiles for age. Data are displayed as cells/ml. Data generated by Helen Su at the NIH.

3.3 T cell lymphopenia in Captain Morgan (*CPM*)

Examination of spleen, mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLNs) revealed a 2-3 fold reduction in $CD4^+$ and $CD8^+$ T cells compared with WT C57BL/6 controls (Fig6A). Total $CD4^+$ and $CD8^+$ T cells were also reduced in the blood as a percentage of total lymphocytes (Fig6B, upper panel). In contrast, numbers of activated $CD44^{hi}/CD62L^{lo}$ $CD4^+$ and $CD8^+$ T cells were found at similar levels in the spleen and PLNs and were significantly increased in the MLN of *CPM* mice

compared to WT (Fig6A). The increased levels of activated CD44^{hi} CD4^{+} and CD8^{+} T cells were also seen in the blood of *CPM* mice (Fig6B, lower panel).

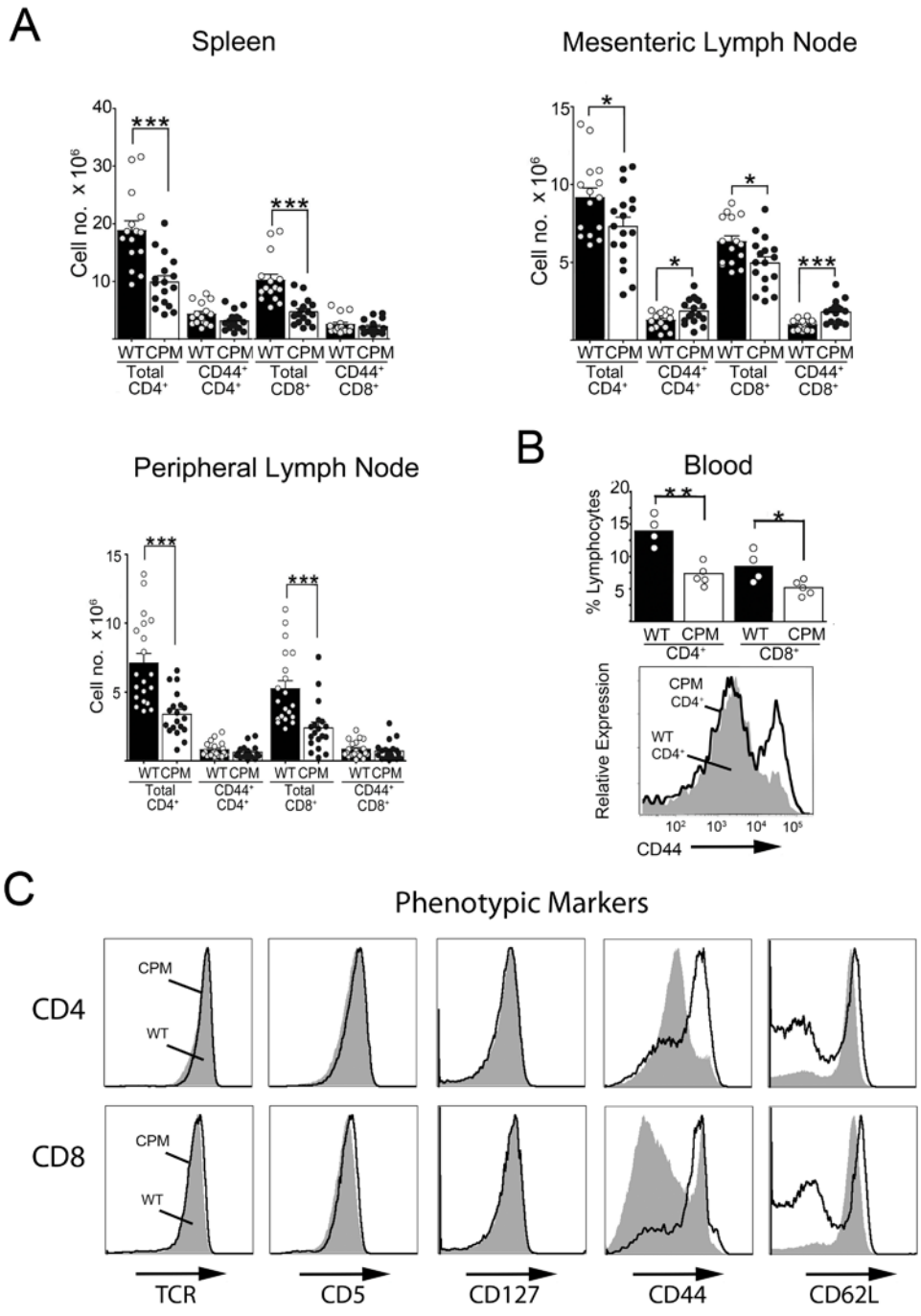


Figure 6: DOCK8 mutation results in reductions of naive peripheral T cells. A) Absolute numbers of total CD4^{+} and CD8^{+} T cell populations and activated CD44^{+}subsets in the peripheral lymphoid organs of WT and *CPM* mice. Dots

represent individual animals aged 9-14 weeks (Data points partly contributed by Teresa Lambe). Bars show arithmetic means and SE with significant differences by t-test *p<0.05, **p<0.01, ***p<0.001. B) Percentage of $CD4^+$ and $CD8^+$ T cells in the blood of WT and *CPM* mice calculated as a fraction of total lymphocytes (t-test *p<0.05, **p<0.01). Lower histograms showing typical surface expression of CD44 on $CD4^+$ T cells in the blood of WT (filled) and *CPM* mice (dark line). C) Histograms depicting representative expression profiles of common T cell phenotypic markers for $CD4^+$ and $CD8^+$ T cells from the spleen of WT (filled) and *CPM* mice (dark line).

Examination of peripheral T cells for further activation and homeostatic markers including the TCR, CD5 and IL7R (CD127) revealed no differences in expression (Fig6C). However, as alluded to by the loss of total $CD4^+$ and $CD8^+$ T cell numbers with relative preservation of $CD44^{hi}$ cells, percentages of $CD62L^{hi}$ T cells were reduced. From these data, I conclude that DOCK8 deficiency in mice leads to peripheral T-cell lymphopenia predominantly affecting the number of naïve rather than activated cells, reminiscent of the phenotype in the human immunodeficiency syndrome.

3.4 T cell development in *CPM* mutants

As previously discussed, guanine nucleotide exchange factors such as DOCK2 and Vav proteins have important roles in the development of T cells by regulating TCR sensitivity to self-peptide MHC molecules and therefore influence processes such as positive and negative selection [4, 5]. Therefore, I looked at the possibility that abnormal thymic development and differences in the positive and negative selection of T cells might explain the reduction in peripheral T cells. Initial profiling of

thymocytes by CD4 and CD8 staining uncovered no significant changes in T cell development although trends were observed in higher proportions of single positive $CD4^+$ T cells (Fig7).

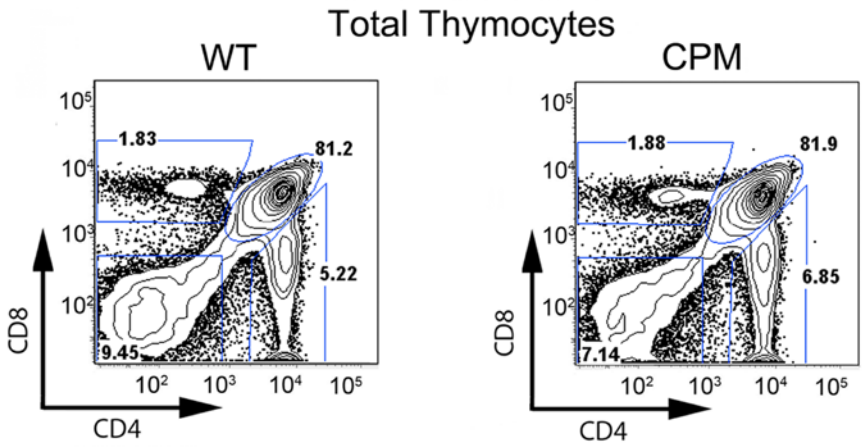
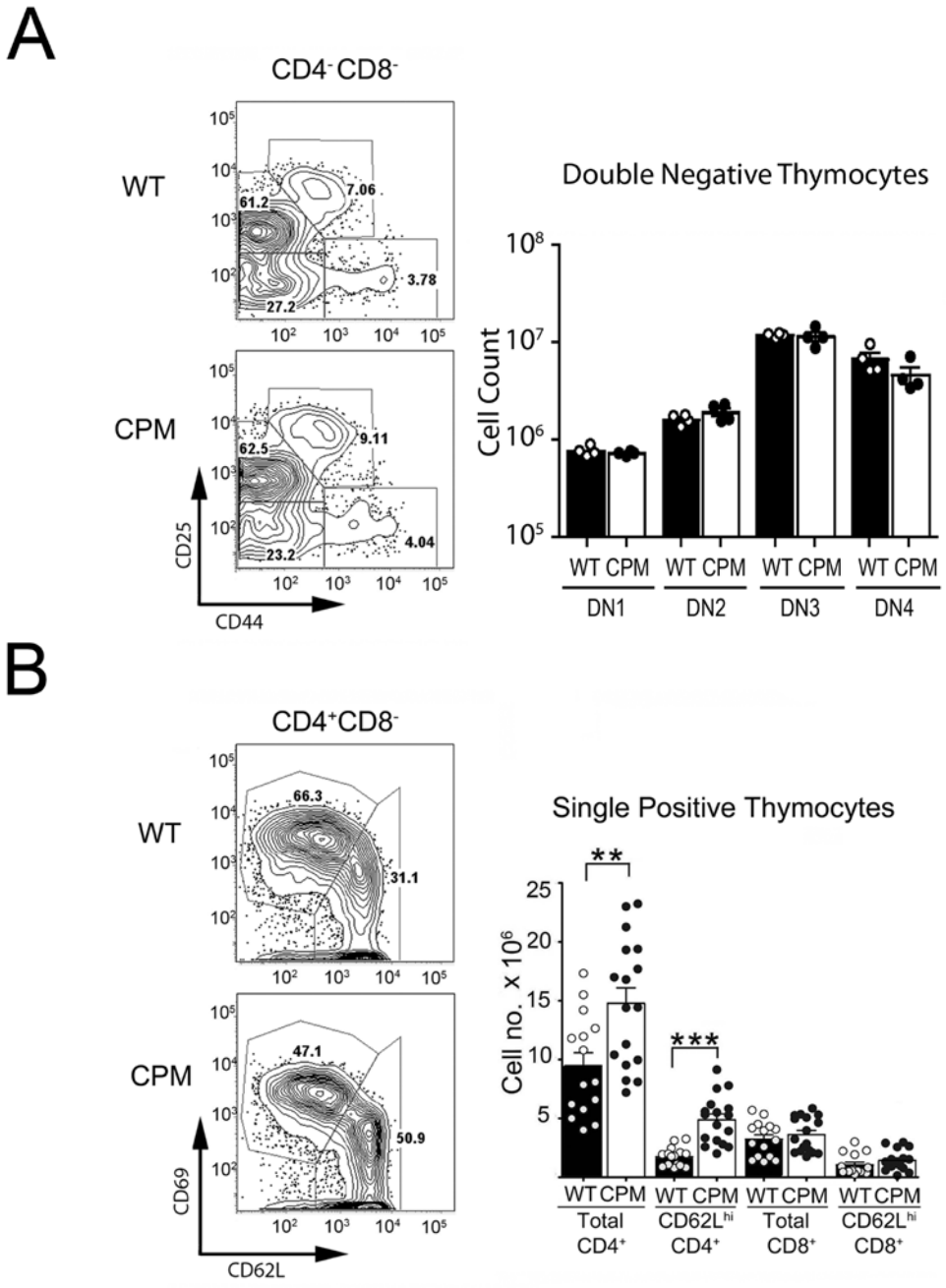


Figure 7: T cell development appears grossly normal in *CPM* mice. A) Typical FACS staining of DN, DP and SP thymocytes from WT and *CPM* mice with percentages of lymphocytes based on CD4 and CD8 staining depicted.

Therefore, I carried out a detailed analysis of the individual stages of T cell development. Numbers of early progenitor T cells at the double negative stage of T cell development were normal through all transitional stages (DN1, $CD44^+$ $CD25^-$; DN2, $CD44^+$ $CD25^+$; DN3, $CD44^-$ $CD25^+$; DN4, $CD44^-$ $CD25^-$) (Fig8A). The sole abnormality observed during T cell development was an increased number of mature, single positive (SP) $CD4^+CD62L^{hi}CD69^{lo}$ T cells (Fig8B). However, this same increase was not observed in the case of SP $CD8^+$ T cells and therefore I do not believe this presents a sufficient explanation for the peripheral lymphopenia in *CPM* mice. Further analysis of SP T cells showed no differences in expression of cell survival factors such as IL7R (CD127) or in other markers related to the selection of

SP T cells such as CD5 and TCR (Fig8C). As these markers are unchanged, it seems unlikely that T cell selection processes within the thymus are significantly affected by the absence of DOCK8.



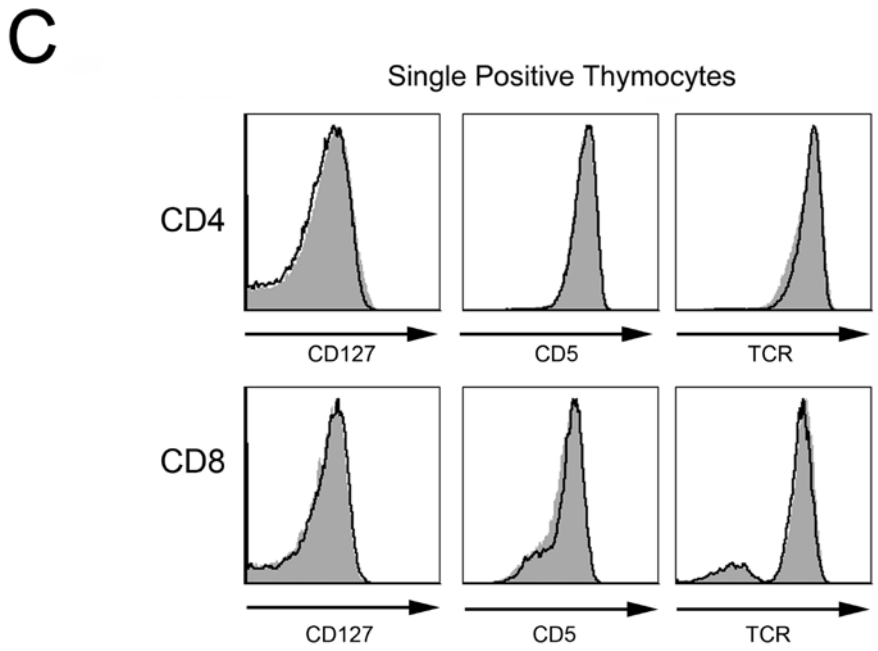


Figure 8: Detailed analysis of thymic development in *CPM* mice. A) Earlier stages of DN thymocyte development are shown with illustrative FACS staining of individual stages based on CD44 and CD25. Cells numbers were enumerated at each stage. B) Late stages of thymocyte development are depicted by representative FACS plots showing CD69 and CD62L staining. Cells numbers were enumerated for both $CD4^+$ and $CD8^+$ SP T cells. Dots represent individual animals aged 9-14 weeks (Data points partly contributed by Teresa Lambe). Bars show arithmetic means and SEM with significant differences by t-test *p<0.05, **p<0.01, ***p<0.001. C) Representative histograms show expression levels of maturation markers of both SP $CD4^+$ and $CD8^+$ T cells with WT (filled) and *CPM* mice (dark line).

3.5 Single positive T cells are generated at normal levels

To investigate the production of single positive T cells in the thymus, I carried out bromodeoxyuridine (BrdU) labelling experiments to analyze cell turnover and transition through different stages of thymic development. Labelling experiments

showed that equivalent levels of BrdU labelling were seen at all stages in T cell development (Fig9A). Notably, no differences in the absolute number of $BrdU^+$ SP $CD4^+$ T cells were seen ($1.00 \times 10^6 \pm$ SE 0.14 in *CPM* vs. $1.33 \times 10^6 \pm$ SE 0.18 in WT, p=0.1674; unpaired t-test), implying that the increased levels of mature *CPM* $CD4^+$ T cells were not due to increased production of SP $CD4^+$ T cells (Fig9B).

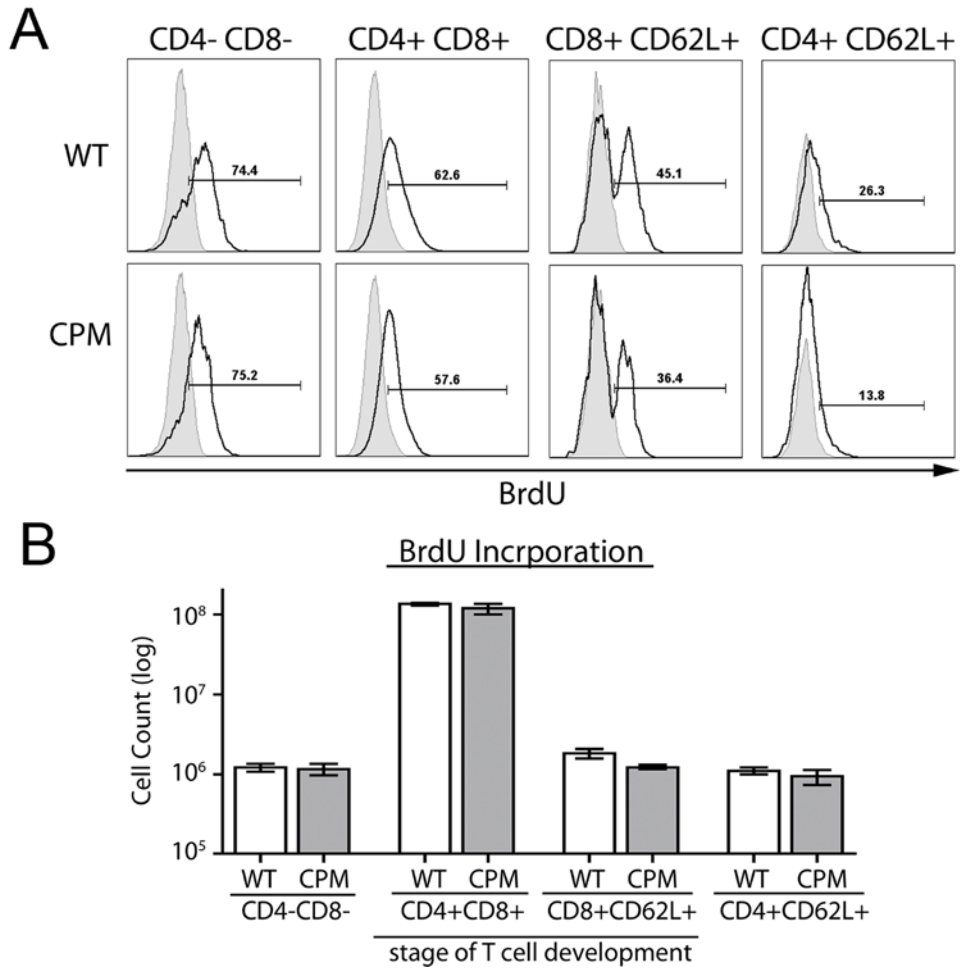


Figure 9: Production of SP thymocytes investigated by BrdU staining. A) Representative FACS histograms depicted different stages of thymocyte development and BrdU incorporation of naïve (filled) and BrdU treated animals (dark line). B) $BrdU^+$ thymocytes were enumerated at each stage of development. Bars show arithmetic means and SEM with significant differences by t-test.

3.6 T cell lymphopenia in *CPM* mice is a cell intrinsic trait

To examine if T cell lymphopenia in *CPM* mice was a cell intrinsic defect I reconstituted lethally irradiated CD45.1 allotype-marked recipients with *CPM* bone marrow (straight chimeras) and with 20:80 mixtures of WT (CD45.1) and *CPM* or WT (CD45.2) bone marrow (mixed chimeras). Normally, 50:50 mixtures are generated for the purposes of such experiments. However, although the expected 1:1 ratio of follicular B cells is generated in mixed chimeras using WT: *CPM* bone marrow, they fail to reconstitute normal numbers of *CPM* T cells due to a block in T cell development prior to the DN stage. This defect was not seen in unmanipulated *CPM* mice or in the absence of competition in chimeric conditions with normal numbers of T cell precursors seen at the DN stages of thymic development (Fig8A and Fig10). Therefore, the loss of DOCK8 only becomes limiting in the early development of T cells the presence of competition. However, the cause of this defect is unknown.

In single bone marrow chimeras, thymic development was normal with equivalent numbers of SP $CD4^+$ and $CD8^+$ T cells produced within the thymus. Interestingly, the accumulation of mature $CD69^{lo}$ $CD62L^+$ SP CD4 T cells was still present at significant levels (Fig10). However, the phenotype was not as prevalent as seen in unmanipulated CPM mice therefore the accumulation of SP $CD4^+$ T cells within the thymus is due to a mixture of cell intrinsic and extrinsic factors. However, loss of naïve $CD4^+$ and $CD8^+$ T cells was still apparent with reduced numbers of *CPM* T cells in the spleen and lymph nodes (Fig10).

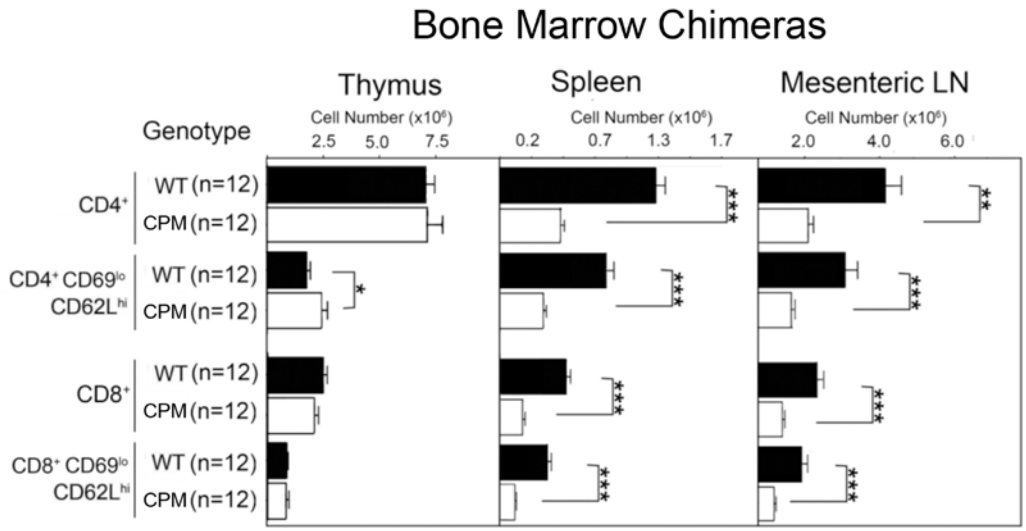


Figure 10: T cell development and survival assessed in straight, BM chimeras. A) CD45.1 transgenic mice were irradiated before reconstitution with donor bone marrow from WT and *CPM* mice. 8-10 weeks post BM reconstitution mice were culled and flow cytometry analysis was carried out. Numbers of T cells were enumerated and are depicted for the thymus, spleen and mesenteric lymph nodes. Bars show arithmetic means and SEM with significant differences by t-test *p<0.05, **p<0.01, ***p<0.001.

A limitation of straight bone marrow chimeras is the lack of WT haemopoetic cells and therefore mixed chimeras can be generated to overcome this problem. Due to competition with WT cells, this model is also more sensitive in testing cell deficiencies. Analysis of 20:80 mixed chimeras showed the same competitive failure to reconstitute thymic development at the DN stage. However, no differences were observed in the transition of *CPM* T cells through the DP and SP stages of development (Fig11A) demonstrating that DOCK8 deficiency is not limiting during T cell selection. However, naïve peripheral T cells were still significantly reduced in

CPM compared with WT cells (Fig11A), which suggests a cell intrinsic defect in peripheral $CD4^+$ and $CD8^+$ survival. This intrinsic loss of both $CD4^+$ and $CD8^+$ T cells may help to explain the immunodeficiency observed in DOCK8 deficient patients. However, additional defects such as the accumulation of SP $CD4^+$ T cells seen in *CPM* mice were not observed in the thymus of mixed chimeras supporting the idea that this is not the principal cause of peripheral lymphopenia (Fig11A). Moreover, the increased prevalence of *CPM* $CD44^{hi}$ T cells was not found in mixed chimeras (Fig11B) implying that this defect is primarily due to increased homeostatic proliferation in unmanipulated *CPM* mice due to lymphopenia and the increased amounts of homeostatic cytokines such as IL7 and IL15.

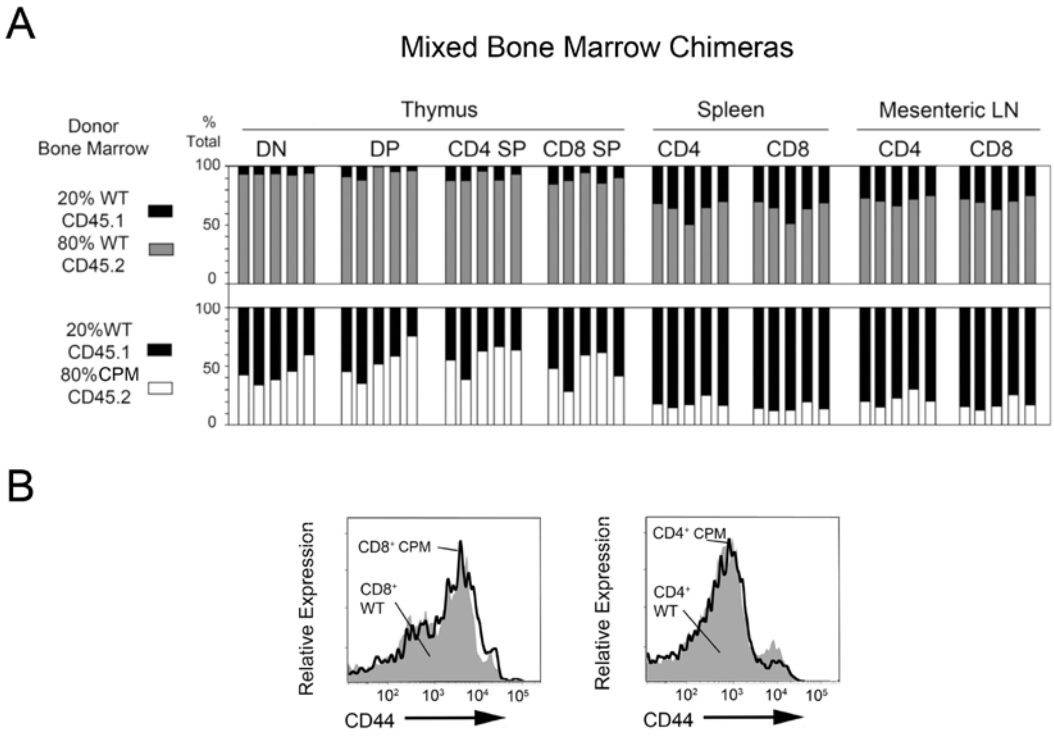


Figure 11: DOCK8 deficiency results in cell intrinsic loss of naive T cells. A) CD45.1 transgenic mice were irradiated before reconstitution with BM derived of 80% WT CD45.2 or 80% *CPM* CD45.2 cells mixed with 20% CD45.1 transgenic cells. 8-10 weeks post BM reconstitution mice were culled and flow cytometry analysis was

carried out on both primary and secondary lymphoid organs (thymus, spleen, mesenteric lymph nodes). Reconstitution levels are depicted and bars represent individual chimeric mice. B) CD44 expression profiles of $CD4^+$ and $CD8^+$ T cells from the spleen are shown for CD45.2 WT and *CPM* mixed chimeras

3.7 Migration and survival of early naïve T cells in the absence of DOCK8

As previously discussed, lymphocytes from DOCK2 knockout mice fail to migrate to a variety of chemoattractants, resulting in the loss of T cell thymic egress in response to S1P, and peripheral lymphopenia [6-8]. I therefore considered whether similar effects could contribute to immunodeficiency in the absence of DOCK8. To assess chemotaxis, I mixed equivalent numbers of mature allotype-marked thymic and lymph node T cells from *CPM* and WT and assessed their ability to migrate in response to constitutively expressed cytokines SDF, SLC and S1P. In contrast to DOCK2 knockout cells, the migration of thymic and peripheral T cells to these chemokines was predominantly normal. However, a consistent increase in the migration of mature *CPM* thymic $CD4^+CD62L^{hi}$ T cells towards CXCL12 was apparent (Fig12; p=0.0224 by ANOVA, combining data from 3 experiments).

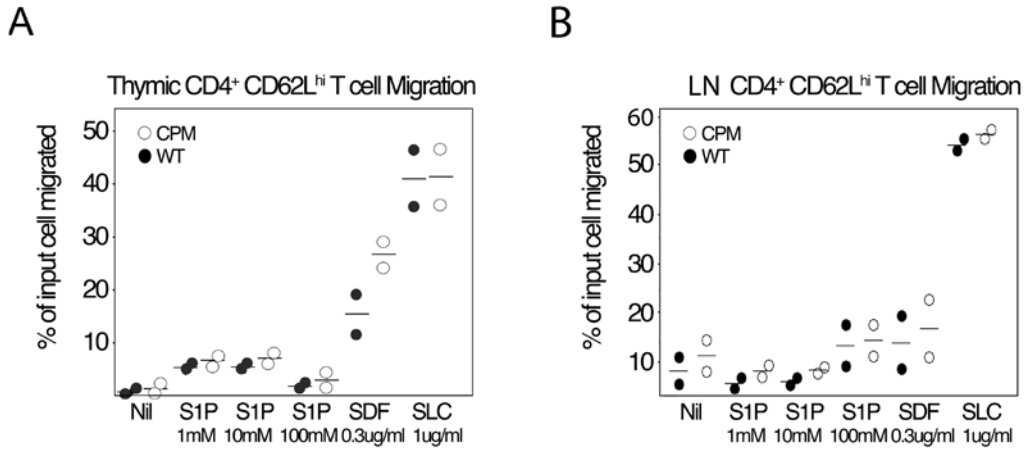


Figure 12: *In vitro* investigation of T cell migratory responses. Transwell migration assays carried out using mixtures of $CD4^{+}CD62L^{hi}$ thymocytes (A) and $CD4^{+}CD62L^{hi}$ derived from peripheral lymph nodes (B) from *CPM* (CD45.2) and WT (CD45.1) in response to various chemoattractants (S1P, SDF and SLC) at indicated concentrations. Samples were set up in duplicates with WT cells (closed circles) and *CPM* (open circles) respresent individual wells.

3.8 T cell migration and localization are intact *in vivo*

Chemokines including CCL19 and CCL21 have dominant roles in the migration and recirculatory behaviour of naïve peripheral T cells [9]. However, the importance of CXCR4 signalling in response to CXCL12 has also been previously documented to facilitate the entry of T cells into the peripheral lymph nodes [10]. Therefore, in collaboration with Jason Cyster from the UCSF (San Francisco, USA) we investigated the CXCR4/CXCL12 signalling axis in more detail *in vivo*. In these experiments, 50:50 mixtures of mature thymic *CPM* & WT T cells were transferred into WT recipients and *plt/plt* recipients, which lack expression of CCL21 and CCL19. In these experiments performed at the UCSF, the proportion of thymic *CPM* $CD4^{+}$ $CD62L^{hi}$ T cells in peripheral lymphoid organs after 90 minutes was increased in comparison to WT cells (Fig13). These findings are consistent with an increased chemotactic response to CXCL12 but also confirm that entry and localisation of cells to peripheral lymphoid organs is normal.

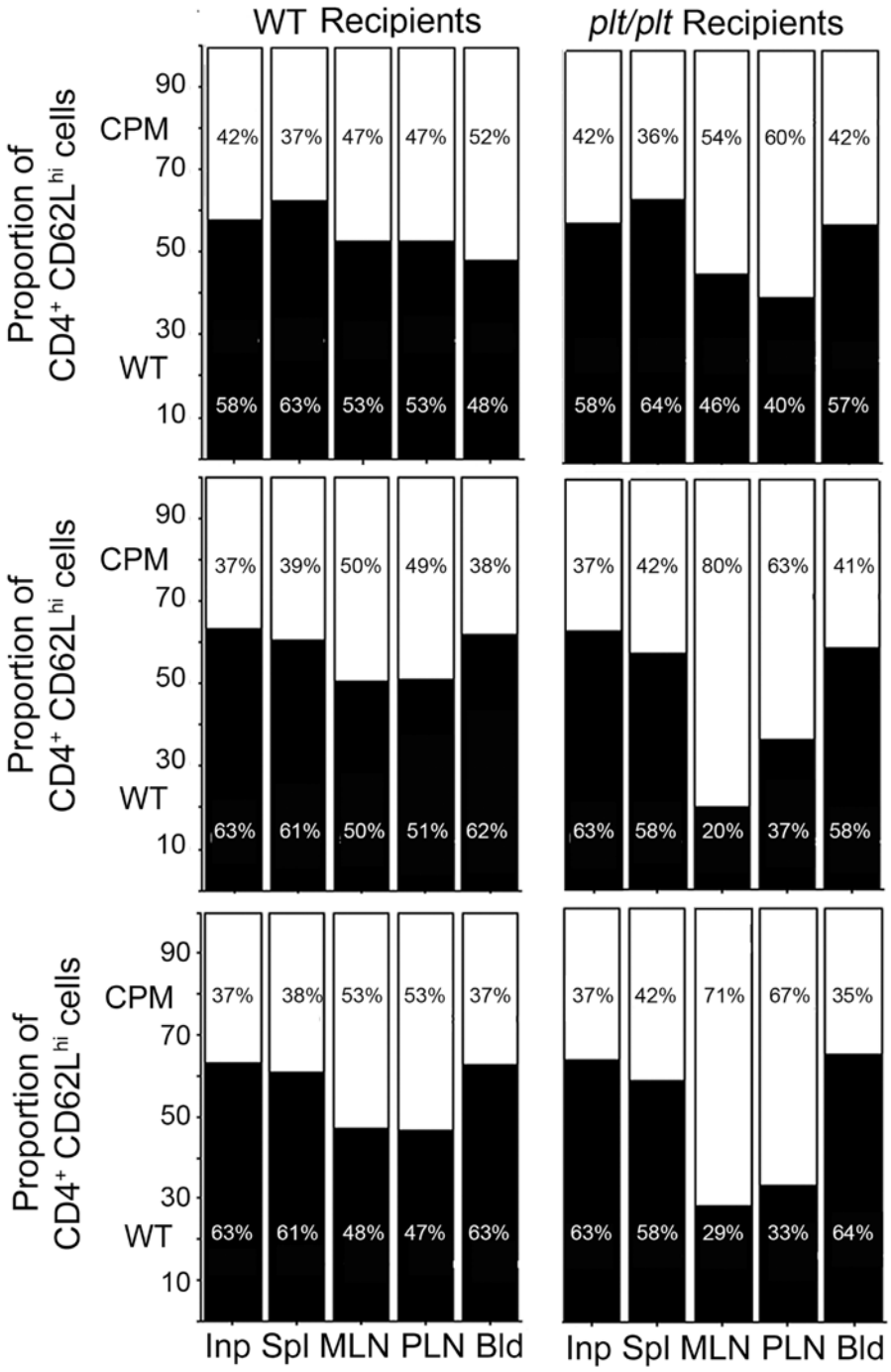
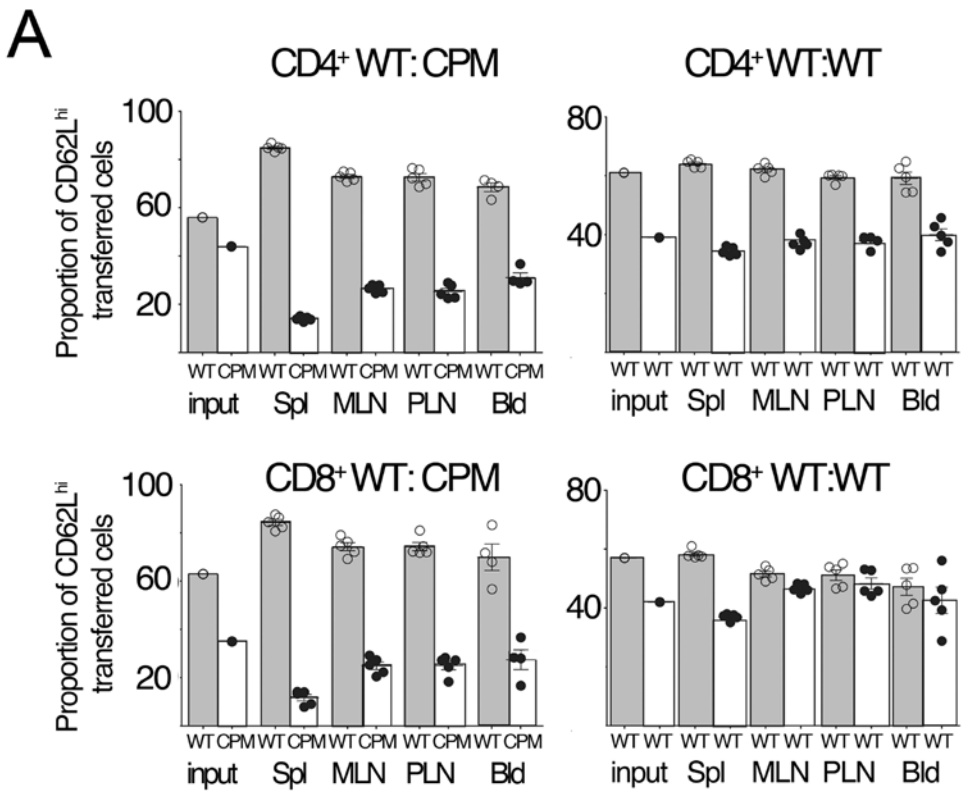


Figure 13: Localization of mature naïve SP T cells into lymphoid organs. A) Co-transfer for 90 min of $1x10^6$ fluorescently labelled thymocytes from *CPM* (CD45.2) and WT (CD45.1) into WT or *plt/plt* recipient mice. Data show proportions of $CD4^+CD62L^{hi}$ cells at input (inp), and after transfer in spleen (spl), MLN, PLN and blood (Bld). **Data generated by Jason Cyster at the UCSF.**

3.9 Single positive thymocytes have intrinsic survival deficiency

As migration and localization of mature thymocytes are normal in the absence of DOCK8, adoptive transfer experiments were carried out to evaluate the survival of mature thymocytes from *CPM* mice. Twenty-four hours after transfer of mixtures of *CPM* and WT SP thymocytes into CD45.1 allotyped hosts, the proportions of mature thymic *CPM* $CD4^+$ and $CD8^+$ T cells were reduced relative to WT (Figure 14A). These data suggest that DOCK8 is important in the maintenance of the survival of mature T cells after their localisation to secondary lymphoid organs. In relation to this, I found that expression of the pro-survival factor Bcl2 was consistently reduced in *CPM* SP $CD4^+$ and $CD8^+$ thymocytes, suggesting that DOCK8 is important in the activation of anti-apoptotic pathways which promote levels of survival factors such as Bcl2 and may contribute to survival of these cells at this late stage in T cell development (Fig14B).



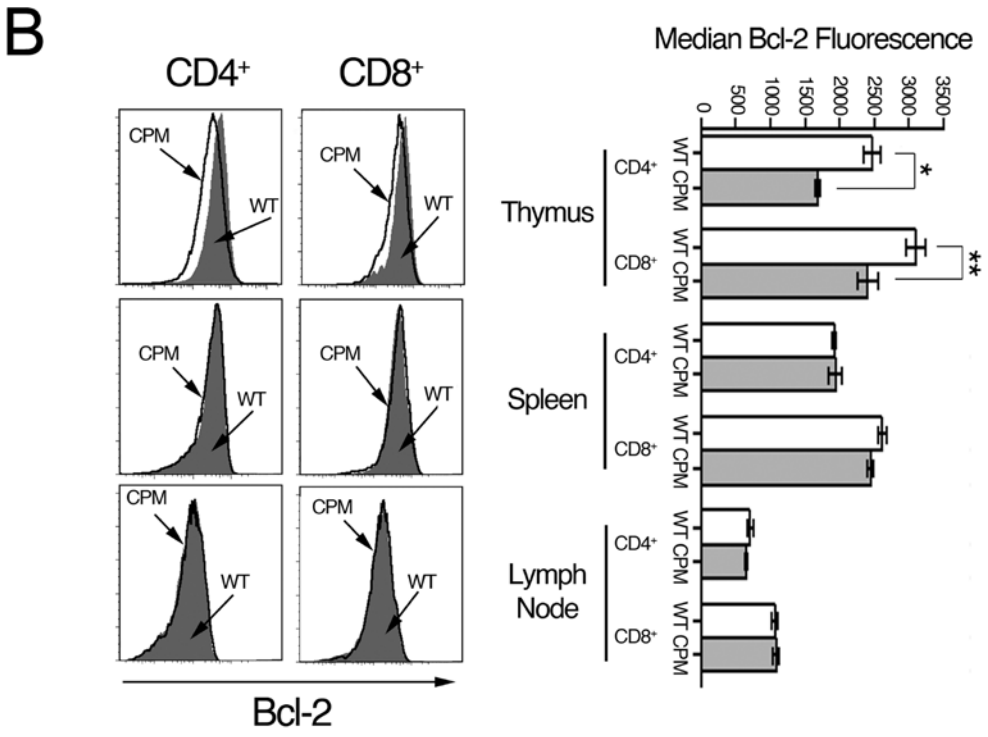


Figure 14: Impaired survival of SP *CPM* T cells. A) Co-transfer for 24hr of fluorescently labelled thymocytes from WT or *CPM* (CD45.2) and WT (CD45.1) mice into WT recipients, adjusted so approximately equal numbers of mature ($CD62L^{hi}$) single positive T cells were transferred. Data show proportions of $CD4^{+}CD62L^{hi}$ cells at input (inp), and after transfer in spleen (spl), MLN, PLN and blood (bld). Data are representative of at least three experiments. (B) Intracellular Bcl2 expression on total $CD4^{+}$ and $CD8^{+}$ T cells in the thymus, spleen and lymph nodes of WT (filled) and *CPM* (dark line) mice shown as representative histograms and median fluorescence, where n=4-5, columns are means, bars are SE and t test * $p<0.05$, ** $p<0.001$.

3.10 Decreased survival of peripheral *CPM* T cells

Together, the above results suggested that defects in cell survival are the principal reason for the loss of mature T cell populations in DOCK8 immunodeficiency. To evaluate this further, *CPM* and WT mice were fed BrdU for 15 days and the survival of labelled cells were followed for a further 30 days. In the first phase, BrdU labelling of $CD4^+$ and $CD8^+$ T cells was equivalent or more rapid in the spleen and lymph nodes of CPM compared with WT (Figure 15B-C). This is consistent with a higher level of homeostatic proliferation with the majority of newly labelled cells displaying a $CD44^{hi}$ phenotype (Fig15A). However, during the washout phase, *CPM* $CD4^+$ and $CD8^+$ T cells appeared to be lost at faster rates compared to WT cells confirming a survival defect in mature T cells (Figure 15B-C).

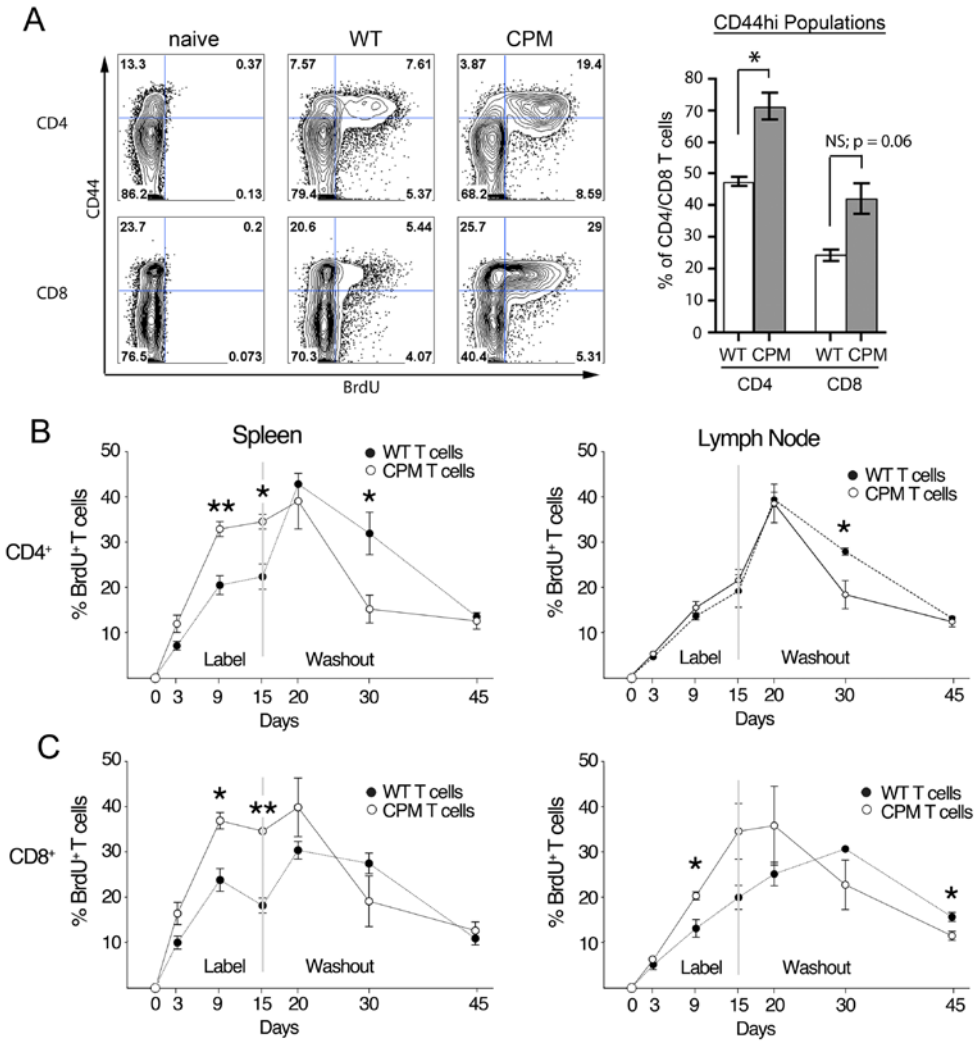


Figure 15: Increased turnover and decreased survival of *CPM* T cells. A) WT and *CPM* mice were given BrdU orally for 9 days by the addition of BrdU at 0.8mg/ml in their drinking water. Mice were then culled and examined by flow cytometry to look at BrdU incorporation. FACS plots depict $CD4^+$ and $CD8^+$ T cells and their relative expression of CD44 and BrdU incorporation. Incorporation was measured as a percentage of parent populations with bars depicting arithmetic means and SEM with significant differences by t-test *$p<0.05$. B)+C) Pulse-chase experiments involved the removal of BrdU from drinking water at 15 days (grey line) and animals were culled at designated time points to analyze relative levels of BrdU incorporation during labelling and washout phases. Percentage of $BrdU^+$ T cells are shown for both spleen and peripheral lymph nodes. Each data point is representative of 3 mice with arithmetic means and SEM presented. Significant differences were tested by t-test *$p<0.05$, **$p<0.01$.

3.11 Conclusions

Through the use of the ENU mutant, Captain Morgan, we have gained insight into the function of the guanine nucleotide exchange factor, DOCK8 and its roles in T cell homeostasis. *CPM* mice, like patients with DOCK8 deficiency, show losses of naïve T cells in the peripheral lymphoid organs including the blood, peripheral lymph nodes and spleen. $CD44^{hi}$ memory-like T cells are relatively conserved which appears to be due to increased homeostatic proliferation induced by lymphopenia.

I have demonstrated that the development of mature thymocytes is intact. However, a significant increase in mature SP $CD4^+$ T cells was observed in unmanipulated mice. Based on BrdU incorporation assays, this did not appear to be due to increased production of mature $CD4^+$ T cells. One possible explanation for this phenotype may be due to altered egress from the thymus. However, this phenotype was not observed for $CD8^+$ T cells and was not present in mixed chimeras. Therefore, this accumulation of mature SP $CD4^+$ T cells does not appear to present the principal mechanism underlying lymphopenia in this model.

Although the accumulation of SP $CD4^+$ thymocytes was not necessarily a cell intrinsic defect, reductions in both naïve $CD4^+$ and $CD8^+$ T cells were seen in both straight and mixed chimeras. The CD44 profiles of *CPM* T cells were also returned to normal in these mice giving further support that this phenotype in unmanipulated mice is due to lymphopenia induced proliferation. Losses of peripheral T cells could be explained by a number of defects including deficiencies in the migration or survival of naïve T cells.

Migration of thymic and peripheral $CD4^+$ T cells was demonstrated to be relatively normal. However, an increased sensitivity to the chemokine, CXCL12, was seen in mature thymic $CD4^+$ T cells. This change in migratory capacity may therefore contribute to the decreased egress of thymocytes from the thymus. However, both $CD4^+$ and $CD8^+$ mature thymocytes were demonstrated to have a shortened lifespan when adoptive transferred into WT mice. Therefore, decreased survival of *CPM* T cells seems the most likely factor in explaining T cell lymphopenia in *CPM* mice.

With the use of BrdU pulse chase experiments, peripheral $CD4^+$ and $CD8^+$ T cells were also shown to undergo increased turnover and decreased survival. This provides further evidence that DOCK8 plays a key role in maintaining T cell populations by promoting cell survival factors.

However, the losses of naïve T cells in *CPM* and the data provided here related to the survival of *CPM* T cells do not appear to be sufficient enough to fully explain the immunodeficiency and susceptibility of patients to recurrent viral infection. Work by Randall et al carried out further analysis of human DOCK8 deficient T cells looking at cell surface markers related to a T cell 'exhausted' phenotype. Higher proportions of patient $CD8^+$ T cells expressed markers including CD57 and CX3CR1 and notably lower expression of CD127 [11]. This is consistent with higher proportions of DOCK8 deficient T cells showing signs of T cell exhaustion due to chronic antigen encounter. Therefore, DOCK8 deficient T cells from patients may have undergone altered differentiation in vivo as a consequence of altered homeostasis and recurrent viral infection.

These data lead to the hypothesis that DOCK8 deficient patients may also carry defects in their ability to respond to pathogen challenge and mount normal immune responses.

3.12 References

1. Zhang, Q., et al., Combined immunodeficiency associated with DOCK8 mutations. N Engl J Med, 2009. 361(21): p. 2046-55.
2. Randall, K.L., et al., Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol, 2009. 10(12): p. 1283-91.
3. Jabara, H.H., et al., DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation. Nat Immunol, 2012. 13(6): p. 612-20.
4. Fujikawa, K., et al., Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. J Exp Med, 2003. 198(10): p. 1595-608.
5. Sanui, T., et al., DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-theta and LFA-1, in T cells. Immunity, 2003. 19(1): p. 119-29.
6. Fukui, Y., et al., Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature, 2001. 412(6849): p. 826-31.
7. Nombela-Arrieta, C., et al., Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. Immunity, 2004. 21(3): p. 429-41.
8. Reif, K. and J. Cyster, The CDM protein DOCK2 in lymphocyte migration. Trends Cell Biol, 2002. 12(8): p. 368-73.
9. Johnston, B., et al., Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. J Immunol, 2003. 171(6): p. 2960-9.

10. Okada, T., et al., Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. J Exp Med, 2002. 196(1): p. 65-75.

11. Randall, K.L., et al., DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. J Exp Med, 2011. 208(11): p. 2305-20.

Chapter 4

The Role of DOCK8 in Effector and Memory T cell Differentiation and Maintenance

4.1 The role of DOCK8 in anti-viral T cell immunity

The individuals with homozygous mutations in DOCK8 suffer from a wide range of bacterial and viral infections and fail to respond normally to vaccination with low levels of protective antibody titres post immunization [1]. However, patients present clinically with recurrent infections of the lungs suggestive of humoral deficiency and infections with Herpes Simplex Virus, Human Papillomavirus (HPV) and Molluscum Contagiosum indicative of T cell abnormalities. In relation to this, work has been undertaken to isolate DOCK8 deficient T cells from patients to carry out a range functional assays to understand the potential role of DOCK8 in T cell activation. Experiments are difficult because of the difficulties in isolating naïve T cells from the peripheral blood and controlling for recurrent infections and different treatment regimes. Some have demonstrated decreased T cell activation and proliferation in *in vitro* assays but results are inconclusive [2, 3].

The importance of $CD8^+$ T cells and their role in anti-viral immunity has been demonstrated in both animals and humans [4-6]. Depletion of $CD8^+$ T cells in mouse models of infection or disruption of $CD8^+$ T cells due to mutations in molecules specifically related to $CD8^+$ T cell function results in susceptibility to viruses [7, 8]. Adoptive transfer of antigen specific $CD8^+$ T cells can also restore host immunity [9]. Therefore, one possible explanation for the increased susceptibility of DOCK8 deficient patients to viral infections may be due to alterations in the activation and differentiation of antigen specific $CD8^+$ T cells.

To address this question, I carried out an examination of the role of DOCK8 in T cell immunity by using our *CPM* strain. *In vitro* activation of $CD4^+$ and $CD8^+$ T cells was carried out to examine T cell differentiation and proliferation. Profiling of $CD4^+$ helper T cell subsets including TH1, TH2 and TH17 was carried out to explore appropriate T cell differentiation in response to cytokines. After this analysis was carried out *in vitro*, I challenged *CPM* mice with attenuated and virulent viruses to measure primary and memory $CD8^+$ T cell responsiveness. These experiments revealed a role for DOCK8 in the maintenance of anti-viral immunity.

4.2 Normal activation and differentiation of peripheral *CPM* T cells

As previously discussed, the ability of DOCK8 deficient T cells to undergo normal activation in response to TCR stimulation is unclear. Therefore, I analyzed the response of *CPM* T cells to TCR stimulation to investigate possible defects in their activation or differentiation. Overnight stimulation of *CPM* T cells with anti-CD3 showed no difference in the up-regulation of activation markers, CD69 and CD25 on *CPM* and WT $CD4^+$ and $CD8^+$ T cells (Figure 16A). In addition to this, *CPM* and WT $CD4^+$ and $CD8^+$ T cells underwent similar proliferative responses after stimulation with anti-CD3 and anti-CD28 *in vitro*, with cells undergoing similar numbers of divisions, after five days in culture (Figure 16B). These findings show that *CPM* T cells are able to respond normally to TCR stimulation and proliferate freely *in vitro*.

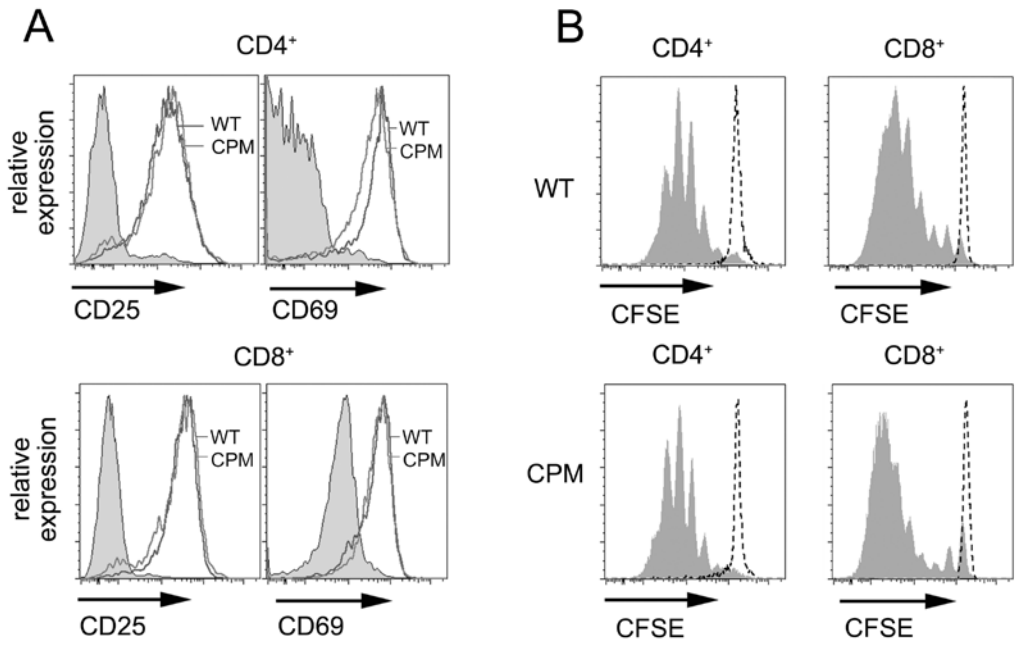


Figure 16: Normal T cell activation and proliferation in *CPM* T cells. A) Histograms showing CD25 and CD69 expression on *CPM* and WT $CD4^+$ and $CD8^+$ splenocytes stimulated overnight with plate-bound anti-CD3 (10ug/ml), and unstimulated controls (filled histograms). Analysis was carried out on live cells and data is representative of 3 experiments. B) Proliferation of WT or *CPM* $CD4^+$ or $CD8^+$ T cells (filled histograms), was assayed by CFSE dilution after stimulation with 10μg/ml plate-bound anti-CD3 and 10μg/ml soluble anti-CD28 for 5 days, compared to unstimulated controls (broken line). Analysis was carried out on live cells. Plots in (A) and (B) are representative of at least five replicates using separate mice.

The susceptibility of AD-HIES patients has been suggested to be due to altered T cell differentiation with patients deficient in the transcription factor, STAT3, lacking TH17 cells [10, 11]. However, this possibility has not been explored in AR-HIES patients. To address this, our collaborator Helen Su (Bethesda, USA) analyzed the cytokine production of activated/memory $CD45RO^+$ $CD4^+$ T cells from the blood of

DOCK8-deficient patients, parents and controls after stimulation *ex vivo* with PMA and ionomycin. Higher proportions of activated cells secreting TH2 cytokines IL4 or IL13 were observed in 3/5 and 4/5 DOCK8-deficient patients respectively, a finding that may be linked to the hyper-IgE phenotype of DOCK8 immunodeficiency. (Fig17). In contrast, levels of IFN-γ and IL17 producing cells were modestly reduced in patients although this did not reach statistical significance. However, the degree to which these phenotypes reflect ongoing infection or the over-stimulation/exhaustion of peripheral T cells is again uncertain.

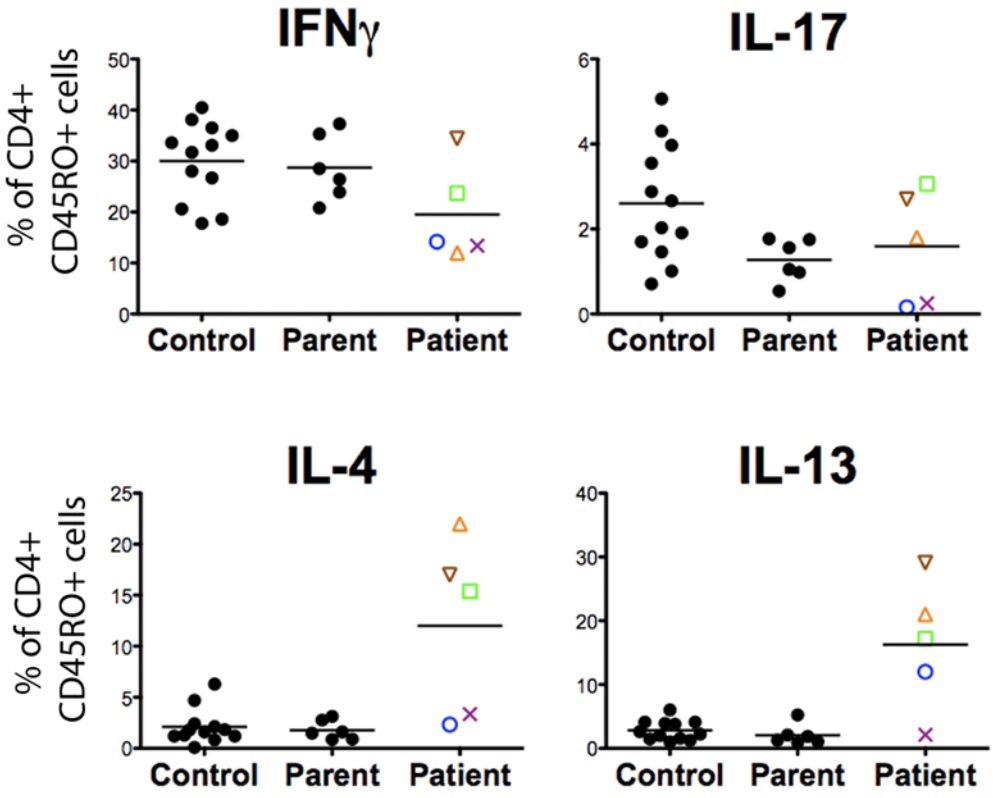
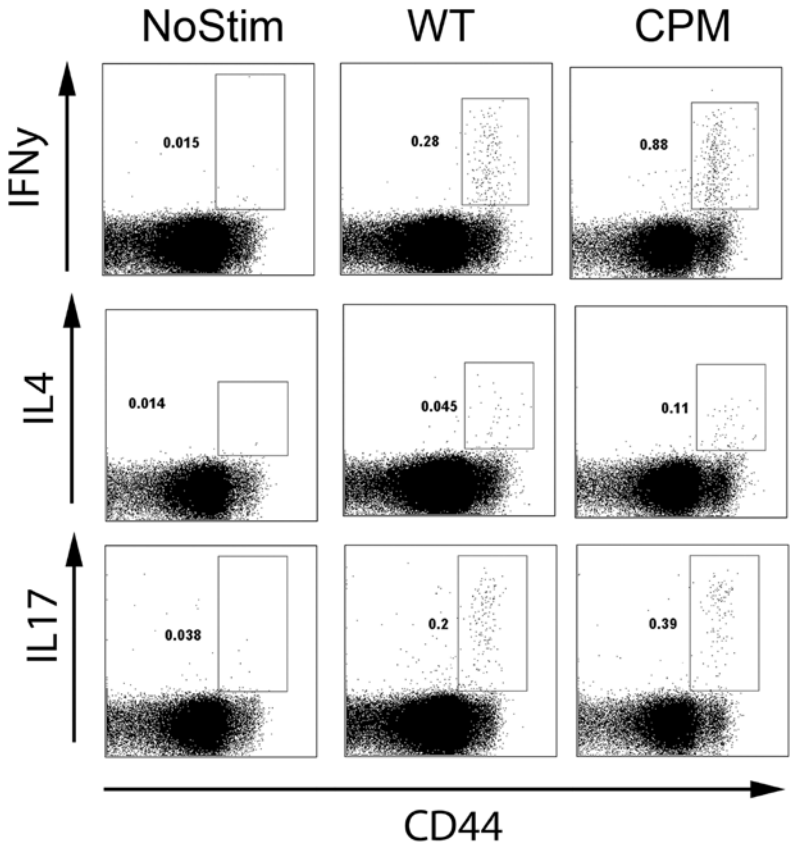


Figure 17: Cytokine profiles of $CD4^+$ T cells from DOCK8 deficient patients A) Intracellular cytokine staining for IFN-γ, IL4, IL13 and IL17 was carried out on live human $CD45RO^+$ $CD4^+$ T cells stimulated for 6 hr with PMA and ionomycin. Symbols are individual patients with different shapes representing individual patients.

Data is presented as a percentage of $CD4^+CD45RO^+$ cells and arithmetic means are depicted. Data generated by Helen Su at the NIH.

In parallel to Helen Su's work, I carried out similar experiments stimulating *CPM* $CD4^+$ T cells with anti-CD3 stimuli to quantify levels of TH1, TH2 and TH17 subsets under homeostatic conditions. Similar to patient samples, cytokine producing cells were isolated to the activated/memory cell populations based on CD44 staining (Fig18). However, no significant differences were seen in the proportions of $CD4^+$ T cells producing IFNγ, IL4 or IL17 indicating no deficiencies in T cell polarization (Fig18).



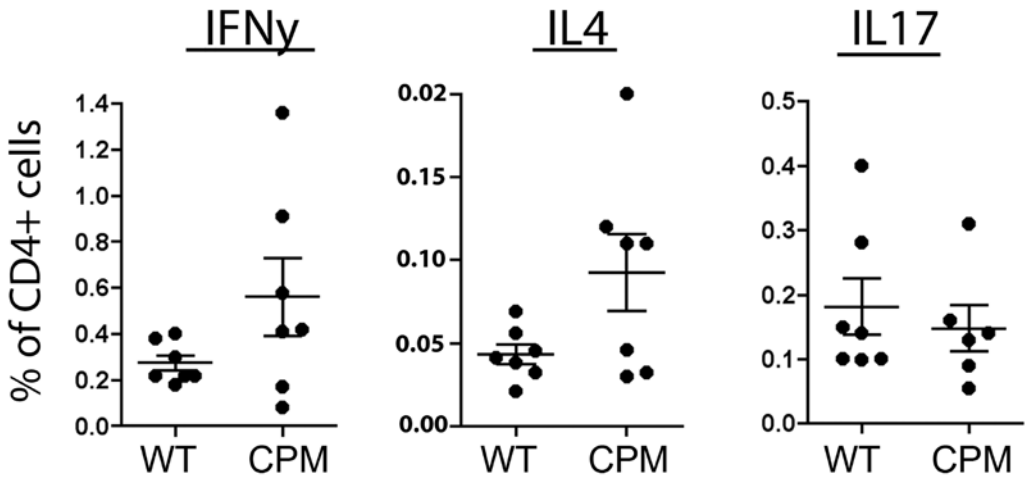


Figure 18: Investigation of T helper subsets in *CPM* mice. A) Intracellular staining of splenocytes from WT and CPM mice was carried out after stimulation for 6hrs with plate-bound anti-CD3 (10ug/ml). Relative expression profiles of CD44 and different cytokines (IFNγ, IL4 and IL17) are depicted in representative FACS plots. Data were then evaluated as a percentage of total $CD4^+$ T cells. Dots represent individual mice with graphs showing arithmetic means and SEM. Significance was tested by t-test.

To investigate T cell differentiation further, I asked if *CPM* $CD4^+$ T cells could differentiate normally under TH1 or TH2 polarising conditions *in vitro*. Under these conditions, Tbet and GATA3 were induced appropriately (Fig19A). IFN-γ production by TH1 cells was equivalent after re-stimulation of cells after the 5 days of culture. However, interestingly, proportions of IL4 secreting cells were higher in *CPM* TH2 T cells (Fig19B). It is possible that DOCK8 and differences in the ability of cells to regulate the cytoskeleton may result in the alteration of the signalling threshold of the TCR or other receptors, or small differences in the basal and induced levels of Tbet and GATA3 might contribute to such a bias in humans and mice. However, the

differentiation of effector cell subsets does not appear to be impaired in the absence of DOCK8 and is therefore unlikely to contribute to the state of immunodeficiency.

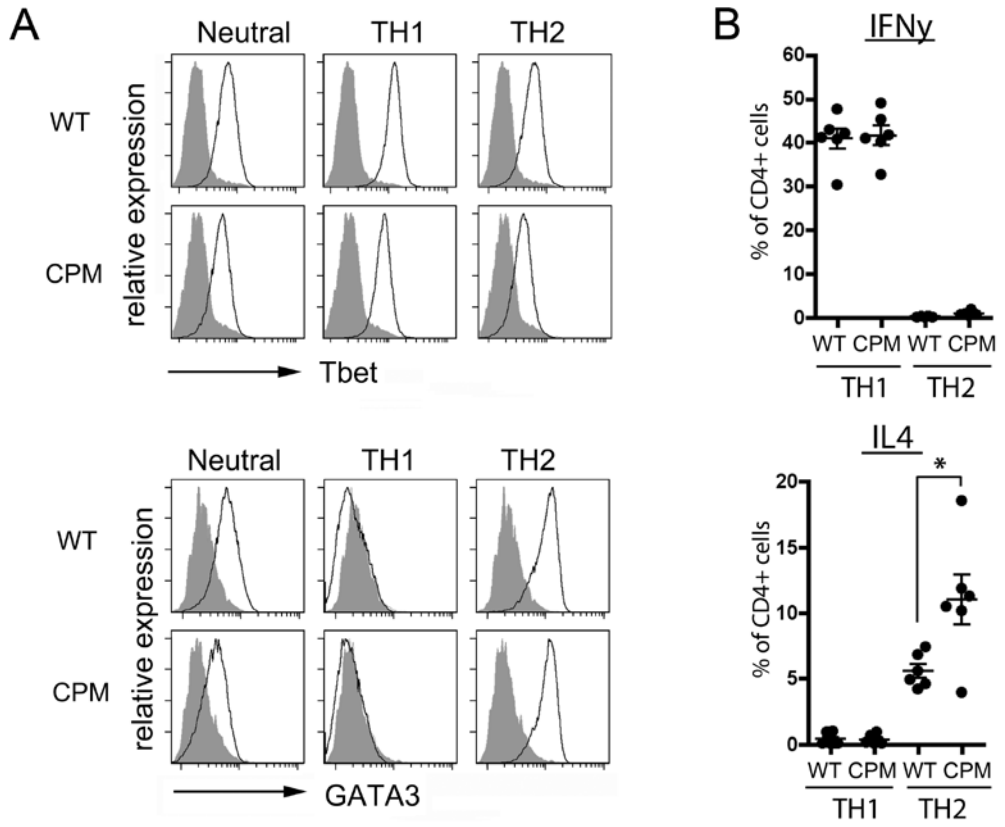


Figure 19: $CD4^+$ T cell differentiation is normal in the absence of DOCK8. A) T cells were stimulated using 10μg/ml plate-bound anti-CD3 for 5 days with cultures supplemented with cytokines/neutralising antibodies conducive to different T helper cell polarizing conditions. Intracellular staining for Tbet and GATA3 (dark line) was carried out on live, $CD4^+$, $CFSE^{low}$ cells. Data show representative plots repeated in 5 mice, filled histograms show isotype controls. B) At the end of the initial 5-day culture, cells were re-stimulated with anti-CD3 and intracellular cytokine staining of $CD4^+$ T cells was carried out for IFNγ and IL4. Data is presented as of percentage of $CD4^+$ T cells. Dots represent individual mice, columns depict arithmetic means with SEM. Significance tested by t test, * $p<0.05$.

4.3 *CPM* mice show losses of memory CD8 T cells

As *CPM* T cells showed no obvious abnormalities in the *in vitro* assays described, I began an assessment of active immune responses *in vivo* in response to viral challenge. Firstly, I examined T-cell responsiveness of *CPM* cells during pathogenic infection by challenging *CPM* and WT mice with mouse adapted influenza strain x31 (A/HK-x31 (H3N2)). Using daily weight loss as a clinical parameter I found no significant difference in disease severity or the ability to survive the acute infection through the nadir of infection at day 6 to recovery by day 10 (Fig20A). 11 of 12 WT mice survived the challenge, while 9 of 12 *CPM* mice survived. Seven days post immunization, mice were bled and antigen-specific $CD8^+$ T cells were enumerated by *in vitro* stimulation of lymphocytes with viral epitopes recognised by T cells. No significant difference between WT and *CPM* in the numbers of $LAMP1^{hi}$ $CD8^+$ T cells specific to T-cell restricted epitopes were observed in these assays (Fig20B). As T cell differentiation and proliferation appeared to be unaffected in *in vitro* assays these findings were not surprising.

To assess the memory phase of the response, mice were bled 56 days after primary infection and $LAMP\text{-}1^{hi}$ $CD8^+$ T cells, responsive to conserved, internal x31 restricted epitopes, were enumerated (Fig20B). Whilst WT mice produced a robust response to x31, memory T cells were not sustained above background in *CPM* mice. Together these data suggest that the functions of DOCK8 are not limiting during primary T cell responses against viral infection but are critical for maintenance of memory to vaccination and viral infection.

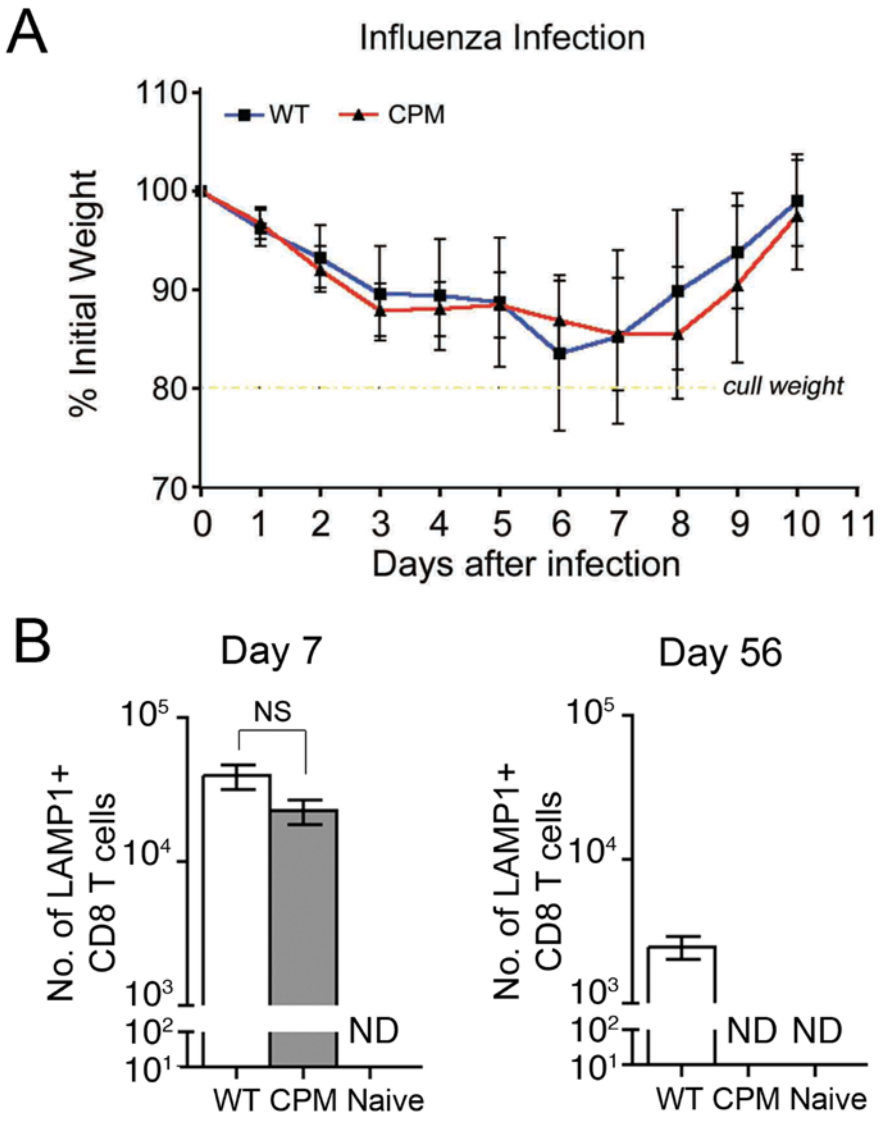


Figure 20: *CPM* mice are capable of controlling live influenza infection. A) Weight loss and recovery in *CPM* and WT mice infected with Influenza strain X-31 (n=12 in each group). Animals were culled if weight fell by >20%. Data show arthmetic means with SE. Data is representative of two experiments. B) The *ex vivo* influenza X-31 antigen specific response in *CPM* and WT mice from (A), showing numbers of LAMP1^{hi+} CD8^{+} T cells 7 days (left graph) and LAMP1^{+} at 8 weeks (right graph). Numbers are corrected for background levels observed in unstimulated controls, (ND, not detectable.)

4.4 Characterization of CD8 T cell responses in the absence of DOCK8

As described previously, the normal immune response to pathogens *in vivo* results in the activation, proliferation and differentiation of antigen specific T cells leading to the formation of short-term effector cells with cytotoxic and cytokine producing capacity to control pathogen levels [8, 12, 13]. In parallel to this, long-lived memory cells are also produced which survive after contraction of the effector population and provide long-term protective immunity against future infections. From the data collected from the *in vitro* experiments and the influenza infections described, it appears that DOCK8 is not limiting in the production of a primary immune response to pathogen. However, the functionality of these cells and their ability to form long term memory populations may be disrupted. To investigate these possibilities I compared the response of *CPM* and WT mice to vaccination with replication deficient Modified Vaccina Ankara virus (MVA). After 9 days no significant difference in the numbers of antigen-specific $CD8^+$ T cells was observed between *CPM* and WT mice (Fig21A). Proportions of IFN-γ, IL2 or TNF-α producing cells directed against MVA were also normal (Fig21E). This data provides strong evidence that the initial phase of the T-cell response is not functionally limited by the lymphopenia in pre-immune animals. Anti-MVA specific $CD8^+$ T cell numbers also remained similar after the contraction phase (Fig21A); therefore, there was no premature collapse of the effector T-cell population in the absence of DOCK8. However, 42 days after the primary immunization, during the phase of long-lived memory cell survival, the number of antigen-specific cells was significantly reduced (Fig21A). It has been previously demonstrated that memory T cells are derived from specific memory precursor populations which are generated during the initial encounter with antigen. These cells can be defined based on cell markers including KLRG1, CD127 and Bcl2 [14, 15].

The loss of surviving T cells did not appear to be due to a block in the differentiation of these memory precursor cells (MPECs) as numbers of MPECs, defined as KLRG1^{low} CD127^{hi}, were unaltered at day9 of the response (Fig21B). Furthermore, expression levels of memory cell markers CD127, CD62L, KLRG1 and Bcl-2 were similar on MVA-specific WT and *CPM* T cells throughout the anti-viral response and after re-challenge supporting this conclusion (Fig21C). After 55 days, animals were re-challenged with MVA and the antigenic response again quantified. This response was reduced in *CPM* mice compared with WT in terms of absolute numbers of antigen specific CD8^{+} T cells (Fig21A). However, the fold-increase in the secondary response was proportionate to the number of surviving memory cells (WT, mean fold increase of 15.83 +/- S.E 1.81 vs *CPM*, mean fold increase of 26.22 +/- S.E 4.98) (Fig21D), again suggesting that the functionality of *CPM* T cells, both *in vitro* and during both primary and secondary responses is normal, but the survival of memory cells is limiting for CD8^{+} T-cell immunity in the absence of DOCK8.

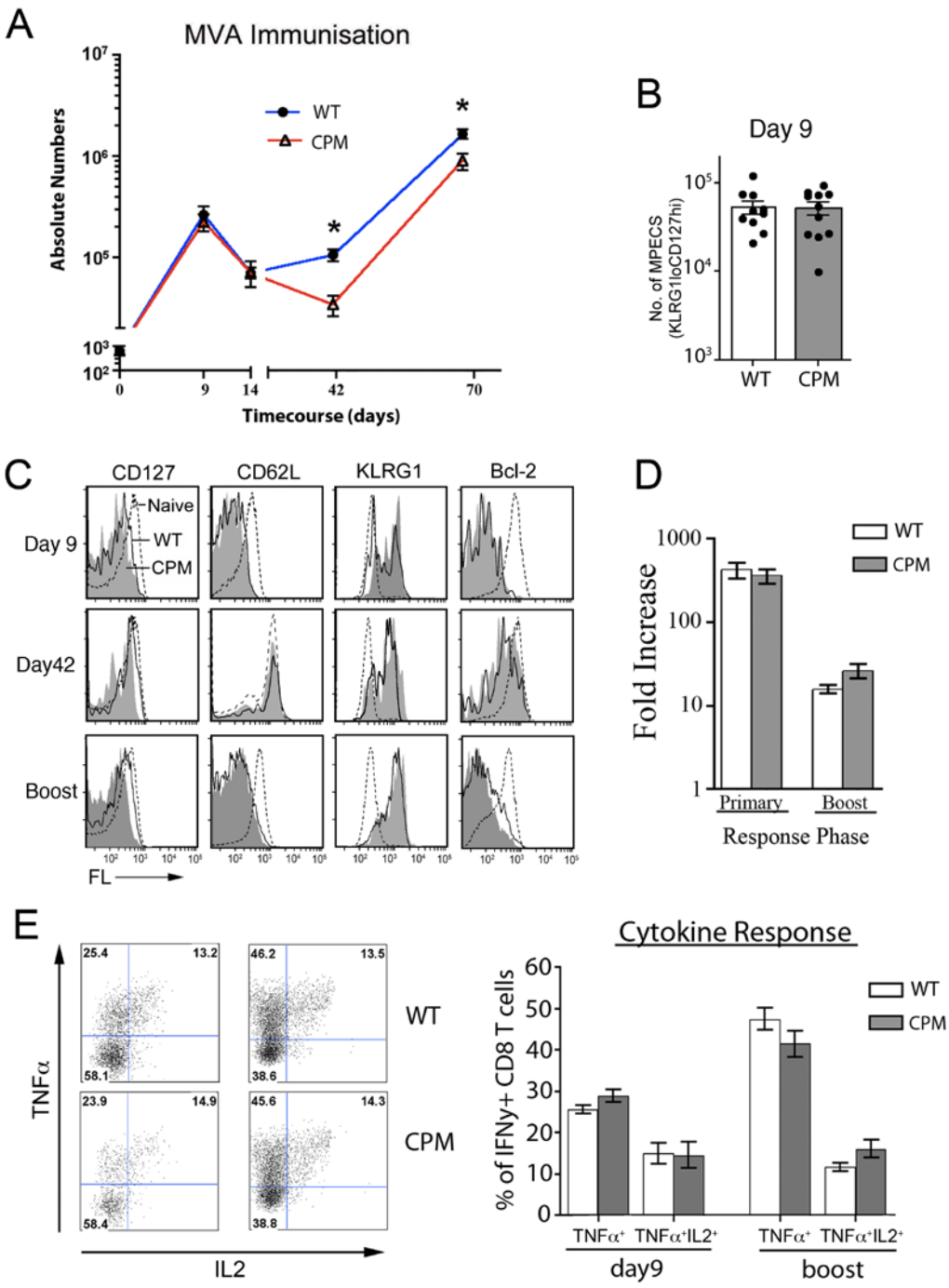


Figure 21: Detailed analysis of *CPM* antigen specific responses to MVA. A) Numbers of splenic $CD8^+$ T cells secreting IFN-γ in response to the MVA specific peptide, B8R, taken at various time points before and after primary and secondary immunisation of mice with intradermal MVA. Data show arthimetic means (WT circles and CPM triangles) combined from 3 experiments where n>4, with SEM and * $p<0.05$. B) Numbers of memory precursor cells (defined as $KLRG1^{low}$ $CD127^{hi}$) at

day 9 post-immunisation from (A). Columns are arthmetic means and bars are SEM. C) CD127, CD62L, KLRG1 and Bcl2 expression on B8R-responsive IFN-γ-secreting $CD8^+$ splenic T cells from WT (thick line) and CPM (filled histogram) mice at day 9 and day 42 post immunization and after re-challenge. Dotted line shows staining on naïve non-responding cells. D) Fold change in the number of antigen responders during the primary and re-challenge response are calculated based on data from (A) for both WT and *CPM*. Data depicts arithmetic means and SEM. E) Functionality of IFNγ secreting $CD8^+$ T cells was investigated by further staining for cytokines IL2 and TNFα. FACS plots depict representative cytokine profiles of antigen specific cells. Data is presented as a percentage of IFNγ secreting $CD8^+$ T cells for day 9 and after re-challenge.

4.5 Loss of Memory CD8 T cells is a cell intrinsic defect

To investigate further the nature of DOCK8 deficiency in the maintenance $CD8^+$ memory T cells, the MVA immunization protocol was repeated with chimeric mice reconstituted as before with 20:80 mixtures of WT (CD45.1) and *CPM* or WT (CD45.2) bone marrow. In this competitive setting, *CPM* antigen specific T cells failed to mount comparable primary $CD8^+$ responses to MVA relative to WT cells (Fig22A, middle panel). Previously I have demonstrated that in the presence of competition, defects due to DOCK8 deficiency become apparent such as the case of the reconstitution of early thymocyte precursors, which was not seen in unmanipulated mice. The loss of *CPM* $CD8^+$ response in this competitive situation may be consistent with an underlying defect in survival, which can normally be compensated for by increased proliferation in the absence of competition. Consistent with the underlying defect in survival, the most profound effect of competition was on

the KLRG1^{hi} short-lived effectors, which are far more susceptible to cell death than the MPEC population which was not as badly affected (Fig22A, middle panel). During the maintenance phase of memory, day 42, *CPM* responders were further affected when compared with MPEC (Fig22A, right panel). Together these data support an intrinsic role for DOCK8 in the maintenance of CD8^{+} T cell memory. Interestingly, WT and *CPM* CD8^{+} memory cells expressed similar levels of markers such as CD127^{+} (IL7R) and KLRG1 as well as the survival factor Bcl2 (Figure 22B).

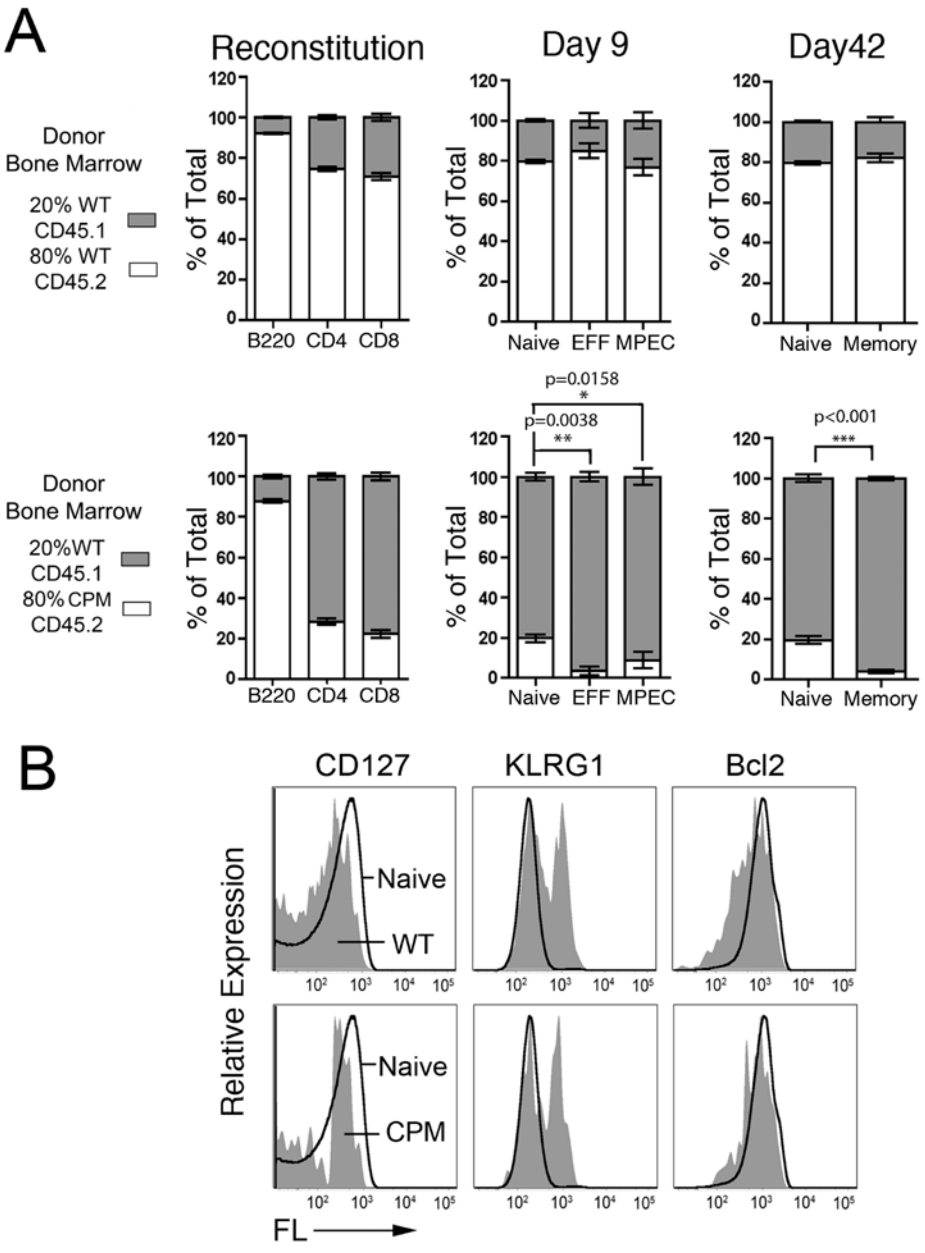


Figure 22: Loss of memory $CD8^+$ T cells is a cell-intrinsic defect. A) The percentage of CD45.1 and CD45.2 B8R specific $CD8^+$ cells in the spleen of chimeric mice reconstituted with an 20:80 mixture of CD45.1 WT and CD45.2 WT T cells (top panels) or CD45.1 WT and CD45.2 CPM T cells (bottom panels). Left panel shows $B220^+$, $CD4^+$ and $CD8^+$ populations in blood of recontituted animals two weeks preimmunization; right panels show B8R antigen-specific CD8 T cells from animals culled 9 days and 42 days post immunization with MVA with the percentage of 'naïve' non-responding $CD8^+$ T cells for comparison. Effector (EFF,) and memory precursor (MPEC) are defined as $KLRG1^{hi}$ $CD127^{low}$ and $KLRG1^{low}$ $CD127^{hi}$ respectively. B8R specific $CD8^+$ T cells at day 42 are defined as 'Memory'. Bars represent means with SE with significant differences by t-test. B) Histograms showing expression of CD127, KLRG1 and Bcl2 on 'Memory' cells on CD45.2 WT (top row, filled) and CD45.2 CPM (bottom row, filled) compared with CD45.2 'Naïve' cells (solid line).

In addition to the above data in mixed chimeras, I also carried out MVA challenge experiments in straight chimeras. In the absence of competition from WT cells, *CPM* T cells mounted normal levels of primary $CD8^+$ T cell responses in this context (Fig23, middle panel). However, levels of memory $CD8^+$ T cells were still reduced (Fig23, right panel). This gives further evidence that DOCK8 deficiency is not limiting in the expansion and differentiation of cytotoxic T cell responses but is important in the long-term maintenance of memory cells.

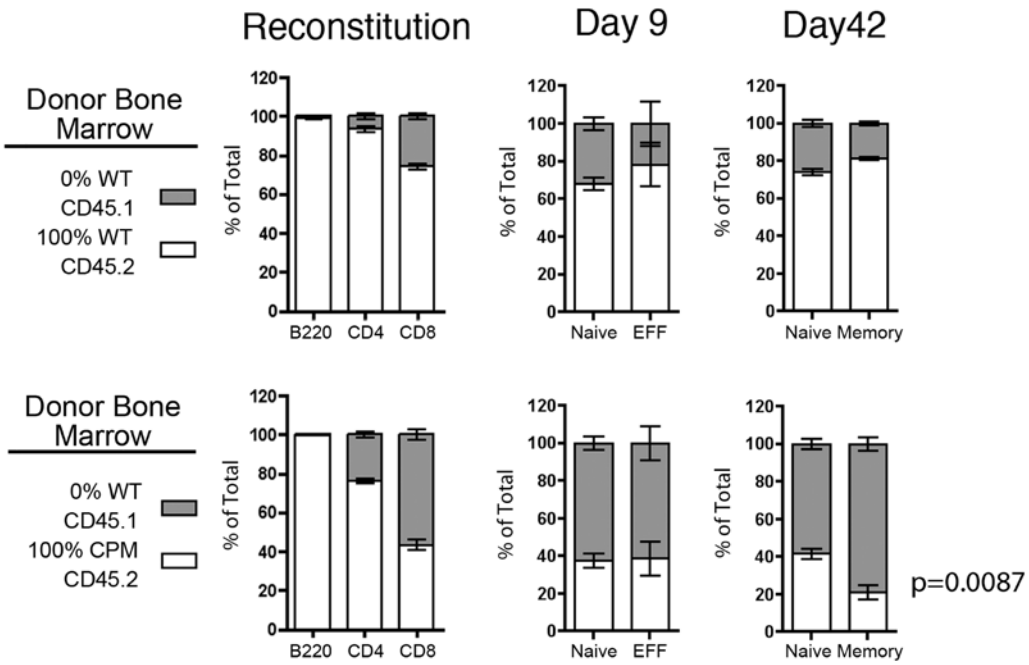


Figure 23: *CPM* primary responses are rescued in the absence of competition while losses in $CD8^+$ T cell memory remain. A) Straight chimera constituted with 100% WT or *CPM* bone marrow were exposed to MVA virus and responses quantified. Left panel shows $B220^+$, $CD4^+$ and $CD8^+$ populations in blood of recontituted animals two weeks preimmunization; right panels show B8R antigen-specific CD8 T cells from animals culled 9 days and 42 days post immunization with the percentage of 'naïve' non-responding $CD8^+$ T cells for comparison. Bars represent means with SE with significant differences by t-test.

4.6 Conclusions

DOCK8 deficient patients are susceptible to a range of bacteria and viruses including herpes simplex virus, human papillomavirus (HPV) and molluscum contagiosum. However, whether this susceptibility is simply a symptom of T cell lymphopenia or is due to a specific role of DOCK8 in the ability of antigen specific T cells to undergo activation, differentiation and expansion has not been investigated.

I have demonstrated here that *in vitro* $CD4^+$ and $CD8^+$ T cells undergo normal activation after TCR stimulation with equivalent up-regulation of activation markers such as CD25 and CD69 compared to WT cells. Proliferation of *CPM* T cells was also intact after 3 days of culture.

DOCK8 deficient patients are also severely atopic and in the case of AD-HIES patients have been demonstrated to have deficiencies in TH17 cells. Analysis of $CD4^+$ helper T cell subsets showed relatively normal levels of IFNγ, IL4 and IL17 producing cells under homeostatic conditions. Therefore, I do not believe DOCK8 has a critical role in the differentiation of TH17 cells in this animal model. More detailed analysis of TH1 versus TH2 differentiation in regard to transcriptional regulation of Tbet and GATA3 respectively showed no clear differences between WT and *CPM* cells. Re-stimulation of *CPM* TH1 and TH2 $CD4^+$ T cells did show a significant increase in the proportion of IL4 producing cells. How significant this result is in explaining the atopic phenotype seen in patients is unclear.

After this examination of *CPM* T cells using the above *in vitro* assays was complete, I carried experiments using both virulent and attenuated viruses to examine the capacity

of $CD8^+$ T cells in vivo to respond to antigen challenge. Using the influenza stain x31 (A/HK-x31 (H3N2)) and the attenuated vaccina virus (MVA), I have demonstrated that primary $CD8^+$ T cell responses are intact in the absence of DOCK8 with normal expansion and cytokine production by cytotoxic T cells. $CD8^+$ T cells also underwent normal differentiation into short-lived responders and memory precursor cells based on CD127 and KLRG1 staining. However, although similar proportions of antigen specific $CD8^+$ T cells died 14 days post infection, antigen specific memory $CD8^+$ T cells were lost after 6 weeks. Interestingly, these memory T cells were still functionally active undergoing equivalent expansion after re-challenge and expressed similar cytokine profiles.

As this loss of memory $CD8^+$ T cells may be due to extrinsic factors such antigen presentation or the availability of exogenous factors such as cytokines or lack of $CD4^+$ T cell help [12, 13], I carried out the immunization experiments with MVA in mixed chimeras containing both WT and *CPM* bone marrow. In the presence of competition, effector $CD8^+$ T cell populations were reduced in the absence of DOCK8. However, the most dramatic effects were still seen in memory $CD8^+$ T cells 6 weeks post infection. Therefore, the presence of WT cells cannot rescue this memory phenotype, providing strong evidence that DOCK8 plays a critical, cell intrinsic role in the maintenance of memory $CD8^+$ T cells.

4.7 References

1. Jabara, H.H., et al., DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation. Nat Immunol, 2012. 13(6): p. 612-20.
2. Engelhardt, K.R., et al., Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. J Allergy Clin Immunol, 2009. 124(6): p. 1289-302 e4.
3. Zhang, Q., et al., Combined immunodeficiency associated with DOCK8 mutations. N Engl J Med, 2009. 361(21): p. 2046-55.
4. Gadola, S.D., et al., TAP deficiency syndrome. Clin Exp Immunol, 2000. 121(2): p. 173-8.
5. de la Calle-Martin, O., et al., Familial CD8 deficiency due to a mutation in the CD8 alpha gene. J Clin Invest, 2001. 108(1): p. 117-23.
6. de la Salle, H., et al., HLA class I deficiencies due to mutations in subunit 1 of the peptide transporter TAP1. J Clin Invest, 1999. 103(5): p. R9-R13.
7. Doherty, P.C., Cytotoxic T cell effector and memory function in viral immunity. Curr Top Microbiol Immunol, 1996. 206: p. 1-14.
8. Harty, J.T., A.R. Tvinnereim, and D.W. White, CD8+ T cell effector mechanisms in resistance to infection. Annu Rev Immunol, 2000. 18: p. 275-308.
9. Walter, E.A., et al., Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med, 1995. 333(16): p. 1038-44.
10. Freeman, A.F. and S.M. Holland, Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. Pediatr Res, 2009. 65(5 Pt 2): p. 32R-37R.

11. Milner, J.D., et al., Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature, 2008. 452(7188): p. 773-6.
12. Kaech, S.M. and E.J. Wherry, Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity, 2007. 27(3): p. 393-405.
13. Wherry, E.J. and R. Ahmed, Memory CD8 T-cell differentiation during viral infection. J Virol, 2004. 78(11): p. 5535-45.
14. Joshi, N.S. and S.M. Kaech, Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. J Immunol, 2008. 180(3): p. 1309-15.
15. Kaech, S.M., et al., Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol, 2003. 4(12): p. 1191-8.

Chapter 5

The Role of DOCK8 in the Development and Survival Of Invariant Natural Killer T cells

5.1 Role of DOCK8 in human iNKT cells

Invariant NKT (iNKT) cells are a rare lymphocyte population with immunoregulatory properties and have been demonstrated to have roles in active immunity to bacteria, viruses and cancers. Growing evidence also suggests that iNKT cells are dysregulated in immune pathologies such as asthma and atopic dermatitis [1-4]. Therefore much research has been carried out to understand the development and activation of these cells with the aim of manipulating such cells for therapeutic purposes. Some success has already been achieved in this respect with analogues of alpha-galactosylceramide (αGalCer) entering clinical trials to treat cancer [5].

In addition to this, patients with autoimmune and primary immunodeficiencies (PIDs) often have defects in iNKT cell numbers and function. For example, individuals with homozygous mutations in the cytoskeletal protein, wiskott-aldrich syndrome protein (WASp), are immunodeficient and prone to autoimmune manifestations and have been shown to have low levels of iNKT cells in the blood [6]. In animal models, iNKT cells deficient in WASp protein underwent altered development and have defects in survival and activation [6, 7].

In collaboration with Helen Su, NIH (Bethesda, USA), we were able to establish levels of iNKT cells in the blood of DOCK8 deficient patients. Similar to Wiskott-Aldrich syndrome, iNKT cells were reduced in the absence of DOCK8 (Fig24). However, iNKT cells are primarily found in the secondary lymphoid organs such as

the liver and spleen and therefore the study of iNKT cells in humans is difficult. Through the use of our ENU strain, Captain Morgan (*CPM*), I examined the role of DOCK8 in the development and maintenance of iNKT cells. The principal outcomes of this work were the discovery of a role for DOCK8 in the development and survival of iNKT cells and the characterization of a novel cell marker during iNKT cell development within the thymus.

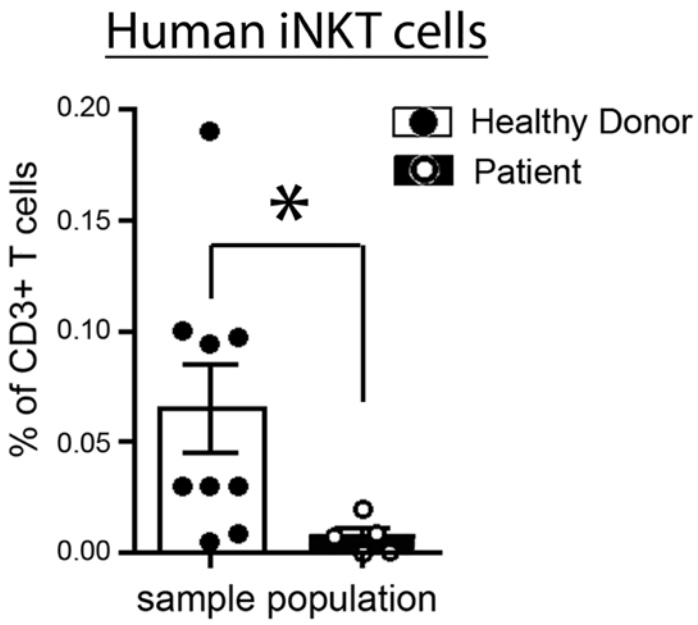


Figure 24: Analysis of iNKT levels in DOCK8 deficient patients. A) Staining of PBMCs with αGC-loaded tetramer to identify iNKT cells was carried out in healthy donors (white bars) and DOCK8 deficient patients (black bars). Data is presented as a percentage of $CD3^+$ T cells with means and SEM depicted. Significance was tested by t test, * $p<0.05$.

5.2 Deficiency in DOCK8 causes the loss of peripheral iNKT cells

As previously mentioned, iNKT cells are predominantly found in the peripheral lymphoid tissues including the liver and spleen. Comparison of peripheral iNKT cell numbers in wildtype (WT), heterozygous and homozygous DOCK8 deficient *CPM* mice showed a 3-4 fold reduction in cells in the liver and spleen of homozygous mutants (Fig25). In additional to this, fewer iNKT cells were also found in the BM and PLNs. Of note, no differences were seen between WT and heterozygous mutants in terms of iNKT cell numbers in the liver and spleen and therefore only homozygous mutants were examined throughout the rest of this body of work.

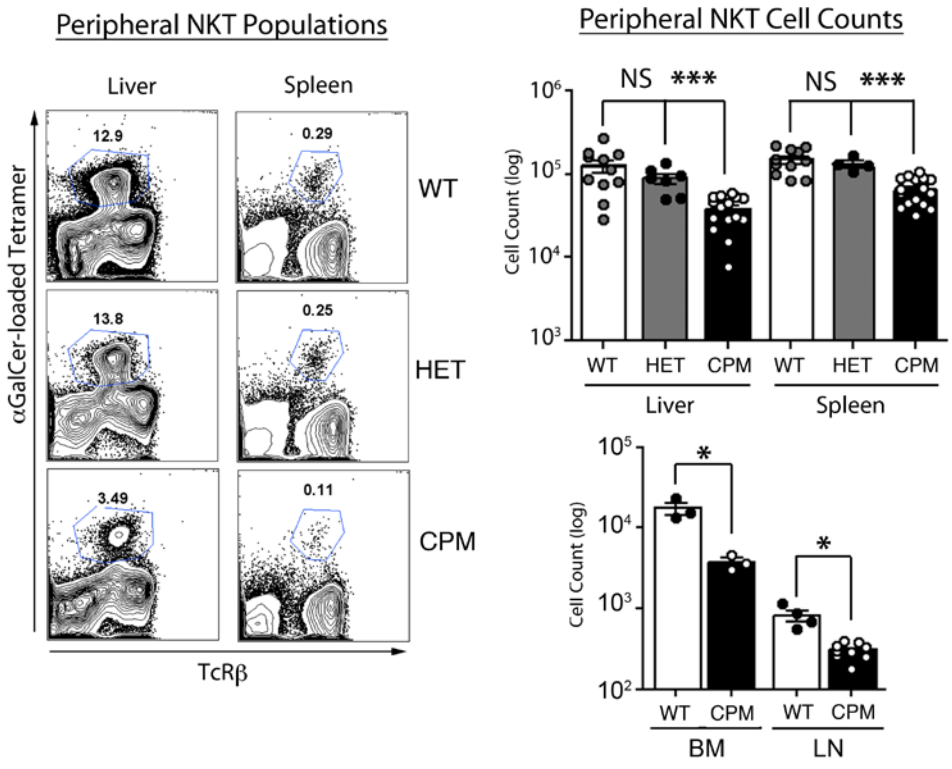
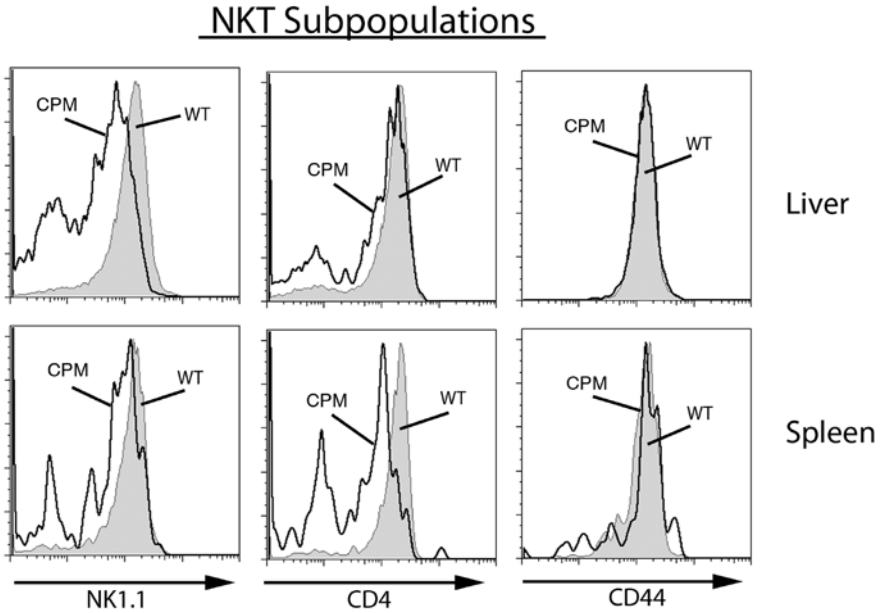


Figure 25: DOCK8 deficiency results in iNKT cell losses in *CPM* mice. A) Flow cytometry analysis of iNKT cells in liver and spleen of WT, heterozygous and homozygous DOCK8 mutants showing representative staining with αGC-loaded tetramer and TCRβ. Cell numbers were enumerated based on staining for liver and

spleen for WT, heterozygous and homozygous mutants. Analysis of bone marrow and lymph nodes was carried out in WT and *CPM* mice only. Data presented depicts arithmetic means with SEM. Statistical significance was tested by t-test, *p<0.05, **p<0.01, ***p<0.001.

Peripheral iNKT cells from WT and *CPM* mice expressed similar high levels of the early developmental marker CD44 (Fig26) but disproportionately fewer *CPM* iNKT cells in the liver and spleen expressed NK1.1, a marker of peripheral differentiation, and CD4 (Fig26). Quantification of $NK1.1^+$ and $NK1.1^-$ stages of development illustrated that the most profound effects were seen in the $NK1.1^+$ compartment, although $NK1.1^-$ iNKT cell numbers were also reduced. These findings demonstrate that loss of DOCK8 causes a deficiency in peripheral iNKT cells, affecting both early $CD44^+NK1.1^-$ and late $CD44^+NK1.1^+$ populations with a greater effect on the more differentiated cells.



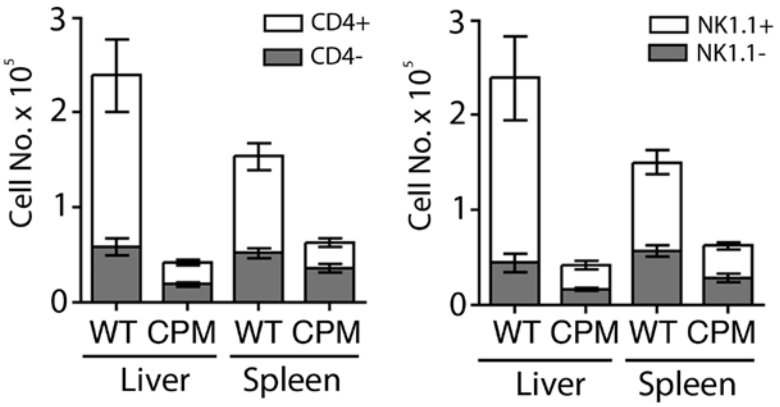


Figure 26: Altered maturation of iNKT cells in the absence of DOCK8. A) Relative expression levels of phenotypic and maturation markers (CD4, NK1.1, CD44) for iNKT from WT (filled) and *CPM* mice (dark line) are depicted. Numbers of CD4 and NK1.1 subpopulations of iNKT were quantified based on flow cytometry staining and cell counts from respective samples. Data are representative of 4 independent experiments showing arithmetic mean and SEM.

5.3 DOCK8 deficiency causes loss of the thymic, $CD44^{+}NK1.1^{+}$, iNKT cells

To address whether the loss of peripheral iNKT cells could in part be explained by deficiencies during iNKT cell development, I compared WT and *CPM* cells in the thymus. The total number of thymic iNKT cells was significantly reduced in *CPM* compared to WT mice ($3.44x10^6$, SD 1.44 vs. $19.8x10^6$, SD 10.1 cells, $p = 0.0002$) (Fig27). As DOCK2 knockout mice show similar losses in thymic iNKT cell numbers (due to a failure in the positive selection of iNKT precursors) this appeared to be a plausible explanation for the loss of iNKT cells [8].

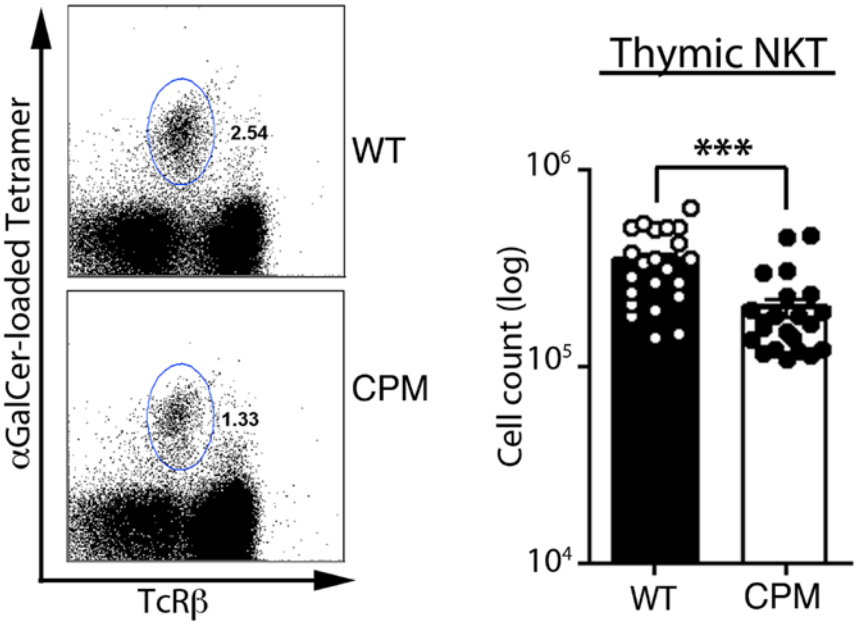


Figure 27: *CPM* mice show reduced numbers of iNKT cells in the thymus. A) Representative FACS staining of thymocytes for αGC-loaded tetramer and TCRβ is shown. Numbers of iNKT cells in the thymus were quantified based on flow cytometry analysis. Data depicts arithmetic means and SEM. Data are representative of at least 4 independent experiments.

However, further analysis of iNKT cell development revealed that DOCK8 was not essential for the earlier stages of iNKT cell development as numbers of $CD44^{-}NK1.1^{-}$ and more mature $CD44^{+}NK1.1^{-}$ cells were comparable between the *CPM* and WT strains (Fig28A). Instead, the reduction of iNKT cells was found to be restricted to the larger $CD44^{+}NK1.1^{+}$ compartment, a resident thymic population with poorly defined function (Fig28A). During development, iNKT cells progressively increase their expression of the transcriptional factor, Tbet, which is important in the maturation and responsiveness of cells to survival factors such as IL15, and down-regulate their

expression of GATA3 [9, 10]. Analysis of these two transcription factors appeared to be normal in the absence of DOCK8 (Fig28B). Together, these data suggest that the development of iNKT cells is intact in the absence of DOCK8. As DOCK8 deficiency does not limit the production of migratory $CD44^{+}$ $NK1.1^{-}$ cells this does not appear to be a possible explanation for the loss of iNKT cells in the periphery.

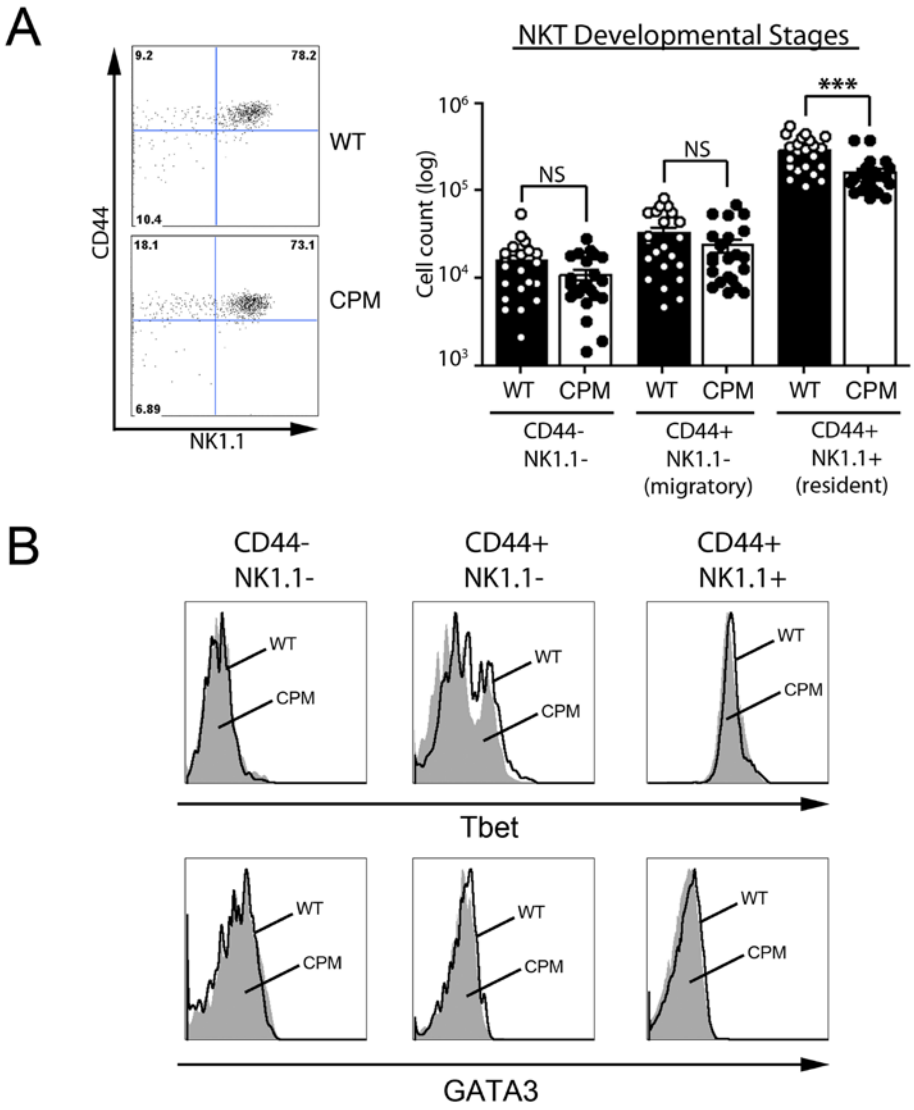


Figure 28: Thymic iNKT cell deficiency is specific to mature, $NK1.1^{+}$ cells. A) αGC-loaded tetramer positive cells were defined by CD44 and NK1.1 expression into the three, linear stages of iNKT cell development. Numbers of cells were calculated for each stage and arithmetic means and SEM are depicted. Statistical significance was

tested by t-test, *p<0.05, **p<0.01, ***p<0.001. B) Histograms representing typical expression profiles of transcription factors, Tbet and GATA3, are shown in the progressive stages of iNKT development. WT (black line) and *CPM* (filled) are shown.

5.4 Increased turnover and decreased survival of *CPM* iNKT cells

To address an alternative explanation, which would be the failure of homeostatic proliferation or increased turnover due to cell death in different cellular compartments, I fed mutant and WT mice bromodeoxyuridine (BrdU) in drinking water for a period of 9 days. Positive selection of iNKT cells in the thymus is accompanied by a proliferative burst which gives rise to the first stage of iNKT cell development ($CD44^{-}NK1.1^{-}$). Equivalent proportions and numbers of stage1 iNKT cells were labelled after nine days of BrdU treatment suggesting that this initial proliferative step is intact and positive selection of iNKT cells is normal in *CPM* mice (Fig29A). The $CD44^{+}NK1.1^{-}$ and $CD44^{+}NK1.1^{+}$ phases of iNKT development also exhibited levels of BrdU similar to WT, implying that the progression of cells through the different stages of development is equivalent (Fig29A). Since fully mature iNKT cells in the thymus are a resting population of cells, this was a strong indication that the loss of iNKT cells in the thymus is primarily due to a problem in survival of these cells. In the periphery, it is more difficult to interpret the BrdU data due to possible differences in the survival of recent thymic emigrants (RTEs) and possible alterations in migration. However, a significantly greater proportion of *CPM* iNKT cells in the liver were BrdU positive, which is also indicative of higher cell turnover and decreased survival, which also appears to be apparent in the thymus (Fig29B).

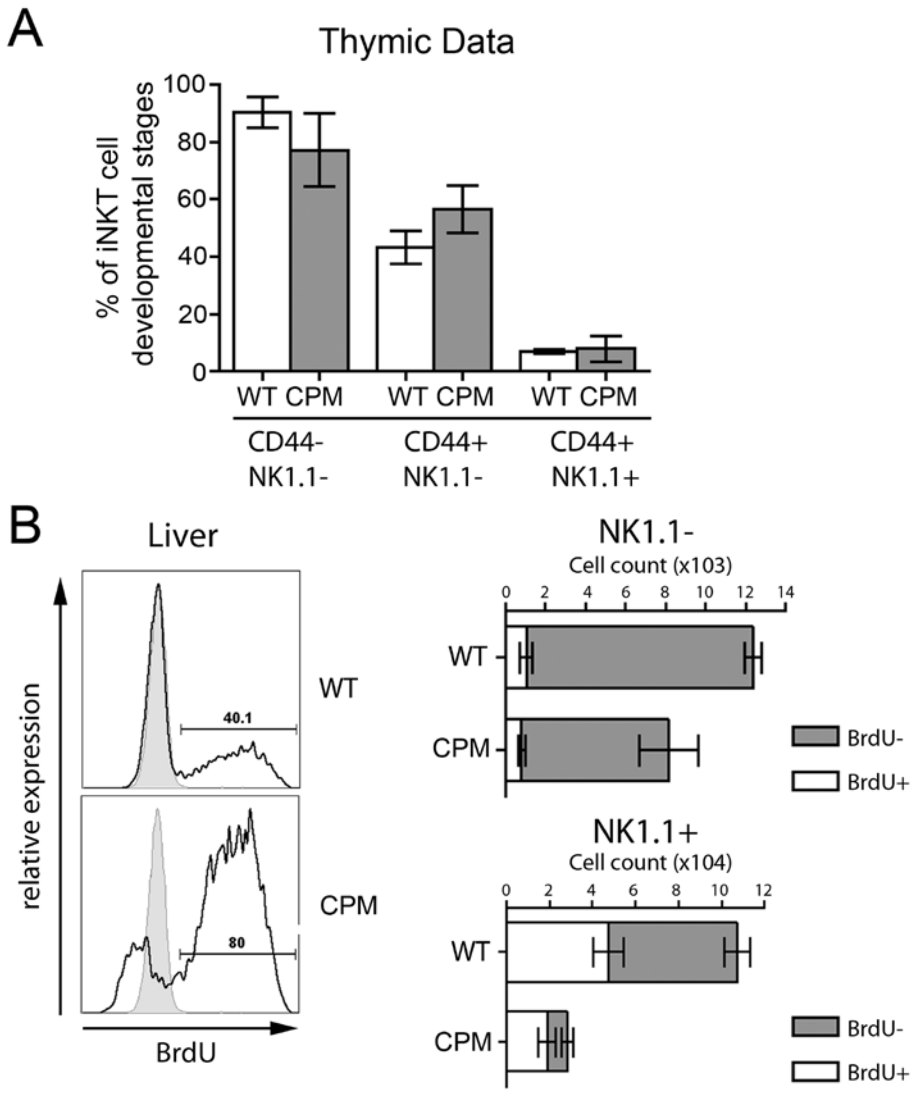


Figure 29: Analysis of iNKT cell development through the use of BrdU. A) WT and *CPM* mice were given BrdU orally for 9 days by the addition of BrdU at 0.8mg/ml in their drinking water. Mice were then culled and examined by flow cytometry to examine BrdU incorporation. Stages of iNKT cell development are depicted showing $BrdU^+$ cells as percentage of parent. B) Relative expression of BrdU is shown for the liver for both WT and *CPM* mice. Cell numbers are calculated for NK1.1- (immature) and NK1.1+ (mature) iNKT cells and $BrdU^+$ and $BrdU^-$ cells are shown. Graphs are representative of 4 mice per experimental group.

5.5 Cell intrinsic and extrinsic effects of DOCK8 deficiency on iNKT cells

To distinguish between the direct and indirect effects of DOCK8 deficiency on iNKT cell development and differentiation, I reconstituted irradiated WT recipient mice (CD45.1 allotype) with 80:20 mixtures of WT or *CPM* (CD45.2 allotype) and WT cells (CD45.1 allotype). This proportion of *CPM* to WT cells was chosen because as previously demonstrated; *CPM* T cells fail to reconstitute the thymus to the same levels as WT cells (Fig11). This phenotype was also true of *CPM* iNKT cells with reconstitution levels for αGalCer-tetramer double-positive iNKT at 47% +/- 10% compared to WT levels of 77% +/- 1.3% (Fig30A).

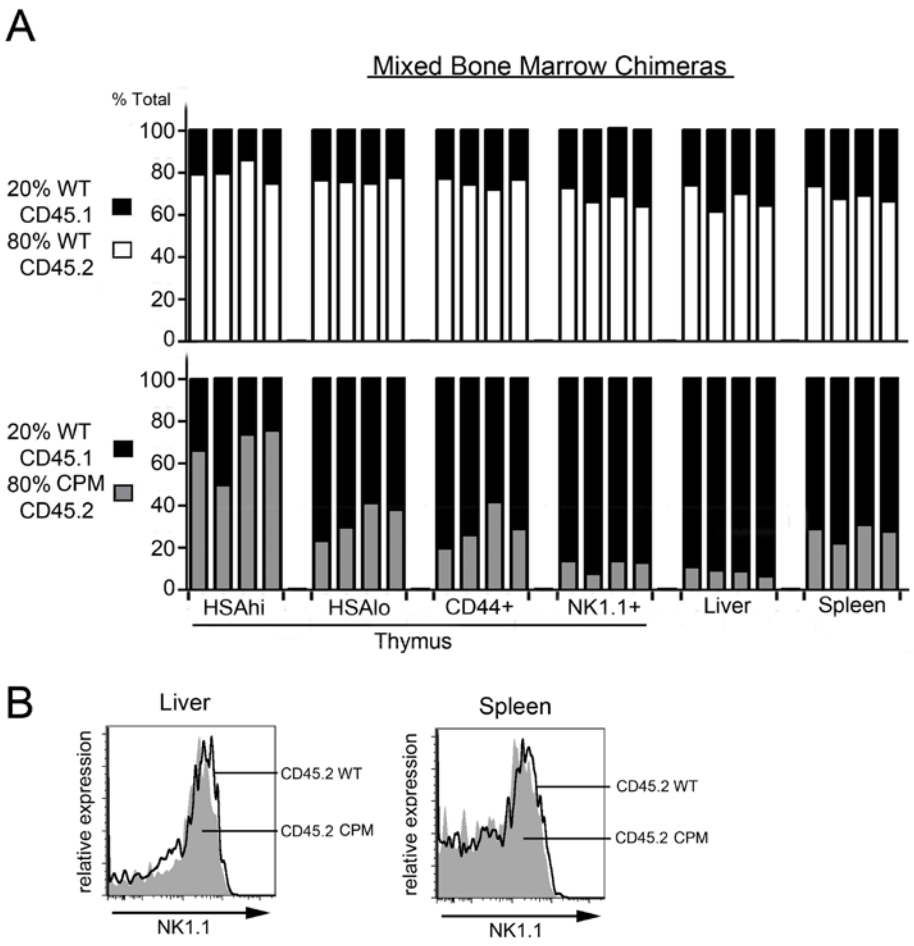


Figure 30: Loss of iNKT cells is intrinsic to liver and thymic iNKT cells. Irradiated mice were reconstituted with BM derived from 80% WT CD45.2 or 80% DOCK8

CD45.2 mice mixed with 20% CD45.1 transgenic cells. Bars depict individual chimeric mice. A) The reconstitution of different thymic selection points in the thymus are shown for αGC-loaded tetramer positive cells as well as peripheral data for the liver and spleen. B) Relative expression levels of the maturation marker NK1.1 on iNKT cells are shown between WT and *CPM* iNKT cells from the liver and spleen.

In this more stringent test of the requirement for DOCK8 in iNKT cell development I found reduced levels of *CPM* cells at the early stage of positive selection, when cells become HSA^{lo} (Fig30A). This point in development is characterized by engagement with antigen and SLAM receptors and a similar defect in iNKT development is apparent in SAP or SLAM receptor deficient mice [11-13]. The fact that this same defect was not observed in unmanipulated *CPM* mice shows this is not normally limiting, possibly because of compensatory proliferation after selection. The loss of mature thymic $NK1.1^+$ iNKT cells persists in *CPM* chimeric mice demonstrating this is a DOCK8 intrinsic defect (Fig30A).

Given the evidence that TCR signalling may play a role in the differentiation of peripheral $NK1.1^+$ iNKT cells from $CD44^+NK1.1^-$ precursors [14], I examined levels of $NK1.1^+$ expression on WT and *CPM* iNKT in the liver and spleen and found that it was restored to normal levels in mixed chimeras, indicating this defect in unmanipulated mice is due to cell extrinsic factors (Fig30B). Interestingly, splenic iNKT cells were also restored to normal levels, being in the same ratio to WT cells as the migratory precursor, $CD44^+NK1.1^-$ cells in the thymus (Fig30A).

In contrast to the situation in the spleen, the reduction in iNKT cells in the liver persisted in mixed chimeras, confirming that this phenotype is principally cell intrinsic **(**Fig30A). The loss of *CPM* liver iNKT cells in mixed chimeras, despite restoration of splenic iNKT cells and NK1.1 expression, shows that loss of this population is due neither to a defect in thymic egress nor to a defect in differentiation to the $CD44^{+}NK1.1^{+}$ state. The findings make a compelling argument that the limiting effects of DOCK8 deficiency are strongly related to the environment inhabited by iNKT cells. Both the resident thymic and liver iNKT cell populations are intrinsically crippled by loss of DOCK8.

5.6 *CPM* iNKT cells fail to survive after adoptive transfer

To separate the generation and the survival of peripheral iNKT cells and to test for possible defects in the migration and localisation of peripheral cells, I transferred mixtures of CD45.2 allotype and GFP expressing WT and *CPM* cells into WT (CD45.1 allotype) recipients for 24hr and for 9 days. After 24 hours, *CPM* iNKT cells localized to equivalent or higher levels than WT counterparts implying no defect in the ability of DOCK8 deficient cells to localise to the liver or spleen (Fig31A). However, when mice were analyzed after 9 days post transfer, levels of *CPM* iNKT cells had dropped significantly in both the liver and spleen (Fig31A). This again supports an intrinsic defect in iNKT cell survival. Decreased expression of the pro-survival factor Bcl-2 in iNKT cells from unmanipulated *CPM* animals provides further evidence of an intrinsic defect (Fig31B).

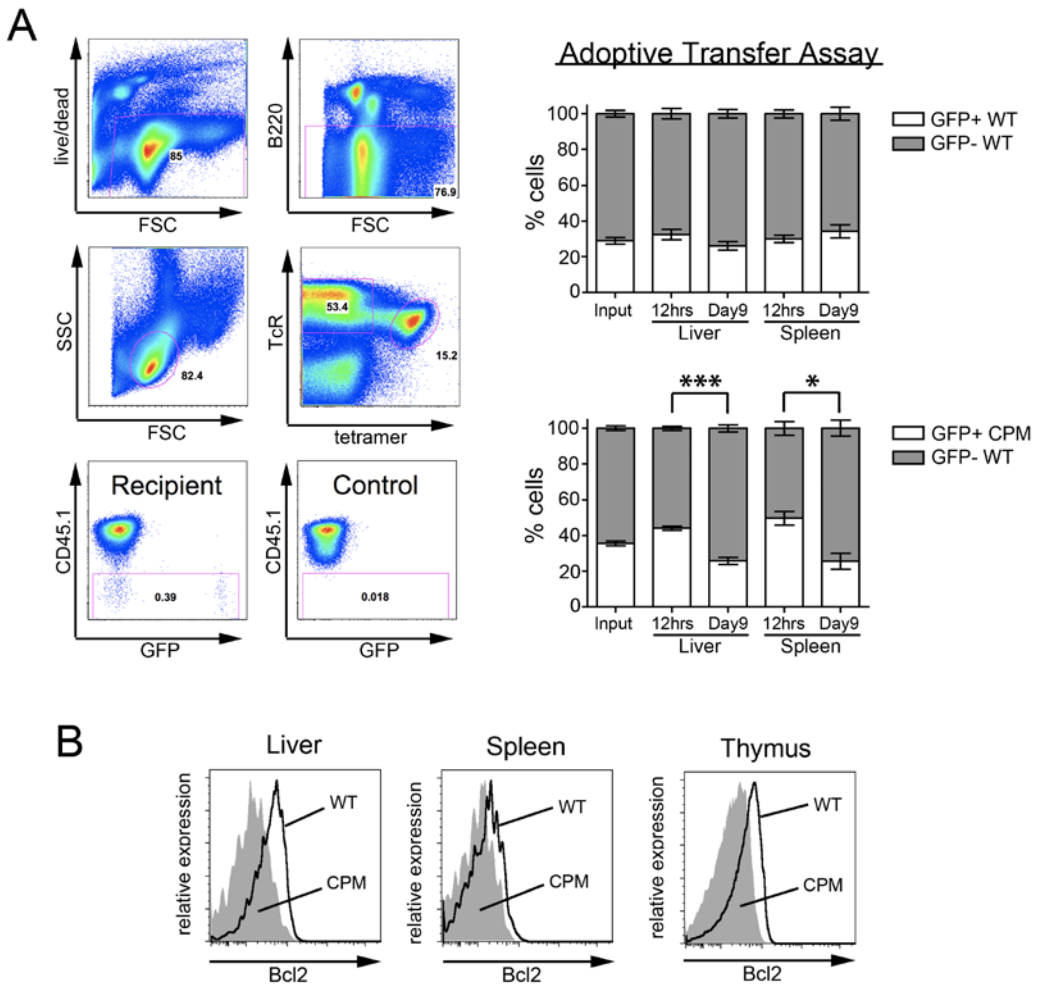


Figure 31: Adoptive transfer of *CPM* iNKT cells demonstrates intrinsic survival defect . A) WT (CD45.2) GFP and *CPM* (CD45.2) GFP iNKT cells derived from spleens were mixed with WT (CD45.2) iNKT cells and adoptively transferred by intravenous injection into CD45.1 transgenics hosts. Animals were culled at designated time points before donor cells were analyzed by FACS. Gating strategy to define donor cells is illustrated showing recipient and control samples. Graphs depict WT and *CPM* (CD45.2 cells) as percentages of adoptively transferred cells from the spleen and liver. Bars depict arithmetic means based on 5 individual animals and SEM. Statistical significance was tested by t-test, *p<0.05, **p<0.01, ***p<0.001. B) Relative expression profiles of Bcl2 are depicted for liver, spleen and thymic iNKT cells with WT (filled) and *CPM* (dark line).

5.7 Activation with αGalCer demonstrates intact TCR signalling in *CPM* iNKT

Studies in DOCK2 and DOCK8 deficient mice have shown defects in formation of the B and T cell immune synapse [15-17]. As antigen-receptor signalling can be involved in the differentiation, maturation and effector function of iNKT cells, I asked if abnormalities in iNKT cell responsiveness to TCR stimuli could account for decreased survival of peripheral *CPM* iNKT cells. Accordingly, I injected *CPM* mice with the glycolipid, αGalCer, and looked at iNKT activation by intracellular cytokine staining (ICS). Despite the reduction in the total number of cells, the responses by iNKT cells in the liver were equivalent in *CPM* mice compared to WT, suggesting TCR signalling is unaffected by the loss of DOCK8 function and that processes such as antigen presentation were not limiting (Fig32A). However, responses in the spleen were significantly weaker in *CPM* mice (Fig32A). As CD1d positive marginal zone B cells are absent in *CPM* mice, and have a well-documented role in iNKT activation in the spleen [18], I considered the possibility that this defect in activation may be due to differences in antigen presentation. To determine more accurately the nature of iNKT activation in the absence of DOCK8, I generated chimeric mice and repeated the experiment, and in this assay the cytokine responses were restored (Fig32B) consistent with the hypothesis that reduced splenic iNKT cell responses were due to the absence of MZ B cells in *CPM* mice.

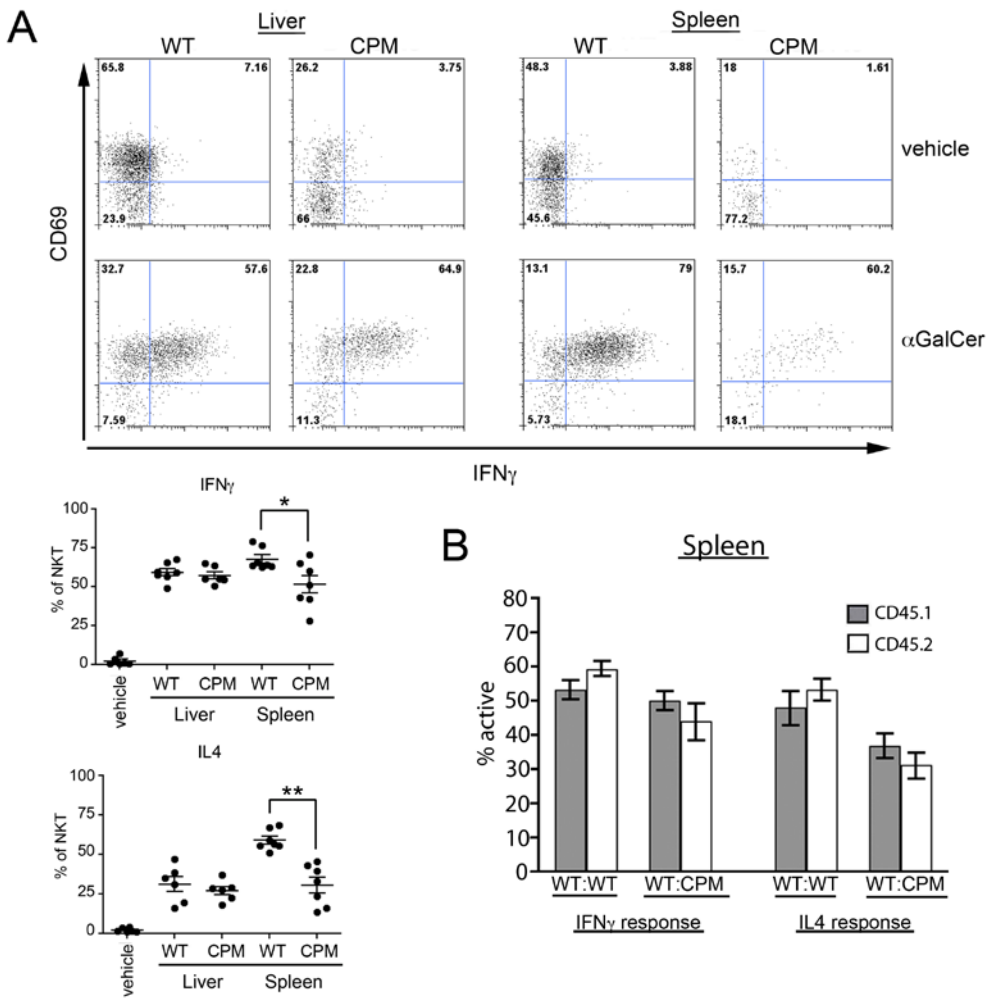


Figure 32: Examination of iNKT cell activation by αGalCer challenge. A) Mice were injected with αGalCer and responses quantified by ICS. Flow cytometry analysis was carried out using CD69, IFNγ and IL4 staining. Plots depicted are representative of αGC-loaded tetramer^{+} cells after treatments indicated. Graphs illustrate responders as a percentage of iNKT cells showing arithmetic means and SEM. Data is representative of 3 independent experiments. B) Irradiated mice were reconstituted with bone marrow derived from 80% WT CD45.2 or 80% *CPM* CD45.2 mice mixed with 20% CD45.1 transgenic cells before injection with αGalCer intravenously as previously described. Percentage of activated iNKT based on IFNγ and IL4 expression is shown. Bars represent the arithmetic means with SEM of responses generated in 5 individual animals.

The same factor may also affect the differentiation of splenic iNKT cells and their survival, however TCR activation and cytokine production in response to αGalCer is normal in *CPM* iNKT cells in the liver. On this basis, as well as on the basis of normal up regulation of NK1.1 in mixed chimeras, I have found no evidence for an involvement of DOCK8 in iNKT cell activation itself and no suggestion that loss of TCR signalling could easily account for the decreased number of resident iNKT cells in the liver or thymus.

5.8 LFA-1 expression on liver iNKT cells is DOCK8 dependent

Current thinking suggests that long term survival of memory type cells, including terminally differentiated iNKT cells, depends on growth factors including homeostatic cytokines and environmental cues that they receive from cell-cell contact, notably via integrins [19-21]. The response of WT and *CPM* iNKT cells from the thymus to 3 day culture with media alone or media supplemented with IL2, IL7, IL15 or IL21 was equivalent, suggesting that defective responses to soluble growth factors is not the principal cause of cell loss (Fig33A). However, LFA-1 expression was reduced in *CPM* iNKT cells in the liver and other organs (Fig33B**)**. Signalling through the LFA-1 integrin has already been shown to be essential for survival or long-term retention of iNKT cells in the liver although it is not required for thymic or splenic iNKT cell numbers [19, 21]. Therefore, a cell intrinsic disruption in the integrin expression, binding and/or signal transduction, (as previously observed in CPM mice in regards to both B and T cell synapse formation), could lead to a failure to gain crucial survival factors to preserve iNKT cell numbers.

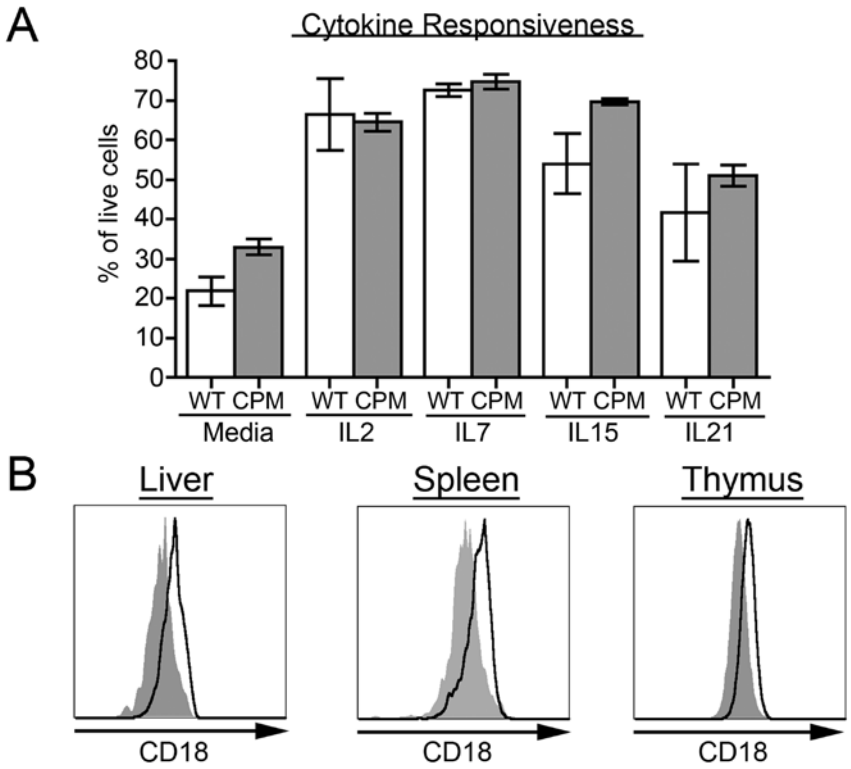


Figure 33: Analysis of cytokine responsiveness and LFA-1 expression. A) iNKT cells derived from the thymus were cultured in 96 well plates and supplemented with a range of cytokines (10μg/ml). Cells were cultured for 3 days before staining for dead cells using Live/dead kit (Invitrogen) to test for cell viability. Data is presented as the percentage of live cells after culture. Bars depict arithmetic means and SEM based on 3 samples. Data is representative of 3 separate experiments. B) Staining profiles of the integrin subunit, CD18 (part of the LFA-1 integrin) are depicted with relative expression of WT (dark line) versus *CPM* (filled).

5.9 Loss of DOCK8 defines a new CD103^{+}NK1.1^{+} iNKT cell subset in the thymus

To try to elucidate the intrinsic defect in survival, I took a genetic approach, comparing gene expression of iNKT cell subsets using RNA microarrays. Analysis of the liver iNKT cell phenotype was complicated by the difficulty of isolating cells and the complexity of controlling for the different rate of generation of new residents. For these reasons I carried out the analysis using cells isolated from the resident NK1.1^{+} population in the thymus. Comparison of TCRβ^{+}Tetramer^{+}CD8^{-}CD44^{+}NK1.1^{+} cells from the thymus of WT and *CPM* mice showed differential expression of a relatively small subset of 52 genes (Fig34).

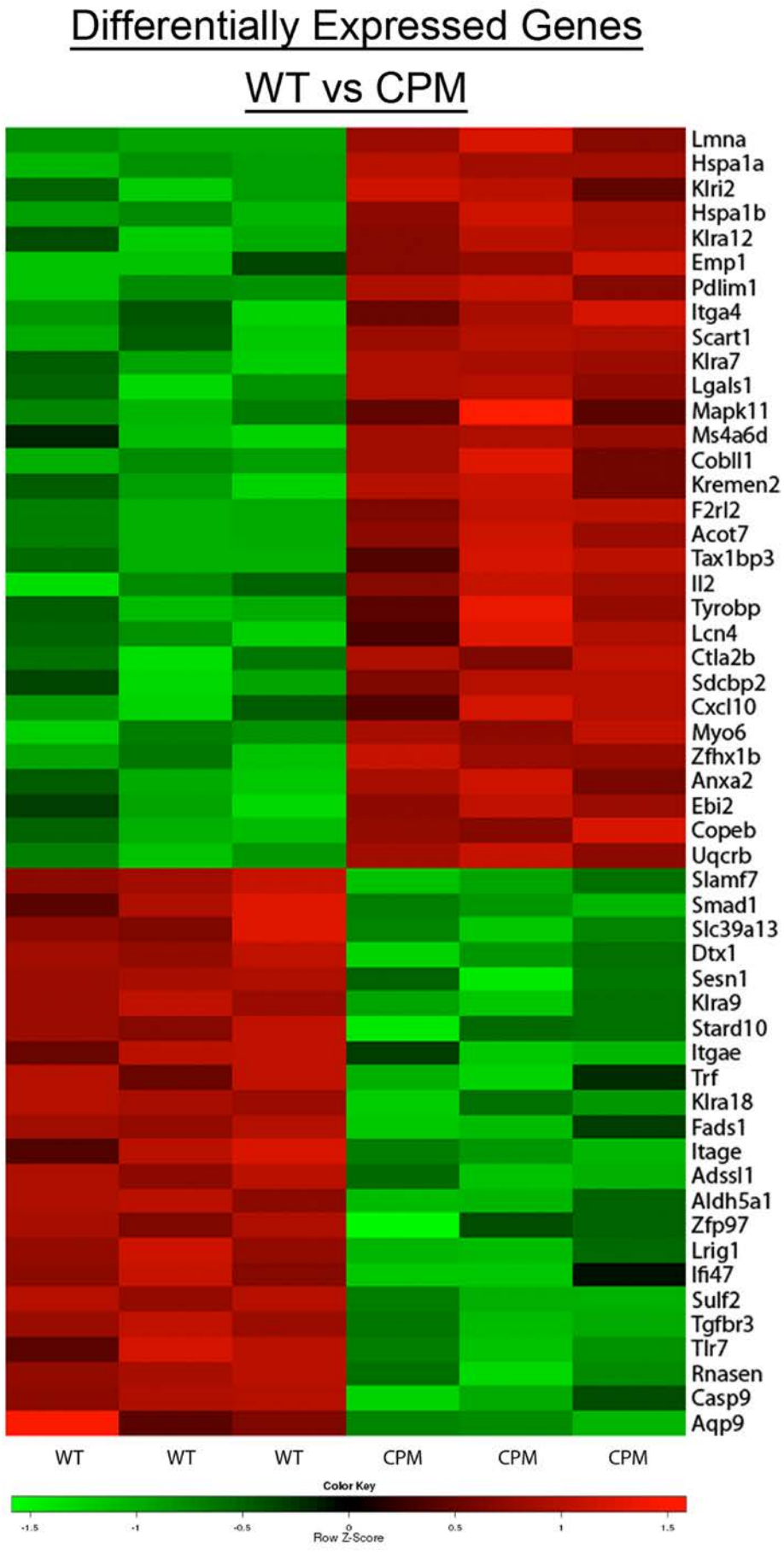

Differentially Expressed Genes
WT vs CPM
Lmna
Hspa1a
Klri2
Hspa1b
Klra12
Emp1
Pdlim1
Itga4
Scart1
Klra7
Lgals1
Mapk11
Ms4a6d
Cobll1
Kremen2
F2rl2
Acot7
Tax1bp3
Il2
Tyrobp
Lcn4
Ctla2b
Sdcbp2
Cxcl10
Myo6
Zfhx1b
Anxa2
Ebi2
Copeb
Uqcrb
Slamf7
Smad1
Slc39a13
Dtx1
Sesn1
Klra9
Stard10
Itgae
Trf
Klra18
Fads1
Itage
Adssl1
Aldh5a1
Zfp97
Lrig1
Ifi47
Sulf2
Tgfbr3
Tlr7
Rnasen
Casp9
Aqp9
WT
WT
WT
CPM
CPM
CPM
Color Key
-1.5
-1
-0.5
0
0.5
1
1.5
Row Z-Score

Figure 34: Genetic analysis of fully mature, thymic *CPM* iNKT cells. A) Fully mature, NK1.1^{+} iNKT cells were sorted to >99% purity from the thymuses of WT (n=3) and CPM (n=3) *mice* by FACS. Prior to sort, thymuses were processed as normal by depletion of CD8^{+} T cells by MACS. FACS samples were sorted based on gating strategies to identify live, CD8^{-}, TCRβ^{+}, αGC-loaded tetramer^{+}, CD44^{+} and NK1.1^{+} cells. After isolation of RNA from cells, samples were hybridized to commercially available MouseWG-6-V2 microarray chips to uncover differences in gene expression profiles. Analysis uncovered 52 differentially expressed genes. The heatmap depicts the relative expression of these genes comparing WT expression profiles to *CPM* mutants.

Noticeable amongst the differentially expressed genes was the two-fold lower overall expression of integrin αE (Itgae), also known as CD103. CD103 binds the integrin subunit β7 to form the αEβ7 complex, whose main ligand is the epithelial cell adhesion molecule E-cadherin. CD103 has not previously been reported as a marker on iNKT cells in the thymus or associated with increased expression of NK1.1; but it has been reported on NK1.1^{-} iNKT cells in the skin and peripheral lymph nodes and is widely expressed on intraepithelial lymphocytes (IELs) in the gut and on some regulatory T cells, as well as dendritic cell subsets. Flow cytometry of WT iNKT cells in the thymus showed progressive up regulation of CD103 expression during development (Fig35A, B), so that a majority of the differentiated thymic resident NK1.1^{+} cells expressed the integrin. In the absence of DOCK8, although CD103 expression was normal during early stages of development and was successfully up regulated on the more mature CD44^{+}NK1.1^{-} subset, CD44^{+}NK1.1^{+} *CPM* iNKT cells

failed to maintain CD103 expression (Fig35B). Analysis of liver iNKT cells demonstrated only a small proportion of $CD103^+$ cells and therefore this integrin does not appear to have a critical role in the periphery (Fig35C).

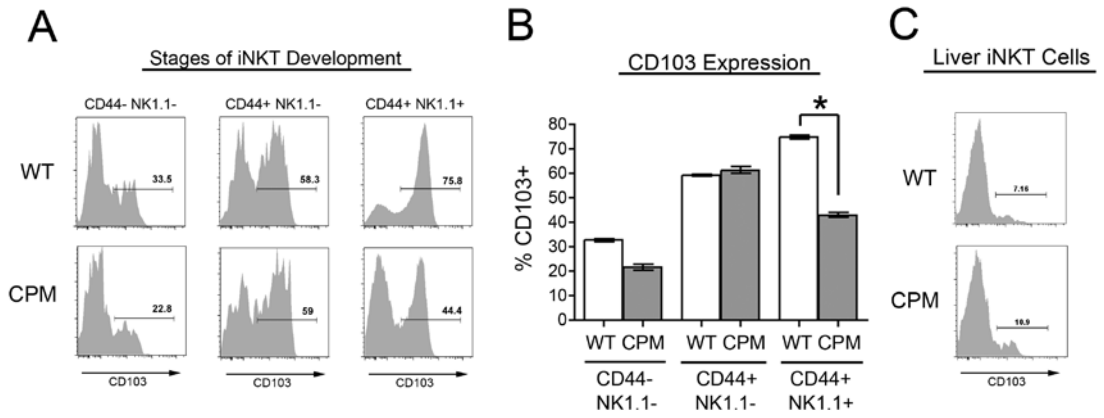


Figure 35: CD103 identified as a novel marker of iNKT cell development. A) Representative profiles of CD103 expression of αGC-loaded $tetramer^+$ cells during development in the thymus from WT and *CPM* mice. B) Percentages of CD103 positive iNKT cells are depicted showing arithmetic means and SEM. Statistical significance was tested by t-test, *$p<0.05$. C) Representative profiles of liver iNKT cells are depicted. Data are representative of at least 3 independent experiments.

5.10 Role of DOCK8 in CD103 regulation is specific to thymic iNKT cells

As previously mentioned, CD103 expression is important in the localization and maintenance of intraepithelial lymphocytes [22]. To assess whether DOCK8 had a critical role in specifically maintaining CD103 expression in other cell types, I isolated IELs from the small intestine and carried out staining of $CD4^+$ and $CD8^+$ T cells for CD103 expression. If CD103 expression or activity were impaired in the absence of DOCK8, reduced numbers of IELs would be expected. Although trends in

reductions of IELs were observed in the numbers of both $CD4^+$ and $CD8^+$ T cells in the small intestine, these data did not reach statistical significance (Fig36A). Expression of CD103 was also normal on *CPM* T cells therefore DOCK8 appears to have a more specialized role in maintaining CD103 expression in thymic iNKT cells (Fig36B).

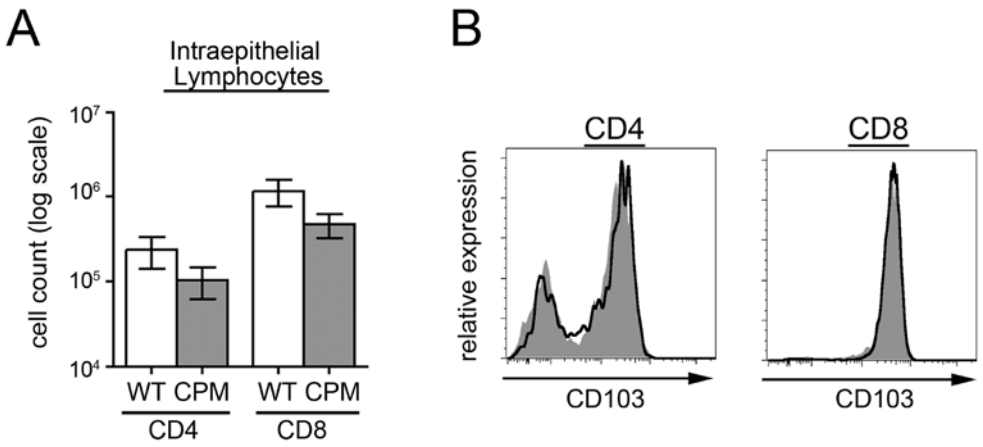


Figure 36: Analysis of intraepithelial lymphocytes (IELs) in *CPM* mice. A) IELs were isolated from the small intestine and stained to analyze expression of CD103. Numbers of cells isolated were quantified and are depicted as arithmetic means and SEM. B) Histograms depict representative expression profiles of CD103 on $CD4^+$ and $CD8^+$ T cells from WT (dark line) and *CPM* (filled).

5.11 DOCK8 regulates integrins and survival factors in a cell intrinsic manner

Focusing on thymic iNKT cells, this defect in CD103 expression is cell intrinsic in mixed bone marrow chimeras as well as decreased expression of the pro-survival factor Bcl2 (Fig37A). In contrast, the expression of other molecules known to be associated with the development iNKT cell population, such as ICOS, were similar to

WT in the $CD44^{+}NK1.1^{+}$ population (Fig37A). Although only a small percentage of liver iNKT cells express the integrin CD103, all liver iNKT cells express the integrin, LFA-1. LFA-1 has been demonstrated to have a critical role in the localization and survival of liver iNKT cells by interfering with the function of this integrin through the use of blocking antibodies [21]. Expression of this integrin is also reduced in the absence of DOCK8 in a cell intrinsic manner and again, similar to *CPM* thymic iNKT cells, the expression of the pro-survival factor Bcl2 was also reduced (Fig37B). Together, these data suggest that DOCK8 has an important role in providing survival factors to iNKT cells both in the thymus and liver. However, iNKT cells from different organs may demonstrate a range of dependence for integrins including LFA-1 and CD103.

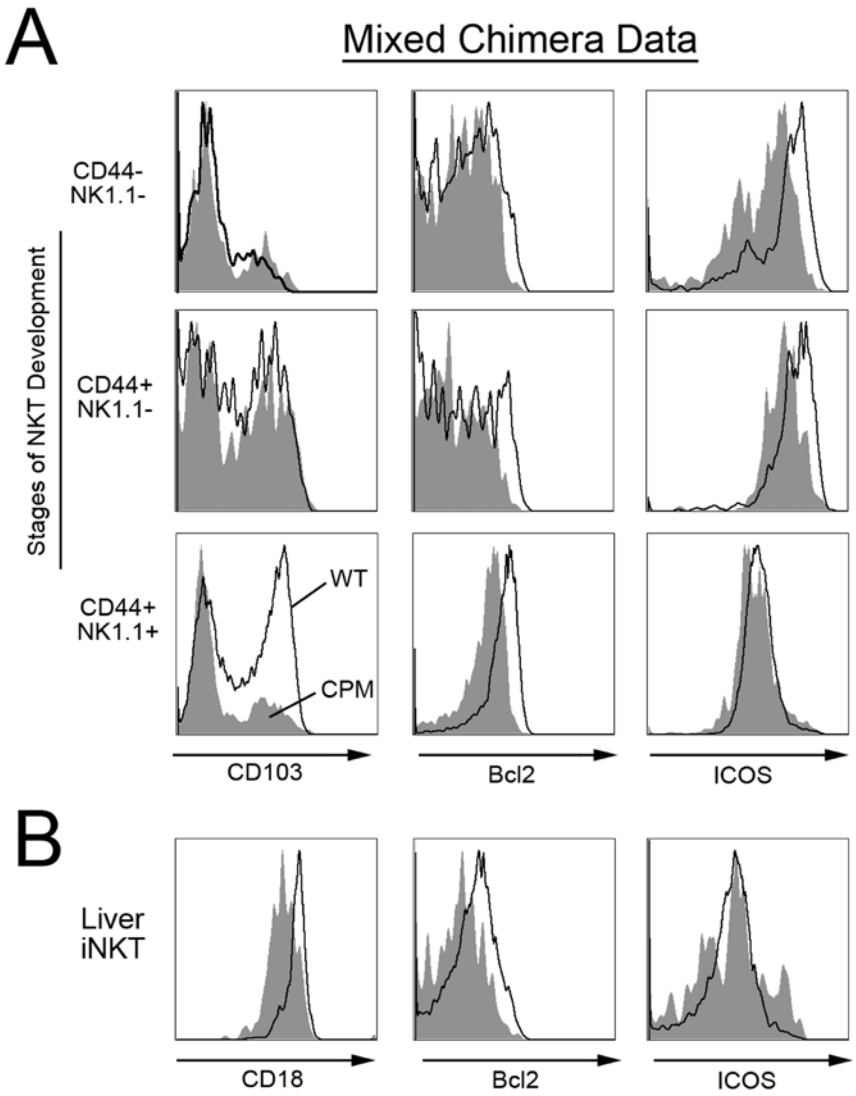


Figure 37: Loss of CD103 expression is an intrinsic defect of *CPM* iNKT cells. A) Irradiated mice were reconstituted with BM derived from 80% WT CD45.2 or 80% DOCK8 CD45.2 mice mixed with 20% CD45.1 transgenic cells. Stages of iNKT development are depicted for WT (dark line) and *CPM* (filled) illustrating different phenotypic markers including CD103, Bcl2 and ICOS. B) Representative histograms are also shown for liver iNKT cells.

5.12 Expression of CD103 is associated with increased expression of a wide range of immune regulated genes

To understand the factors affecting the resident iNKT cells I compared gene expression in precursor CD103 negative and more differentiated CD103 positive cells from WT mice (Fig38). In this analysis the normal differentiation to the CD103^{+} subset was characterised by increased expression across a wide range of targets, representing 220 genes. A much smaller subset of 66 genes were downregulated, including the Slamf1 receptor, which is involved in the earlier stages of iNKT cell differentiation, and Bcl2.

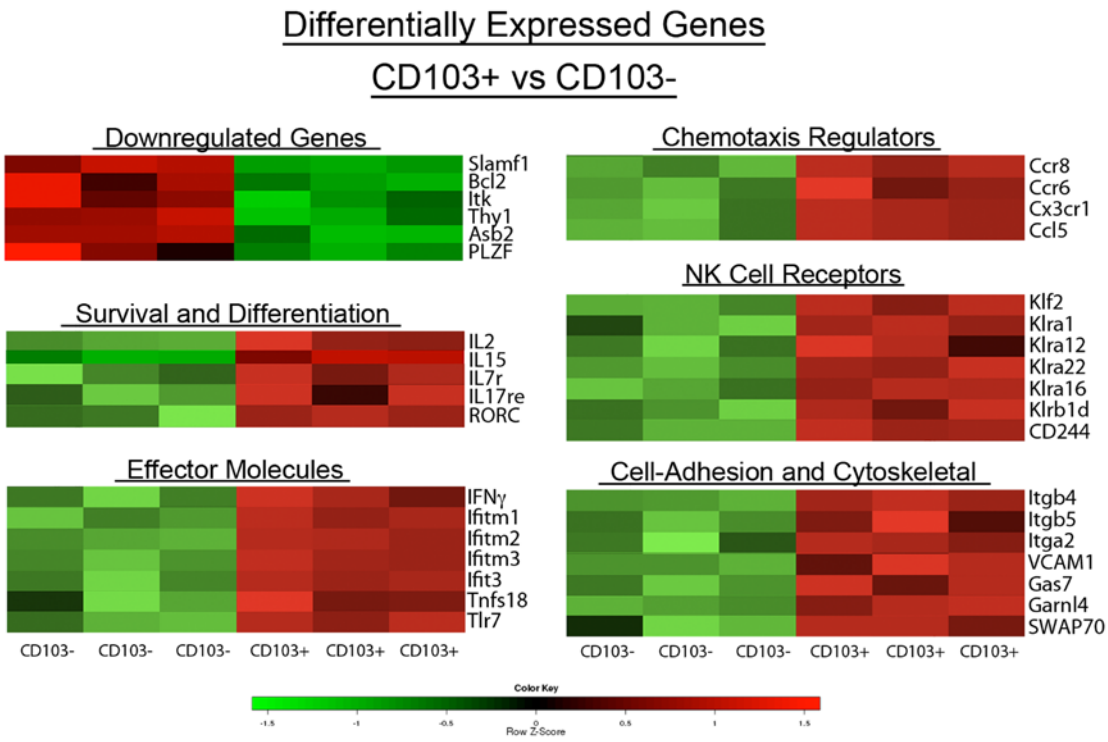


Figure 38: Genetic analysis of the role of CD103 in iNKT cell development. A) iNKT cells were sorted from WT mice based on CD103 expression. Live, CD8^{-}, TCRβ^{+}, αGC-loaded tetramer^{+}, CD44^{+} and NK1.1^{+} cells were subdivided into CD103^{+} and CD103^{-} fractions before isolation of RNA samples. Comparisons of gene expression were then carried out using 3 biological replicates. This analysis uncovered a vast

array of genes depicted in the heat maps. Genes were further subdivided based on their documented roles in cellular immunity.

Reduced Bcl2 expression coincided with up-regulation of receptors and cytokines involved in pro-survival signals including the IL-7 and IL-17 receptors, IL-2 and IL-15. Differentiation also coincided with expression of the nuclear hormone receptor RORC, up-regulation of effector molecules, including TLR7 and chemokine receptors and other markers including CCR6, which may be required for trafficking to CCL20 expressed by thymic epithelial cells. As well as CD103, the differentiated cells express high levels of genes involved in cell-cell contact and cytoskeletal regulation, including E-cadherin itself, $\alpha 2$ and $\beta 4$ integrins, and growth arrest specific gene 7 (Gas7); an adaptor protein involved the formation of neurites through direct interactions with actin and an association with N-WASP. The mRNA of the actin binding protein, SWAP70, was also increased 3 fold in $CD103^+$ cells. SWAP70 deficiency results in the loss of germinal centre and MZ B cells and impaired activation of LFA-1 in B cells, features already reported in *CPM* mice [23]. Thus, proteins such as Gas7 and SWAP70 may be part of signalling pathways downstream of DOCK8 activation. The role of these proteins in the function and survival of resident cell populations will be the subject of future work.

5.13 CD103 expression alone is not critical for thymic iNKT cell survival

To examine the role of CD103 for iNKT cell survival in the thymus, I analyzed CD103 knockout mice. This study shows that CD103 itself is not essential for the survival of the resident iNKT cells in the thymus, since normal numbers of $NK1.1^+$ iNKT cells are present in the thymus of CD103 knockout mice (Fig39A). In

agreement with the microarray data, Bcl2 expression levels were higher in CD103 knockout iNKT cells (Fig39B). In the absence of the integrin CD103, other integrins such as LFA-1 may take on a compensatory role providing pro-survival factors such as Bcl2. These hypotheses will need to be tested in future work.

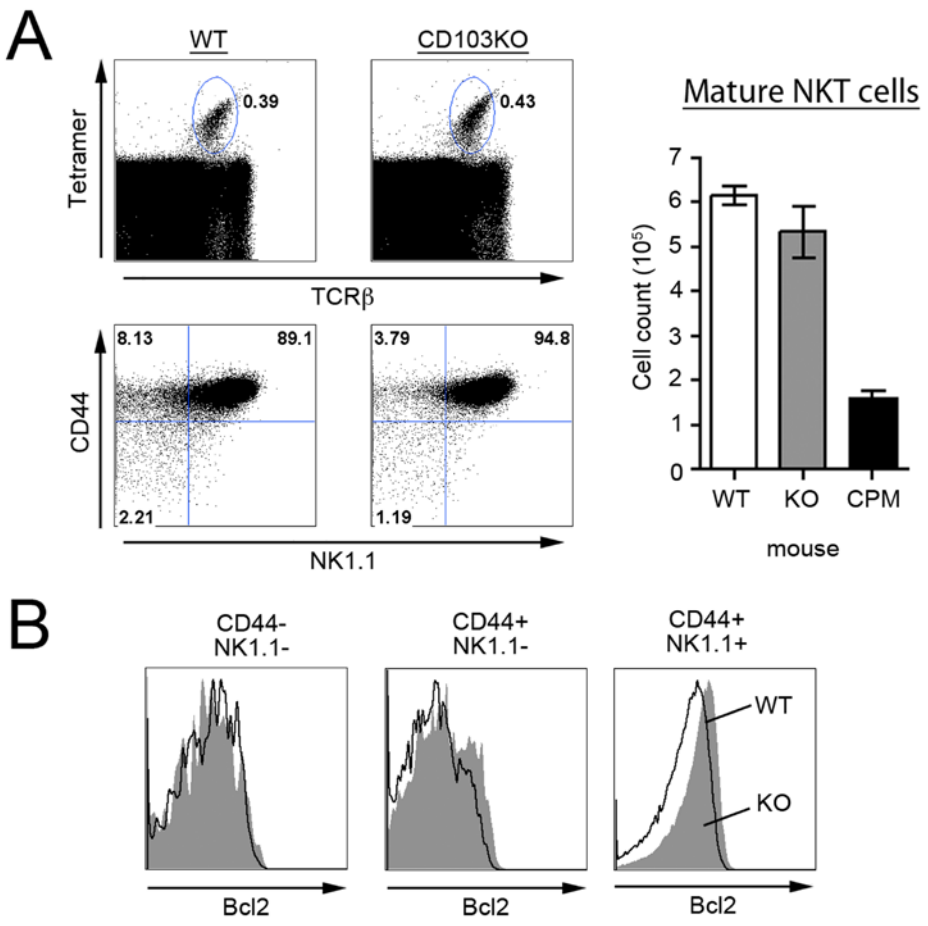


Figure 39: CD103 knockout mice show no alterations in thymic iNKT cell development. A) Flow cytometry analysis of thymic iNKT cells development in WT and CD103KO mice showing representative staining with αGC-loaded tetramer and TCRβ. αGC-loaded tetramer^{+} cells were further analyzed based on CD44 and NK1.1 expression. Numbers of mature, NK1.1^{+} iNKT cells were calculated in WT, *CPM* and CD103KO mice. Bars show arithmetic means and SEM. B) Bcl2 expression profiles of all stages of iNKT cell development comparing WT and CD103KO are depicted. Data are representative of 2 independent experiments.

5.14 Conclusions

The role of DOCK8 has now been shown to be critical for the survival of marginal zone B cells, germinal centre B cells and memory T cells, which are all cell types with innate/activated qualities. Perhaps not surprisingly, loss of DOCK8 also results in the disruption of innate-like lymphocytes such as invariant NKT cells (iNKT). Similar to the T cell lymphopenia seen in mouse and human studies, homozygous mutations/deletions of the DOCK8 gene result in loss of iNKT cells in the blood of patients and in the peripheral lymphoid organs of animal models respectively.

Through the use of the ENU mutant, Captain Morgan (*CPM*), I have been able to show that DOCK8 is required for the maintenance and survival of iNKT cells. In patients with DOCK8 deficiency, like Wiskott-Aldrich syndrome patients, iNKT cells are reduced in the blood. However, difficulties in isolating these rare cells from patients make further analysis difficult. Analysis of *CPM* mice shows peripheral iNKT cells are reduced in the liver, spleen, bone marrow and lymph nodes. Interestingly, peripheral iNKT cells also show clear differences in expression of the maturation marker, NK1.1, implying decreased survival of the most differentiated cells. This pattern of loss of iNKT cell numbers and specifically $NK1.1^+$ cells is repeated in the thymus where it is not due to reduced differentiation into the $NK1.1^+$ population. Deficiencies in iNKT cells in the liver and thymus were seen to be cell intrinsic whereas splenic iNKT cells were restored in mixed chimeras (possibly due to the restoration of the $CD1d^+$ MZ B cell population).

Survival of iNKT cells, like memory T cells, has been shown to be independent of TCR signalling and more dependent on factors such as cytokines. However, full

maturation of iNKT cells in the periphery is dependent on TCR stimulation outside of the thymus. Activation of iNKT cells in liver was intact after injection with αGalCer, suggesting that TCR signalling is not impaired in the absence of DOCK8. Although responses were weaker in the spleen, when experiments were carried out in the setting of mixed chimeras, where antigen presentation by MZ B cells is intact, responses returned to normal.

Adoptive transfer of *CPM* iNKT cells showed that chemotactic responses were unchanged but long-term transfer demonstrated a survival deficiency. This survival defect was accompanied by lower expression of the pro-survival factor Bcl2 and the integrin LFA-1, later confirmed to be cell intrinsic defects due to loss of DOCK8. However, although LFA-1 knockout models show losses of liver iNKT cells, no losses are seen in the thymus [24].

Genetic analysis of thymic iNKT cells from WT and *CPM* mice was able to identify a number of genes differentially expressed. One gene of interest was the integrin CD103. Expression of this integrin was intrinsically and specifically lost on $CD44^{+}NK1.1^{+}$ *CPM* iNKT cells. Up-regulation of this gene is associated with changes in a number of immune regulated genes including factors including cytokines such as IL2 and IL15, integrins and cytoskeletal proteins. Further work will be carried out to understand the roles of these genes in maintaining thymic iNKT cells and to further understand the role of DOCK8 in iNKT cell homeostasis and function.

5.15 References

1. Iwamura, C. and T. Nakayama, Role of NKT cells in allergic asthma. Curr Opin Immunol, 2010. 22(6): p. 807-13.
2. Stock, P. and O. Akbari, Recent advances in the role of NKT cells in allergic diseases and asthma. Curr Allergy Asthma Rep, 2008. 8(2): p. 165-70.
3. Gyimesi, E., et al., Altered peripheral invariant natural killer T cells in atopic dermatitis. J Clin Immunol, 2011. 31(5): p. 864-72.
4. Simon, D., E. Kozlowski, and H. Simon, Natural killer T cells expressing IFN-gamma and IL-4 in lesional skin of atopic eczema. Allergy, 2009. 64(11): p. 1681-4.
5. Nieda, M., et al., Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood, 2004. 103(2): p. 383-9.
6. Locci, M., et al., The Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function. J Exp Med, 2009. 206(4): p. 735-42.
7. Astrakhan, A., H.D. Ochs, and D.J. Rawlings, Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. J Immunol, 2009. 182(12): p. 7370-80.
8. Kunisaki, Y., et al., DOCK2 is required in T cell precursors for development of Valpha14 NK T cells. J Immunol, 2006. 176(8): p. 4640-5.
9. Das, R., D.B. Sant'Angelo, and K.E. Nichols, Transcriptional control of invariant NKT cell development. Immunol Rev, 2010. 238(1): p. 195-215.
10. Townsend, M.J., et al., T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity, 2004. 20(4): p. 477-94.

11. Griewank, K., et al., Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity, 2007. 27(5): p. 751-62.

12. Nunez-Cruz, S., et al., Differential requirement for the SAP-Fyn interaction during NK T cell development and function. J Immunol, 2008. 181(4): p. 2311-20.

13. Pasquier, B., et al., Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med, 2005. 201(5): p. 695-701.

14. McNab, F.W., et al., The influence of CD1d in postselection NKT cell maturation and homeostasis. J Immunol, 2005. 175(6): p. 3762-8.

15. Randall, K.L., et al., DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. J Exp Med, 2011. 208(11): p. 2305-20.

16. Randall, K.L., et al., Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol, 2009. 10(12): p. 1283-91.

17. Sanui, T., et al., DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-theta and LFA-1, in T cells. Immunity, 2003. 19(1): p. 119-29.

18. Bialecki, E., et al., Role of marginal zone B lymphocytes in invariant NKT cell activation. J Immunol, 2009. 182(10): p. 6105-13.

19. Emoto, M., et al., Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. J Immunol, 1999. 162(9): p. 5094-8.

20. Gordy, L.E., et al., IL-15 Regulates Homeostasis and Terminal Maturation of NKT Cells. J Immunol, 2011. 187(12): p. 6335-45.

21. Thomas, S.Y., et al., PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. J Exp Med, 2011. 208(6): p. 1179-88.

22. Schon, M.P., et al., Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. J Immunol, 1999. 162(11): p. 6641-9.

23. Pearce, G., et al., Signaling protein SWAP-70 is required for efficient B cell homing to lymphoid organs. Nat Immunol, 2006. 7(8): p. 827-34.

24. Ohteki, T., et al., Cutting edge: LFA-1 is required for liver NK1.1+TCR alpha beta+ cell development: evidence that liver NK1.1+TCR alpha beta+ cells originate from multiple pathways. J Immunol, 1999. 162(7): p. 3753-6.

Chapter 6

Discussion

This thesis describes the characterisation of a novel ENU mutant, Captain Morgan (*CPM*), containing a mutation in the novel guanine nucleotide exchange factor, dedicator of cytokinesis 8 (DOCK8). The function and role of DOCK8 within the immune system was unknown before the discovery of this ENU mutant. Previously, the critical role of DOCK8 in the maturation and generation of humoral immunity was described by our research group demonstrating that an intrinsic defect in the formation of GC B cells after immunization consequently lead to losses in protective antibody titres and memory B cells. However, *CPM* mice also show other phenotypes including reductions in the naïve T cell compartment. In addition to this, studies involving cohorts of patients suffering from hyper-IgE syndrome (HIES) revealed deletion/mutation in the human DOCK8 gene to be a key contributor to these diseases. Patients are vulnerable to both recurrent bacterial and viral infections indicating a possible deficiency in T cell mediated immunity. Therefore, the work presented here was carried out to further characterize this DOCK8 deficient mouse strain to reveal the role of DOCK8 in T cell immunity and in other cell types including rare lymphocyte populations such as invariant NKT cells.

6.1 Future of the ENU strategy

The use of forward genetics approaches involving N-ethyl-N-nitrosouria (ENU) has provided new insights into the function of novel genes and in mendelian models of human disease [1-4]. The approach has been criticised due to the high costs in maintaining mutated pedigrees with no identifying causative mutations, although WGS will soon relieve bottlenecks in analysis and make ENU mutagenesis more economical [5]. Conventional methods of identifying causative mutations in ENU strains have involved the out-crossing of affected animals to mapping strains to obtain linkage regions associated with phenotypes of interest. This out-breeding strategy carries limitations including the introduction of modifiers resulting in the loss of phenotypes and the need to carry out extensive genotyping (up to 200 markers) on large numbers of mice to identify linkage regions containing candidate mutants. A new approach in our lab has successfully identified causative mutations in a number of previously characterized strains by the use of WGS. This method can successfully identify 1-2 candidate genes through the sequencing of genomes from as little as 3 G3 animals for recessive traits and 6 G3 animals for dominant traits respectively (Bull et al, unpublished data). This approach will be far more economical with falling costs of WGS and reduced animal costs compared to conventional methods.

The identification of DOCK8 as a key regulator of humoral immunity demonstrates the success of the ENU approach and has led to the further characterization of DOCK8 function in T cell immunity and in iNKT cell development and survival. Through conventional genetic approaches, the role of DOCK8 in these processes would have been difficult to decipher without any prior knowledge of its function. Its

discovery has also been important in assisting in understanding the genetic factors underlying hyper-IgE syndromes.

6.2 *CPM* as a model of DOCK8 deficiency syndrome

In terms of explaining human disease, the results presented in this thesis contribute to our understanding of how deficiency in DOCK8 can increase susceptibility to disease, particularly viral infection. The conclusion is that DOCK8 operates at multiple steps in cellular immunity, importantly in the survival of memory $CD8^+$ T cells [6, 7]. The inability to generate high affinity antibodies to pathogens due to a failure to sustain GC B cells, shown previously [8], and the absence of T-cell memory presented in this thesis helps to explain the increased susceptibility of human DOCK8 immunodeficiency syndrome patients to recurrent infections.

The loss of peripheral T cells in *CPM* mice could be explained by a number of factors including altered T cell development, defective cellular migration and reduced lifespan. The relative paucity of peripheral T cells in *CPM* mice is not due to defective thymic development. Processes such as positive and negative selection appear to be normal, based on numbers of SP $CD4^+$ and $CD8^+$ T cells produced in *CPM* mice and normal expression levels of related molecules including CD5, CD127 and TCR. It has been previously demonstrated that CD5 is regulated by TCR engagement with MHC molecules and directly correlates with TCR signalling strength and avidity for ligand during development [9]. Therefore, loss of DOCK8 does not appear to be limiting during thymocyte development and the generation of mature SP T cells.

Although *CPM* T cells have a selective disadvantage in populating early thymic development relative to WT cells in mixed chimeras this is not limiting in natural mutants or single bone marrow chimeras. B-cell development is normal in the mixed chimeras and so competitive effects on the early thymus might be due to the defective survival, proliferation and migration of T cell precursors. As DOCK180 and DOCK2 have been previously demonstrated to be important in the migration of thymic precursors [10], DOCK8 may also have a similar role. In relation to this, *CPM* SP $CD4^+$ T cells accumulate in the thymus and these $CD4^+$ cells show increased migration to SDF-1/CXCL12. Emigration from the thymus is mediated primarily by trafficking to S1P, and whilst CXCL12-dependent signals have been suggested to play a role in thymic egress it remains to be demonstrated formally in physiological circumstances [11-13].

6.3 WHIM syndrome

The discovery that mature naive thymic $CD4^+$ T cells show increased migration to CXCL12 does raise the interesting possibility of a connection between DOCK8 and the human WHIM syndrome, which is a combined immunodeficiency defined by warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis causing neutropenia [14]. WHIM and DOCK8 deficient patients share a similar spectrum of recurrent respiratory and cutaneous viral infections [14] although not all clinical features are comparable. T cells from patients with the WHIM syndrome also show augmented T-cell responses to CXCL12, and a substantial number of these patients have mutations in the CXCR4 chemokine receptor gene [14, 15]. DOCK8 may, therefore, be a regulator of CXCR4 signalling and speculatively, WHIM patients with

no obvious abnormality in CXCR4 dependent signalling may have mutations in DOCK8-dependent signalling pathways [15, 16].

6.4 Differences in migrational patterns

Although SP thymic $CD4^+$ T cells show increased migrational responses to CXCL12, under physiological conditions the increased sensitivity does not alter $CD4^+$ T cell migration into peripheral lymphoid organs. Peripheral T cells also show no differences in chemotactic responses to a range of stimuli, most notably CCL19 and CCL21. CCL19 and CCL21 have been demonstrated to be crucial in the survival and recirculation of naïve T cells with severe lymphopenia described in CCL19 and CCL21 knockout mice [17-19]. Therefore, loss of naïve T cells in the absence of DOCK8 does not appear to be related to chemotactic responses and re-circulatory behaviour.

6.5 Role of DOCK8 in T cell survival

In theory, the decreased number of naïve T cells in the peripheral T-cell compartment could be due to defects in thymic egress, differentiation and proliferation or the increased death of cells. However, the data presented in this thesis suggests that decreased survival is the principal reason for the loss of T cells in DOCK8 deficient animals. The increased turnover of *CPM* T cells *in vivo* and the increase in activated or memory-like ($CD44^{hi}/CD62L^{lo}$) T cells is likely to be caused by lymphopenia-induced proliferation, which occurs when T cells in lymphopenic hosts divide and irreversibly acquire a 'memory-like' phenotype [20]. The increase in $CD44^{hi}/CD62L^{lo}$ T cells is not present in mixed chimeras where cell numbers are normal, and so, in this case, the loss of naive peripheral *CPM* T cells must be due to

an intrinsic defect in survival rather than forced differentiation and division. Specifically, mature SP T cells in the thymus showed reduced levels of the pro-survival factor Bcl2 and showed reduced lifespan when transferred into WT hosts. This loss of T cells does not appear to be related to any deficiencies in proliferative capacity as *CPM* T cells proliferated freely *in vitro* when stimulated with anti-CD3 antibodies and also incorporated high levels of BrdU under homeostatic conditions *in vivo*. As *CPM* cells also localize normally to the peripheral lymphoid organs, these data are indicative of the importance of DOCK8 in the maintaining T cells by promoting pro-survival factors such as Bcl2.

Importantly, lymphopenia affecting the primary repertoire does not seem to be limiting in the primary response to vaccination or infectious challenge, when proliferation can presumably compensate for the reduction in T cells. Therefore, lymphopenia alone cannot explain the susceptibility of DOCK8 deficient patients to recurrent infection. However, although primary responses are normal in *CPM* mice, the most striking effect of DOCK8 deficiency is on the survival of memory $CD8^+$ T cells [6, 7].

6.6 Role of DOCK8 in T cell immunity

The $CD8^+$ T cell immune response can be divided into three phases: the first phase is the growth and differentiation of naïve $CD8^+$ T cells into effector cells with lytic activity and cytokine production; the second is a phase of 10-20 fold contraction leading to a smaller population of long lived memory cells, which arise by the differentiation of effectors [21, 22]; and the third, a phase in which the memory cells are maintained in the host [22, 23]. Long-lived $CD44^+CD62L^{hi}CD127^+$ central

memory T cells (T_{CM}) arise after 5 days following a typical infection and survive within secondary lymphoid organs where they can respond swiftly in a recall response [22-25]. The generation of the memory cells and the expansion of the immune response depend on TCR signalling as well as co-stimulatory molecules including CD28, CD40, 4-1BB, CD27, ICOS, and/or OX40, IL2 and inflammatory cytokines including IL-12 and IFN-α [26-31]. The normal response following MVA vaccination or influenza infection and the initial generation of memory cells suggests that DOCK8 is not limiting downstream of these pathways. The long-term survival of memory $CD8^+$ T cells does not require the presence of antigen but is dependent on cytokines, particularly IL-15 and IL-7 [32-35]. However, the absence of defects in lymphopenia-induced homeostatic expansion and NK cell development (data not shown) which depend on IL-7 and IL-15 [20] argues against a deficiency in cytokine signalling as the cause of $CD8^+$ T-cell memory failure in the absence of DOCK8. In support of this conclusion, iNKT cells from *CPM* mice also showed normal responsiveness to IL7 and IL15 as well as IL2 and IL21.

Losses in $CD8^+$ T cell memory may also be explained by other cell extrinsic factors including deficiencies in $CD4^+$ T cell help [36, 37]. In relation to this possibility, autosomal dominant presentations of HIES are evident with 60-70% of patients possessing mutations/deletions in the cytokine signalling protein STAT3 which has important roles in the activation and differentiation of $CD4^+$ helper T cells [38]. Notably, the importance of STAT3 in the differentiation of TH17 cells is well documented and therefore the consequent loss of TH17 cells in the $CD4^+$ T cell repertoire of STAT3 deficient patients has been put forward as an explanation for the susceptibility of patients to recurrent infections. However, work carried out by our

collaborator Helen Su (Bethesda, USA) performing *ex vivo* stimulation of $CD4^+$ T cell derived from the blood of DOCK8 deficient patients demonstrated no significant differences in IL17 production. Trends towards higher IL4 and IL13 production were observed in this data, which may be related to the atopic phenotype seen in patients. However, due to ongoing infection and treatment it is difficult to interpret these results fully. Similar experiments carried out with *CPM* $CD4^+$ T cells presented here showed no significant differences in cytokine profiles of activated cells supporting the data from Helen Su. Thus, the susceptibility of DOCK8 deficient patients to recurrent infection at this time does not appear to be related to any impairment in $CD4^+$ T helper cell activation and differentiation. The ability of these cells to interact and form stable interactions with antigen specific $CD8^+$ T cells during the initial stages of priming may have important consequences in providing optimal T cell help. However, although this hypothesis was not stringently tested during this period of work, the ability of *CPM* cells (most notably GC B cells) to interact with CD4 T cells has been demonstrated previously. Although losses of GC B cells are observed in *CPM* mice, processes such as immunoglobulin class switching which are CD4 T cell dependent are intact in these animals [8]. Therefore, the reductions in memory seen in *CPM* mice are unlikely to be due to extrinsic factors such as CD4 T cell help. This conclusion is further supported by the experiments carried out using mixed chimera mice. In this setting, the most profound effects of DOCK8 deficiency were found in the memory $CD8^+$ T cell compartment confirming the intrinsic role of DOCK8 in memory T cell formation.

6.7 Role of DOCK8 in integrin biology

Since *CPM* B cells are unable to cluster ICAM-1 following antigen-receptor signalling it is tempting to speculate that similar defects in integrin-dependent signalling might affect the memory T-cell response. In support of this, work carried out by our collaborator Chris Goodnow (Australian National University, Canberra) demonstrated that DOCK8 deficient OT-1 transgenic T cells fail to form coordinated synapses upon interaction with antigen pulsed dendritic cells [7]. Although ICAM-1-dependent interactions between T cells and DCs play a role in the formation of $CD8^+$ T cell memory cells [39, 40], the absence of ICAM-1/LFA-1 interactions has already been described as having its greatest effect on the survival of memory cells [41-43]. However, it is difficult to interpret these results in an *in vivo* setting and how limiting these defects may be. It is also interesting to speculate that a dependence on external signals from molecules such as integrins within cellular niches might also account for the low Bcl2 expression in *CPM* single positive thymocytes and their decreased survival on transfer into the periphery.

6.8 Similarities between DOCK8 deficiency and Wiskott-Aldrich Syndrome

How DOCK8-dependent reorganization of the cytoskeleton could affect lymphocyte survival is also not yet clear. However, a decreased memory response to viral infections is reminiscent of Wiskott-Aldrich Syndrome (WAS), which is an X-linked, immunodeficiency syndrome principally caused by mutations in the WAS protein (WASP) gene [44]. WASP regulates the actin cytoskeleton during a variety of immunological functions including cell migration and the formation and the preservation of T-cell memory [45]. WASP and WASP-family verprolin-homologous (WAVE) proteins are fundamental actin-cytoskeleton reorganizers found in

eukaryotes. The conserved function across species is to receive upstream signals from Rho-family GTPases and then activate the actin-related protein (ARP) 2/3 complex, leading to rapid actin polymerization [46, 47]. There are a number of similarities between WAS and DOCK8-deficiency including peripheral T-cell lymphopenia, a lack of marginal zone B cells and poor immunological synapse formation in WASP-deficient B cells as a consequence of impaired BCR and integrin signalling [48, 49]. Cdc42, the target of DOCK8 is an important regulator of WASP function [50]. Therefore it is an intriguing possibility that DOCK8 augments the formation of active Cdc42, at the membrane interface thereby signalling through WASP. Further investigation of such pathways and their relationship to the human immunodeficiency syndromes will provide important insights into how T-cell memory is sustained.

6.9 Role of DOCK8 in iNKT biology

In this thesis, I have also described how deficiency in DOCK8 leads to a failure to sustain normal numbers of iNKT cells in humans and in mice. The findings presented here show that DOCK8 is required at several stages in iNKT cell development, though the principal cell-intrinsic effect of DOCK8 deficiency is a failure to maintain normal numbers of sessile and terminally differentiated $CD44^{+}NK1.1^{+}$ cells in the thymus and liver. This data mirrors that of the role of DOCK8 for the maintenance of antigen selected and long-lived memory cells and marginal zone B cells [6-8].

6.10 iNKT cells in primary immunodeficiency diseases (PIDs)

Reduced numbers of iNKT cells will contribute to the susceptibility to infectious disease and cancer seen in the human DOCK8 immunodeficiency. Several other immunodeficiency syndromes have been associated with iNKT cell deficiency

including WAS and XLP syndromes [51, 52]: iNKT cells play an important role in the immediate response to pathogens through the recognition of foreign lipids and the release of inflammatory cytokines [53]: they function in cellular immunity via IFNγ secretion and in humoral immunity through the generation of IL4 and direct collaboration with B cells by interactions via CD40L [54-56]. Their importance in anti-viral immunity is highlighted by the various strategies deployed by viruses to avoid CD1d-mediated antigen presentation [57].

The absence of iNKT cells in DOCK8 deficiency is therefore likely to affect the quality and amplitude of immunity to the recurrent viral and bacterial pathogens that beset these patients. There is also compelling evidence that iNKT cells play a role in cancer surveillance [58]. Interestingly, given the clinical phenotype of DOCK8 deficient patients, low levels of circulating iNKT cells predict a poor outcome in patients with head and neck squamous cell carcinoma [59]; and in mice, iNKT cells from the liver provide the best protection against cancer compared to iNKT cells from the spleen or thymus [60, 61].

6.11 Early stages of iNKT cell development

The earliest iNKT cell progenitors arise from $CD4^{+}CD8^{+}$ double positive thymocytes carrying the invariant NKT cell receptor. These $CD24^{hi}$ cells, which stain with αGalCer tetramer, undergo positive selection requiring TCR activation by an unidentified CD1d/lipid self-antigen, which is expressed on thymocytes rather than cortical epithelial cells, and co-stimulation via SLAM receptors [62]. The SLAM receptors signal via the adaptor protein SAP and the Src-family Kinase Fyn [63]. Deficiency in SAP causes X-linked Lymphoproliferative disease (XLP), which is

characterised by a block in iNKT cell development at positive selection, as well as a failure to generate humoral immunity due to loss SLAM dependent signalling in follicular helper T cells from T-B collaborations in the germinal centre [64, 65]. There are parallels between XLP and DOCK8 deficiency in the clinical effects of defects in both B and iNKT cell immunity. However, whilst DOCK8 deficiency has a marked effect on the positive selection of iNKT cells in the presence of competing WT cells, it is not limiting in their absence. This may reflect a quantitative effect of DOCK8 on tuning TCR activation and survival during positive selection, which is analogous to but less profound than that affecting B cell selection and survival in the germinal centre.

Interestingly, DOCK2 deficiency induces an almost complete block in iNKT cell development at the stage of positive selection, without affecting conventional T cell development in the thymus [66]. A similar defect in early iNKT cell selection is also seen in the absence of the Wiskott-Aldrich Syndrome protein (WASp), which is also associated with cytoskeletal rearrangements and integrin signalling [51]. Cdc42, the probable target of DOCK8, is an important regulator of WASp; and in plants, the DOCK8 homologue SPIKE1 controls actin polymerization via the WAVE and ARP 2/3 complex [67]. Therefore, DOCK2 and DOCK8 may operate in parallel to pathways involving factors already described in iNKT cell development, such as SAP, Fyn, IKKβ and WASp [63, 68, 69].

6.12 DOCK8 becomes limiting in late stage, fully mature, thymic iNKT cells

In unmanipulated DOCK8 deficient mice, the principal thymic defect is not in the positive selection of iNKT precursors but a reduction in the resident $CD44^+$ $NK1.1^+$

population. These cells are resident for many months and play an as yet undefined role in the thymic environment. As the development of conventional SP T cells in *CPM* mice appears normal in the absence of this resident population of iNKT cells, this is suggestive that these cells have no significant role in T cell development.

The selection and coincident proliferation of the $CD44^{+}NK1.1^{-}$ precursors depends on antigen presentation by CD1d, SLAM4, co-stimulation by B7-CD28 and ICOS-ICOSL interaction and expression of the transcription factor Tbet leading to up-regulation of CD122 (IL15R), which plays an essential role in survival [61, 69-72]. mRNA expression of these factors is similar in WT and *CPM* thymic $CD44^{+}NK1.1^{+}$ iNKT cells, implying that these are not differentially regulated at the level of transcription. Moreover, in contrast to the situation in mice deficient in these pathways, the rate of generation of new thymic $CD44^{+}NK1.1^{+}$ cells was found to be similar in WT and *CPM* mice, implying that the function of DOCK8 resides in cell survival rather than selection. Survival of the $CD44^{+}NK1.1^{+}$ cells is independent of CD1d signalling but does require IL15 [73, 74]. *CPM* cells respond normally to IL15 stimulation, suggesting that DOCK8 operates in a separate survival pathway. It is possible that that this pathway is upstream of Bcl2, since this is shown to be reduced in all iNKT cell populations in the absence of DOCK8. Our data show that a fall in Bcl2 expression during the normal development of thymic resident iNKT cells coincides with increased expression of a variety of soluble survival factors and receptors, including IL7R and IL15, and integrins such as CD103, which are linked to the cytoskeleton and cell-cell contact. The ligand of CD103, E-cadherin, forms adherens junctions with other cells through homophilic interactions, as well as its heterophilic interactions with $\alpha E\beta 7$, via αE/CD103, and the lymphocyte inhibitory

receptor KLRG1 and modulates signals via Akt, Wnt/βcatenin, NFkB and Rho GTPase dependent pathways [75]. Since DOCK family members are recruited to the plasma membrane by binding to PIP2/PIP3, such a pro-survival pathway could relate to the canonical PIP3-dependent AKT and MAPK dependent cell survival pathways. In this way, the long term survival of iNKT cells within the thymic niche may depend on signals from adjacent cells, which in this case would be thymic epithelial cells expressing E-cadherin [76], as well as soluble growth factors. Further analysis of the $CD103^+$ subset of resident thymic $CD44^+NK1.1^+$ iNKT cells will be required to establish the role of DOCK8 in cell survival and to elucidate the role of these cells in normal physiology.

6.13 Liver iNKT cells survival is regulated intrinsically by DOCK8

Similar patterns of iNKT cell development and survival to those seen in the thymus are repeated in the periphery. Although the differentiation of $NK1.1^+$ cells remains CD1d dependent, antigen-presentation has not been found to be required for iNKT cell survival [74]. The data presented here demonstrate that iNKT cell activation is normal in the liver, and also in the spleen when antigen presentation in not limited by loss of MZ B cells. This suggests that TCR/CD1d interaction and signalling are broadly intact and less likely to represent an explanation for loss of iNKT cells in the periphery. As in the thymus, survival requires stimulation by cytokines such as IL-15 and to a lesser extent by IL-7, except in the lymph nodes where subsets of NKT cells lack CD122 [61]; and long term survival of iNKT cells is perhaps analogous to the situation in memory T cells, which also survive and divide slowly in the presence of IL-15 without obvious requirement for antigen. In the case of DOCK8 deficiency, IL-15 induced proliferation is normal and the numbers of splenic iNKT cells are not

reduced in mixed chimeras. The cell intrinsic defect in iNKT cell survival in the liver due to loss of DOCK8 is therefore more specific. It is possible to speculate that this is due to the interplay between the cells and their immediate environment. Like MZ B cells, which are absent in DOCK8 deficiency, iNKT cells depend on integrin signals via LFA1 for tissue retention [77]. It is therefore possible that survival signals downstream of integrin receptors play a key role iNKT cell survival and in MZ and GC B cells [78, 79]. WASp-deficient iNKT cells are also reduced in the liver, where their deficiency is associated with decreased expression of LFA-1 (CD11a) [51]. The level of dependence on these signals might vary according the presence of other survival signals. In relation to this, the importance of LFA-1 in iNKT homeostasis has been demonstrated in integrin blocking studies using parabiotic mice [77]. Loss of LFA-1 expression using knockout mice has also demonstrated the dependence of iNKT cells on LFA-1 is predominantly in the liver with no differences in splenic iNKT cells [78].

6.14 References

1. Cook, M.C., C.G. Vinuesa, and C.C. Goodnow, ENU-mutagenesis: insight into immune function and pathology. Curr Opin Immunol, 2006. 18(5): p. 627-33.
2. Nelms, K.A. and C.C. Goodnow, Genome-wide ENU mutagenesis to reveal immune regulators. Immunity, 2001. 15(3): p. 409-18.
3. Papathanasiou, P. and C.C. Goodnow, Connecting mammalian genome with phenome by ENU mouse mutagenesis: gene combinations specifying the immune system. Annu Rev Genet, 2005. 39: p. 241-62.
4. Vinuesa, C.G. and C.C. Goodnow, Illuminating autoimmune regulators through controlled variation of the mouse genome sequence. Immunity, 2004. 20(6): p. 669-79.
5. Wienholds, E., et al., Efficient target-selected mutagenesis in zebrafish. Genome Res, 2003. 13(12): p. 2700-7.
6. Lambe, T., et al., DOCK8 is essential for T-cell survival and the maintenance of CD8+ T-cell memory. Eur J Immunol, 2011. 41(12): p. 3423-35.
7. Randall, K.L., et al., DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. J Exp Med, 2011. 208(11): p. 2305-20.
8. Randall, K.L., et al., Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol, 2009. 10(12): p. 1283-91.
9. Azzam, H.S., et al., CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. J Exp Med, 1998. 188(12): p. 2301-11.

10. Lei, Y., et al., Role of DOCK2 and DOCK180 in fetal thymus colonization. Eur J Immunol, 2009. 39(10): p. 2695-702.
11. Matloubian, M., et al., Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature, 2004. 427(6972): p. 355-60.
12. Poznansky, M.C., et al., Thymocyte emigration is mediated by active movement away from stroma-derived factors. J Clin Invest, 2002. 109(8): p. 1101-10.
13. Poznansky, M.C., et al., Active movement of T cells away from a chemokine. Nat Med, 2000. 6(5): p. 543-8.
14. Diaz, G.A. and A.V. Gulino, WHIM syndrome: a defect in CXCR4 signaling. Curr Allergy Asthma Rep, 2005. 5(5): p. 350-5.
15. Diaz, G.A., CXCR4 mutations in WHIM syndrome: a misguided immune system? Immunol Rev, 2005. 203: p. 235-43.
16. Gulino, A.V., et al., Altered leukocyte response to CXCL12 in patients with warts hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome. Blood, 2004. 104(2): p. 444-52.
17. Luther, S.A., et al., Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. Proc Natl Acad Sci U S A, 2000. 97(23): p. 12694-9.
18. Nakano, H. and M.D. Gunn, Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. J Immunol, 2001. 166(1): p. 361-9.

19. Vassileva, G., et al., The reduced expression of 6Ckine in the plt mouse results from the deletion of one of two 6Ckine genes. J Exp Med, 1999. 190(8): p. 1183-8.

20. Surh, C.D. and J. Sprent, Homeostasis of naive and memory T cells. Immunity, 2008. 29(6): p. 848-62.

21. Kaech, S.M. and E.J. Wherry, Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity, 2007. 27(3): p. 393-405.

22. Obar, J.J. and L. Lefrancois, Memory CD8+ T cell differentiation. Ann N Y Acad Sci, 2010. 1183: p. 251-66.

23. Lefrancois, L. and J.J. Obar, Once a killer, always a killer: from cytotoxic T cell to memory cell. Immunol Rev, 2010. 235(1): p. 206-18.

24. Kaech, S.M., et al., Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol, 2003. 4(12): p. 1191-8.

25. Obar, J.J. and L. Lefrancois, Early signals during CD8 T cell priming regulate the generation of central memory cells. J Immunol, 2010. 185(1): p. 263-72.

26. Bachmann, M.F., et al., Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. Eur J Immunol, 2007. 37(6): p. 1502-12.

27. Dai, Z., B.T. Konieczny, and F.G. Lakkis, The dual role of IL-2 in the generation and maintenance of CD8+ memory T cells. J Immunol, 2000. 165(6): p. 3031-6.

28. Jelley-Gibbs, D.M., et al., Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. J Immunol, 2000. 165(9): p. 5017-26.

29. Mescher, M.F., et al., Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev, 2006. 211: p. 81-92.

30. Weinberg, A.D., et al., The generation of T cell memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. J Leukoc Biol, 2004. 75(6): p. 962-72.

31. Xiao, Z., et al., Programming for CD8 T cell memory development requires IL-12 or type I IFN. J Immunol, 2009. 182(5): p. 2786-94.

32. Berard, M., et al., IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. J Immunol, 2003. 170(10): p. 5018-26.

33. Leignadier, J., et al., Memory T-lymphocyte survival does not require T-cell receptor expression. Proc Natl Acad Sci U S A, 2008. 105(51): p. 20440-5.

34. Murali-Krishna, K., et al., Persistence of memory CD8 T cells in MHC class I-deficient mice. Science, 1999. 286(5443): p. 1377-81.

35. Schluns, K.S., et al., Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol, 2000. 1(5): p. 426-32.

36. Clarke, S.R., The critical role of CD40/CD40L in the CD4-dependent generation of CD8+ T cell immunity. J Leukoc Biol, 2000. 67(5): p. 607-14.

37. Masopust, D., et al., The role of programming in memory T-cell development. Curr Opin Immunol, 2004. 16(2): p. 217-25.

38. Engelhardt, K.R., et al., Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. J Allergy Clin Immunol, 2009. 124(6): p. 1289-302 e4.

39. Camacho, S.A., et al., A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nat Immunol, 2001. 2(6): p. 523-9.

40. Scholer, A., et al., Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. Immunity, 2008. 28(2): p. 258-70.

41. Hanninen, A., et al., Ly6C supports preferential homing of central memory CD8+ T cells into lymph nodes. Eur J Immunol, 2011. 41(3): p. 634-44.

42. Parameswaran, N., et al., Lack of ICAM-1 on APCs during T cell priming leads to poor generation of central memory cells. J Immunol, 2005. 175(4): p. 2201-11.

43. Ye, Z., et al., LFA-1 defect-induced effector/memory CD8+ T cell apoptosis is mediated via Bcl-2/Caspase pathways and associated with downregulation of CD27 and IL-15R. Mol Immunol, 2010. 47(14): p. 2411-21.

44. Ochs, H.D. and A.J. Thrasher, The Wiskott-Aldrich syndrome. J Allergy Clin Immunol, 2006. 117(4): p. 725-38; quiz 739.

45. Lutskiy, M.I., et al., Wiskott-Aldrich syndrome in a female. Blood, 2002. 100(8): p. 2763-8.

46. Kurisu, S. and T. Takenawa, The WASP and WAVE family proteins. Genome Biol, 2009. 10(6): p. 226.

47. Ramesh, N. and R. Geha, Recent advances in the biology of WASP and WIP. Immunol Res, 2009. 44(1-3): p. 99-111.

48. Meyer-Bahlburg, A., et al., Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis. Blood, 2008. 112(10): p. 4158-69.

49. Zhang, H., et al., Impaired integrin-dependent function in Wiskott-Aldrich syndrome protein-deficient murine and human neutrophils. Immunity, 2006. 25(2): p. 285-95.

50. Torres, E. and M.K. Rosen, Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. Mol Cell, 2003. 11(5): p. 1215-27.

51. Astrakhan, A., H.D. Ochs, and D.J. Rawlings, Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. J Immunol, 2009. 182(12): p. 7370-80.

52. Bassiri, H., et al., X-linked lymphoproliferative disease (XLP): a model of impaired anti-viral, anti-tumor and humoral immune responses. Immunol Res, 2008. 42(1-3): p. 145-59.

53. Tupin, E., Y. Kinjo, and M. Kronenberg, The unique role of natural killer T cells in the response to microorganisms. Nat Rev Microbiol, 2007. 5(6): p. 405-17.

54. Cerundolo, V., et al., Harnessing invariant NKT cells in vaccination strategies. Nat Rev Immunol, 2009. 9(1): p. 28-38.

55. Lang, M.L., How do natural killer T cells help B cells? Expert Rev Vaccines, 2009. 8(8): p. 1109-21.

56. Nagarajan, N.A. and M. Kronenberg, Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J Immunol, 2007. 178(5): p. 2706-13.

57. Diana, J. and A. Lehuen, NKT cells: friend or foe during viral infections? Eur J Immunol, 2009. 39(12): p. 3283-91.

58. Swann, J.B., et al., CD1-restricted T cells and tumor immunity. Curr Top Microbiol Immunol, 2007. 314: p. 293-323.

59. Molling, J.W., et al., Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol, 2007. 25(7): p. 862-8.

60. Crowe, N.Y., et al., Differential antitumor immunity mediated by NKT cell subsets in vivo. J Exp Med, 2005. 202(9): p. 1279-88.

61. Godfrey, D.I., S. Stankovic, and A.G. Baxter, Raising the NKT cell family. Nat Immunol, 2010. 11(3): p. 197-206.

62. Egawa, T., et al., Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity, 2005. 22(6): p. 705-16.

63. Nunez-Cruz, S., et al., Differential requirement for the SAP-Fyn interaction during NK T cell development and function. J Immunol, 2008. 181(4): p. 2311-20.

64. Pasquier, B., et al., Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med, 2005. 201(5): p. 695-701.

65. Qi, H., et al., SAP-controlled T-B cell interactions underlie germinal centre formation. Nature, 2008. 455(7214): p. 764-9.

66. Kunisaki, Y., et al., DOCK2 is required in T cell precursors for development of Valpha14 NK T cells. J Immunol, 2006. 176(8): p. 4640-5.

67. Basu, D., et al., A SPIKE1 signaling complex controls actin-dependent cell morphogenesis through the heteromeric WAVE and ARP2/3 complexes. Proc Natl Acad Sci U S A, 2008. 105(10): p. 4044-9.

68. Cen, O., et al., The adaptor molecule signaling lymphocytic activation molecule-associated protein (SAP) regulates IFN-gamma and IL-4 production

in V alpha 14 transgenic NKT cells via effects on GATA-3 and T-bet expression. J Immunol, 2009. 182(3): p. 1370-8.

69. Das, R., D.B. Sant'Angelo, and K.E. Nichols, Transcriptional control of invariant NKT cell development. Immunol Rev, 2010. 238(1): p. 195-215.

70. Sille, F.C., et al., Critical role for invariant chain in CD1d-mediated selection and maturation of Valpha14-invariant NKT cells. Immunol Lett, 2011. 139(1-2): p. 33-41.

71. Townsend, M.J., et al., T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity, 2004. 20(4): p. 477-94.

72. Williams, J.A., et al., Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. J Immunol, 2008. 181(2): p. 907-17.

73. Gordy, L.E., et al., IL-15 Regulates Homeostasis and Terminal Maturation of NKT Cells. J Immunol, 2011. 187(12): p. 6335-45.

74. McNab, F.W., et al., The influence of CD1d in postselection NKT cell maturation and homeostasis. J Immunol, 2005. 175(6): p. 3762-8.

75. Van den Bossche, J., et al., Regulation and function of the E-cadherin/catenin complex in cells of the monocyte-macrophage lineage and DCs. Blood, 2012. 119(7): p. 1623-33.

76. Kutlesa, S., et al., E-cadherin-mediated interactions of thymic epithelial cells with CD103+ thymocytes lead to enhanced thymocyte cell proliferation. J Cell Sci, 2002. 115(Pt 23): p. 4505-15.

77. Thomas, S.Y., et al., PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. J Exp Med, 2011. 208(6): p. 1179-88.

78. Emoto, M., et al., Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. J Immunol, 1999. 162(9): p. 5094-8.

79. Ohteki, T., et al., Cutting edge: LFA-1 is required for liver NK1.1+TCR alpha beta+ cell development: evidence that liver NK1.1+TCR alpha beta+ cells originate from multiple pathways. J Immunol, 1999. 162(7): p. 3753-6.

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