Consequences of oncogenic CARD11 signalling for B cells

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Statement of originality

This thesis presents research undertaken in the Immunogenomics laboratory, Department of Immunology at the John Curtin School of Medical Research, Australian National University. The original research work presented here was conducted under the supervision of Professor Christopher Goodnow and Dr Keisuke Horikawa.

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Abstract

Diffuse large B cell lymphoma (DLBCL) is the most common form of Non-Hodgkin’s lymphoma, accounting for 40% of all newly diagnosed cases. Approximately 13% of DLBCL patients have somatic mutations in CARD11. CARD11 functions as a scaffold protein and relays signals from the antigen receptor to the JNK and NF-κB signaling pathways, which contribute to the survival, proliferation and differentiation of lymphocytes. The consequences of acquiring these lymphoma-derived CARD11 mutations in normal B cells, in isolation from the other ~30 mutations accumulated on average in lymphoma cells had not been addressed. By introducing lymphoma CARD11 mutations into antigen activated B lymphocytes using retroviral vectors, we found that gain-of-function CARD11 mutations result in proliferation, growth and survival in vitro. The CARD11 mutants also increased signalling to NF-κB and JNK pathways to varying degrees.

Moreover, when the genetically modified B cells were then transferred in vivo to investigate the consequences of the acquisition of the CARD11^{Mut10} version of CARD11, we observed that the B cells proliferate rapidly and differentiate into plasmablasts. We found that CARD11^{Mut10} induced BLIMP1-mediated plasmablast differentiation and that autocrine production of IL-6 plays a significant role in driving plasmablast differentiation in CARD11^{Mut10}-expressing B cells. However, this initial rapid population expansion is self-limiting: it peaked at day 10 and by 20-days in vivo the numbers of CARD11^{Mut10} expressing B cells decreased drastically. The primary self-limiting circuit in CARD11^{Mut10} – induced proliferation is the induction of the NF-κB negative regulator Tnfaip3/A20, which potentially reduces the signals required for the survival and proliferation of the B cells.

By introducing CARD11 mutations from human lymphomas into antigen activated mature B lymphocytes in mice, we tested the hypothesis that lymphoma somatic mutations corrupt the normal response to self-antigens. Here, we show that all lymphoma-derived CARD11 mutations tested, but not NF-κB activating mutations in lkbkb, block self-antigen induced death and cooperate with chronic antigen receptor stimulation to induce extensive T cell-independent proliferation, BLIMP1-mediated plasmablast differentiation and autoantibody secretion. Our findings
demonstrate that regulation of CARD11 signalling is a critical switch governing the
decision between death and proliferation in antigen-stimulated mature B cells, and
that mutations in this switch represent a powerful initiator for aberrant B cell
responses in vivo.

Recently, patients with germline activating mutations in CARD11 were reported.
These patients are characterised by a B-cell lymphoproliferative disorder: B-cell
expansion with NF-κB and T-cell anergy (BENTA). We show that E127G CARD11
(Mut12), discovered in patients with BENTA, increased NF-κB signalling and, akin
to lymphoma-derived CARD11 mutants, E127G CARD11 protected the B cells from
death and induced accumulation of self-reactive B cells. The analysis of the B cells
in mice expressing an ENU-induced Card11 mutation (M365K) revealed no
BENTA-like disease. In vitro assays in B cells showed that CARD11\textsuperscript{M365K} has
stronger NF-κB and growth-inducing effects than CARD11\textsuperscript{E127G}. Together, these
results reveal an intriguing difference between the effects of overactive CARD11
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Life on Earth is thought to have evolved more than 3.5 billion years ago with the emergence of single cell microorganisms (Line 2002). The ensuing burst of diverse archaea, bacteria and eukaryotes shaped an environment where organisms engaged in a continuous competitive 'struggle for existence' (Darwin 1859). In order to survive early organisms evolved the ability to protect themselves from parasitic pathogens capable of killing and converting other organisms into copies of themselves (Danilova 2006). In response to these threats, eukaryotes have constantly evolved means to disarm attacks from pathogens, which in turn, continually evolve new ways to evade host defense tactics (Danilova 2006). This continuum of host-pathogen arms race is often compared to the Red Queen in the sequel to Alice's Adventures in Wonderland, who must keep moving just to avoid falling behind: 'Now, here, you see, it takes all the running you can do, to keep in the same place' (Carroll 1871, Muraille 2013). Similarly, pathogens and hosts need to constantly adopt new strategies to just survive.

One of the earliest forms of protection or ‘innate immunity’ was the use of cell surface membranes by single cell organisms as a protective barrier between the inside of the cell and the external environment. With time, the more complex single cell organisms evolved receptors on their cell surface membrane or intracellular receptors that could recognise specific patterns present on common pathogens (Beutler 2004). The use of germline-encoded pattern recognition receptors for pathogens, such as the Toll-like receptors, nucleotide-binding domain leucine-rich repeat-containing receptors and C-type lectin receptors constitute a central part of the innate immune system (Janeway and Medzhitov 2002, Akira et al. 2006). The engagement of these generic receptors with conserved patterns on different types of pathogens triggers an inflammatory response that limits both pathogen invasion and damage to self (Janeway 1989, Janeway and Medzhitov 2002, Akira et al. 2006).

In addition to the innate defense mechanisms, jawed vertebrates have evolved an adaptive immune system, which is mediated by lymphocytes in cooperation with cells of the innate immune system (Cooper and Alder 2006). By virtue of re-
arrangeable immunoglobulin V, D, and J gene segments, lymphocyte receptor repertoires of sufficient diversity can be generated such that the antigenic components of any potential pathogen can be recognised. Thus, all jawed vertebrates, including cartilaginous fish, rearrange their V(D)J gene segments to assemble complete genes for the antigen receptors expressed by lymphocytes. Through these specialised cells of adaptive immunity, the system can recognize and initiate a protective response against potentially lethal pathogens, including bacteria, viruses, fungi, and parasites. This specific defense system also remembers previous pathogen encounters and can either repel a second invasion or quickly eliminate the invader by mounting a faster and more efficient immune response.

T and B lymphocytes are two major lineages of lymphocytes that can recognise and respond to pathogens (Cooper et al. 1965, Cooper et al. 1966, Miller and Mitchell 1968, Mitchell and Miller 1968). Akin to other cells of the immune system, T and B lymphocytes are derived from multipotent hematopoietic stem cells. T lymphocytes complete their maturation in the thymus, while B cells are produced in the bone marrow (Anderson and Jenkinson 2001, Hardy and Hayakawa 2001, Miller 2002, Hardy et al. 2007). The mature populations of T and B lymphocytes subsequently enter the bloodstream to patrol the body. The T cell receptors (TCR) recognise peptide fragments of antigens presented by other cells within cell-surface molecules encoded by the major histocompatibility complex (MHC) class I and class II genes (Anderson and Jenkinson 2001). Therefore, T lymphocytes recognise antigens that have been partially digested by the antigen-presenting cells, primarily dendritic cells, phagocytic cells, and B lymphocytes. By contrast, the B cell receptors (BCR) expressed by B lymphocytes recognise exposed epitopes of intact molecules, including surface protein and carbohydrate moieties of invasive microbes. Following engagement of TCRs and BCRs by antigen, the activation of transmembrane and intracellular proteins are triggered, which transduce signals through well-regulated signaling pathways leading to the expression of genes required for immune responses (Hardy and Hayakawa 2001, Hardy et al. 2007).
B cells: a brief historical perspective

The first description of the anatomical structures, which contained B lymphocytes, could be attributed to Malpighi as he identified lymphoid follicles in the spleen in 1687 (Nieuwenhuis and Opstelten 1984). About two centuries later, Hodgkin provided the initial descriptions of deformations of these same splenic follicular structures by what was later identified to be malignant B lymphocytes (Hodgkin 1832). Subsequently, in 1884 Flemming observed proliferating cells in lymph nodes, which he called germinal centers as he proposed that they were the source of lymphocytes that circulated the body (Nieuwenhuis and Opstelten 1984). However, we now know that germinal centers contain proliferating B lymphocytes that form part of antibody responses (MacLennan 1994). Towards the end of the 19th century, Emil von Behring, Kitasato Shibasaburo and Paul Ehrlich all contributed to the discovery of antibodies, for which von Behring was awarded the first Nobel Prize in Physiology or Medicine in 1901 (Kantha 1991, Triendl 2004, Raju 2006). Interestingly, it was the 1938 discovery by Tiselius and Kabat of the biochemical nature of antibodies in the serum of immunised rabbits and horse, which provided the launching pad for the identification of the immune cell type that produced them (Tiselius and Kabat 1938). Thus, plasma cells, a terminally differentiated form of B lymphocytes, were found by Fagraeus to be the antibody-producing cells in 1947-1948 (Fagraeus 1947, Fagraeus 1948).

Following the identification of the antibody producing cells, Burnet and Jerne proposed their competing hypothesis, the ‘clonal selection’ theory and the ‘natural selection’ theory respectively, to explain antibody production in the context of an immune response (Jerne 1955, Burnet 1957, Nossal 2007). Burnet’s theory was subsequently supported by experimental evidence from Nossal who showed that individual cells could produce a single ‘species of antibody’ (Nossal and Lederberg 1958). Following this seminal finding, Porter and Edelman elucidated the chemical structure of the antibody molecule in 1963-1964 (Porter 1963, Edelman and Gally 1964).

Despite the considerable progress made following the discovery of antibodies in the late 1930s, the actual characterisation of B cells only occurred forty years later.
Glick characterised the bursa of Fabricius as a key organ central for antibody production in chicken, and Miller found that the thymus was required for lymphocyte production (Glick et al. 1955, Miller 1961, Ribatti et al. 2006). Subsequently, Cooper and Good suggested a ‘two-cell system’ of lymphocytes as a way of functionally delineating the bursa of Fabricius derived cells responsible for antibody production and the thymus derived cells that are required for delayed-type hypersensitivity and the rejection of skin homografts (Cooper et al. 1965, Cooper et al. 1966). Miller and Mitchell then provided evidence for the distinction between B (bone marrow-derived) and T (thymus-derived) lymphocytes, and demonstrated that cells derived from the bone marrow mediated antibody responses by using mouse transplantation experiments (Miller and Mitchell 1968, Mitchell and Miller 1968).

In the late 1960s and early 1970s, Coombs and Fröland performed elegant studies on human blood samples showing that detection of surface immunoglobulin could be used as a marker of B lymphocytes (Coombs et al. 1969, Froland et al. 1971). Studies in mouse hematopoietic tissues by Raff and Cooper described a population of precursor B cells which expressed cytoplasmic μ chains, but lacked surface IgM expression (Raff et al. 1976). These findings indicated that expression of IgM regulated the development and maturation of diverse number unique B cell clones. Subsequently, in 1978 Tonegawa and Brack provided evidence for the combinatorial rearrangement of V, D and J gene segments in the heavy chain locus and the V and J segments for the light chain loci, revealing how the diversity of IgM receptors were generated during B cells development (Brack et al. 1978). About a decade later, the mechanistic explanation for the initial steps of VDJ rearrangements in lymphocytes was provided by the discovery and characterisation of recombination activating genes 1 and 2 (Rag1 and Rag2) by Baltimore and colleagues (Schatz et al. 1989, Oettinger et al. 1990).

However, how randomly rearranged B cell receptors are selected to avoid the production of autoantibodies- a scenario that Ehrlich described as “Horror autotoxicus” in the early 1900s- remained unclear until the characterisation of in vivo transgenic mice models in the late 1980s (Ehrlich and Morgenroth 1901, Goodnow et al. 1990). In 1948, Burnet and Fenner attempted to address this
question in their 1949 monograph on “The production of antibodies”, where they provided a framework for the interpretation of the results from Traub and Owen, and hypothesised that animals would not produce antibodies against foreign antigens if they were exposed to the same antigens in utero (Traub 1938, Owen 1945, Burnet and Fenner 1949). In 1953, Medwar and Billingham provided experimental evidence for Burnet’s and Fenner’s hypothesis and coined the term ‘actively acquired tolerance’ by showing that neonatal mice that had been exposed to (and engrafted) by hematopoietic cells from a different mouse strain were able to accept skin grafts from the latter mouse strain as adults (Billingham et al. 1953).

**Development, activation and function of B cells**

B lymphocytes are generated from self-renewing hematopoietic stem cells (HSCs) in the bone marrow of adult humans and mice, and in the liver during mid-to-late fetal development (Mikkola and Orkin 2006, Hardy et al. 2007). The hematopoietic stem cell precursors are derived from the aorta-gonad-mesonephros during embryonic development (Ogawa et al. 1991, Muller et al. 1994, Medvinsky and Dzierzak 1996). Hematopoietic stem cells comprise a small fraction of bone marrow cells (~1/30,000), with as few as six cells being sufficient to generate all blood cell lineages and repopulate the entire immune system (Morrison and Weissman 1994, Morrison et al. 1995). HSCs give rise to the B lymphocyte lineage, and all other hematopoietic cell types, through well-characterised stepwise differentiation stages (Hardy and Hayakawa 2001, Hardy et al. 2007).

The immediate differentiation progeny of HSCs are multipotent progenitors (MPPs), which have limited self-renewal capacity but retain the potential to differentiate multiple hematopoietic cell lineages (Adolfsson et al. 2001). MPPs subsequently differentiate into lymphoid-primed multipotent progenitor cells, which express high levels of Flt3 receptor (Adolfsson et al. 2005). These cells then give rise to early lymphoid progenitors (ELPs), which have been proposed to be multipotent cells that are biased to differentiating into lymphoid cells as a fraction of these cells express genes important for lymphocyte development such as transcription of ‘terminal deoxynucleotidyl transferase’ (*TdT*) and *Rag1* (Igarashi
et al. 2002). In turn, ELPs differentiate to generate the more lineage-restricted common lymphoid progenitors (CLPs), which express the receptor for IL-7 (Kondo et al. 1997). The initial characterisation of CLPs indicated that these cells could give rise to T cells, B cells and NK cells, but were unable to generate myeloid cells (Kondo et al. 1997). In addition, the findings that 50% of CLPs contain immunoglobulin heavy-chain DJ rearrangements and the cells are able to rapidly differentiate into B cells, suggested that these cells are early B cell progenitors, which retain the potential for generation of other lymphoid cell lineages (Rolink et al. 1991, Rumfelt et al. 2006).

The first B cell lineage committed progenitors that differentiate from CLPs in the bone marrow are pre-pro B cells (Hardy et al. 1991, Li et al. 1996, Rumfelt et al. 2006). Pre-pro B cells express the B cell-associated marker B220 and B cell-lineage genes such as mb1 (Cd79a) and lambda5, which are regulated by the transcription factor EBF1 (Lin and Grosschedl 1995, Li et al. 1996). Despite a strong bias to the B cell lineage commitment, pre-pro B cells retain the ability to give rise to T cells, but cannot differentiate into myeloid cells (Li et al. 1996). Strict commitment to the B cell lineage occurs with the expression of the transcription factor Pax5 at the pro-B cell stage (Nutt et al. 1997, Nutt et al. 1999). These cells can be identified by the surface expression of CD19, a direct target of Pax5 (Kozmik et al. 1992, Nutt et al. 1998). The Pax5 transcriptional regulator is expressed stably throughout the B cell lineage from the pro-B cell stage of differentiation up to its downregulation in plasma cells (Fuxa and Busslinger 2007). In the absence of Pax5, B cell differentiation is blocked at the pre-pro to pro-B cell stage, and the arrested cells are capable of generating multiple hematopoietic cell types (Nutt et al. 1999, Rolink et al. 1999). Thus, Pax5 maintains B cell lineage identity by repressing a number of genes such as Mcsfr (associated with macrophages) and Notch1 (associated with T cells), while promoting the expression of B cell receptor associated genes such as CD19, Blnk, CD79a and lambda5, as well as the transcription factors SPIB, IKZF3 and IRF8, which play important roles in B cell differentiation and function (Souabni et al. 2002, Tagoh et al. 2006, Cobaleda et al. 2007, Nutt and Kee 2007, Pridans et al. 2008).
Moreover, it is at the pro-B cell stage that the process of immunoglobulin gene recombination is initiated, with ‘diversity’ (D) to ‘junction’ (JH) rearrangements (Alt et al. 1984, Hardy et al. 1991). Owing to the random addition and loss of nucleotides, D segments can be joined to JH segments in any one of the three possible reading frames, thus giving rise to a diverse number of rearranged DJH segments (Desiderio et al. 1984, Jung et al. 2006). However, the DJH segment diversity is limited by a phenomenon referred to as ‘reading frame 2’ counterselection as DJH rearrangements in reading frame 2 encode a truncated form of membrane immunoglobulin that associates with CD79a and CD79b and surrogate light chains to produce a defective pre-BCR that inhibits VH to DJH recombination and is unable to support further development of B lymphocytes (Reth and Alt 1984, Meek 1990, Gu et al. 1991). Following DJH rearrangement on both IgH alleles, VH genes become accessible to the V(D)J recombinase and complete V(D)J segments are assembled (Tonegawa 1983, Alt et al. 1984).

Expression of IgM on the B cell surface (mIgM) results in the assembly of the pre-BCR, and marks a key checkpoint in the transition from the pro-B cell to the pre-B cell stage of development (Reth et al. 1987, Karasuyama et al. 1990, Tsubata and Reth 1990, Kitamura et al. 1992, Blunt et al. 1995). The assembly of the pre-BCR triggers B cell differentiation, clonal expansion and allelic exclusion (Kitamura et al. 1991, Kitamura and Rajewsky 1992, Mostoslavsky et al. 2004). Evidence of allelic exclusion was provided by Nossal and Lederberg as they confirmed the one cell-one antibody hypothesis, in line with Burnet’s clonal selection theory (Nossal and Lederberg 1958, Burnet 1976). Subsequently, the study of transgenic mouse models showed that further V(D)J recombination was inhibited by the expression of the membrane form of IgM in pre-B cells (Nussenzweig et al. 1987, Manz et al. 1988). The inhibition of V(D)J recombination in pre-B cells is still unclear, but mIgM-mediated down-regulation of the enzymes RAG-1 and RAG-2 and decreased accessibility of VH genes to the recombinase machinery seem to contribute to allelic exclusion (Grawunder et al. 1995, Stanhope-Baker et al. 1996, Constantinescu and Schlissel 1997). Similar experiments also suggested that the membrane-bound form of IgM directs the progression from the pro-B to the pre-B cell development stage, where light chain assembly occurs (Reth et al. 1987).
A role for membrane form of IgM in directing pre-B cell development was initially suggested from observations in transgenic mice that overexpressed a human form of mIgM where cMyc driven pre-B cell lymphoma was disrupted (Nussenzweig et al. 1988). The further study of gene-targeted disruptions in mice and naturally occurring genetic lesions in humans showed that the components of the pre-BCR such as CD79a, CD79b, and mIgM were essential for pre-B cell development (Gong and Nussenzweig 1996, Yel et al. 1996, Minegishi et al. 1999). In addition, SCID mice that have a defect in the DNA-PK repair complex and fail to rejoin DNA efficiently during V(D)J recombination have a block at the pro-B cell to pre-B cell stage (Blunt et al. 1995). A similar phenotype results from the disruption of Rag-1 and Rag-2 genes that encode for enzymes that are involved in the generation of double-stranded breaks in DNA during Ig rearrangement (Oettinger et al. 1990, Mombaerts et al. 1992, Shinkai et al. 1992). The defect in B cell development in the SCID and Rag-deficient mice was corrected by the introduction of a rearranged heavy chain transgene, indicating the critical role of mIgM in pre-B cell development (Spanopoulou et al. 1994, Young et al. 1994).

The expression of the pre-BCR has also been associated with the clonal expansion of the pre-B cells (Hardy et al. 1991). As exemplified by the 81X VH gene, it is thought that the assembly of mIgM, the surrogate light chain (λ5 and VpreB), and the accessory proteins CD79a and CD79b acts as a means of screening for heavy chain V regions such that IgVH having appropriate structure for folding with the surrogate light chain are selected for by the induction of proliferation signals (Yancopoulos et al. 1984, Hardy et al. 1991, Decker et al. 1995, Keyna et al. 1995, Karasuyama et al. 1997, Kline et al. 1998, Bannish et al. 2001). This checkpoint could be important for selecting IgVH following the action of deoxynucleotidyl transferase (TdT) which contributes to increased CDR3 diversity by adding non-templated nucleotides at V-D and D-J junctions (Gilfillan et al. 1993, Komori et al. 1993). In addition, the activity of BCR signaling molecules such as Syk, Blnk and PI3K are critical for B cell development, while the Src kinases Lyn, Fyn and Blk seem to be dispensable to B cell development, as mice deficient in genes encoding for the latter have no defect in B cell development (Cheng et al. 1995, Hibbs et al. 1995, Nishizumi et al. 1995, Turner et al. 1995, Fruman et al. 1999, Jumaa et al. 1999, Minegishi et al. 1999, Pappu et al. 1999, Texido et al. 2000).
The clonal expansion of mIgM expressing pre-B cells is followed by arrest of the cells in the G1 phase of cell cycle, during which RAG and Igκ germline transcripts are expressed to initiate light chain gene rearrangement (Reth et al. 1987, Schlissel and Baltimore 1989, Melchers et al. 2000). Appropriate light chain rearrangement results in the replacement of the surrogate light chain by either Igκ or Igλ and the expression of the B cell receptor (Melchers et al. 2000). The regulation of light chain rearrangement is thought to be mediated by asymmetric demethylation of the Igκ genes and by signals from the B cell receptor, which induce the downregulation of the RAG genes (Grawunder et al. 1995, Mostoslavsky et al. 1998, Cherry and Baltimore 1999, Monroe et al. 1999). Moreover, the notion that BCR signaling regulates the transition from the pre-B cell to the immature B cell stage of development is supported by findings of impaired B cell maturation in transgenic mice where the cytoplasmic tail of CD79a is absent (Torres et al. 1996).

Immature B cells are the first B cell lineage cells that express surface antigen receptors as IgM, with little or no IgD (Hardy and Hayakawa 2001). Developing B cells populate the immature compartment for an average of $3^{1/2}$ days during which selection of the mature B cell repertoire occurs (Carsetti et al. 1995). This process has been studied in a number of transgenic mouse models which have indicated that the encounter of the BCR with self-antigen results in (i) receptor editing, where B cells revise the BCR to reduce self-reactivity; (ii) clonal deletion, where the cells are eliminated; (iii) anergy, where the cells become unresponsive to antigen and are short-lived (Goodnow et al. 1988, Erikson et al. 1991, Hartley et al. 1991, Russell et al. 1991, Hartley et al. 1993, Chen et al. 1994, Chen et al. 1995, Pelanda et al. 1997, Healy and Goodnow 1998, Retter and Nemazee 1998, Benschop et al. 1999). The interaction of BCR to self-antigen seems to govern which tolerance mechanism is elicited as high-affinity interactions with membrane-bound antigen are likely to result in deletion, whereas lower-affinity interactions and soluble antigens result in editing or anergy (Goodnow et al. 1988, Hartley et al. 1991, Hartley and Goodnow 1994, Treger 1996, Healy and Goodnow 1998, Benschop et al. 1999).

Immature B cells migrate from the bone marrow to the periphery as transitional B cells, which can be distinguished from mature B cells by differential expression of
cell surface markers such as CD93 and low expression of RAG (Fulop et al. 1983, Rolink et al. 1998, Monroe et al. 1999, Yu et al. 1999). Transitional B cells are short-lived and it is estimated that only 10% to 30% of these cells enter the long-lived mature peripheral B cell compartment (Forster and Rajewsky 1990, Osmond 1991, Sprent and Tough 1994, Rolink et al. 1998). These findings are supported by a number of reports suggesting that the size of the transitional B cell compartment is variable and can be influenced by immunization or infection (Forster and Rajewsky 1990, Allman et al. 1992, Cyster et al. 1994, Agenes and Freitas 1999, Nagaoka et al. 2000). Thus, homeostasis in the B cell lineage is thought to be maintained by regulating the number of transitional B cells entering the mature compartment through the modulation of thresholds for positive and negative selection (Gu et al. 1991, Hartley et al. 1991, Agenes and Freitas 1999, Sandel and Monroe 1999, Levine et al. 2000).

Signals from the B cell receptor seems to be an important determinant for the selection of transitional B cells into the mature B cell compartment as conditional loss of IgM expression aborts all stages of development from the transitional stage (Lam et al. 1997). Mutations affecting BCR signaling components have been shown to have defects in transitional B cell maturation (Loder et al. 1999, Martin and Kearney 2000). In addition, mice lacking functional CD79a, Syk, Btk, Vav, Lyn, CD45 and CD19, all components of the proximal BCR signaling network, fail to induce differentiation of transitional cells into mature B cells (Tarakhovsky et al. 1995, Zhang et al. 1995, Torres et al. 1996, Wang et al. 1996, Chan et al. 1997, Turner et al. 1997, Loder et al. 1999, Martin and Kearney 2000). PI3K signaling downstream of the BCR seems to provide signals important for B cell maturation and survival (Deane and Fruman 2004, Verkoczy et al. 2007, Aiba et al. 2008, Herzog et al. 2008, Srinivasan et al. 2009).

Mature B cells are typically comprised of the follicular, marginal zone and B-1 subsets (Allman and Pillai 2008). These B cell sub-populations can be categorised based on the differential expression of a variety of cell surface molecules such as IgM, IgD, CD23, CD21/35 and CD5 (Hardy et al. 1982, Hayakawa et al. 1983, Waldschmidt et al. 1988, Takahashi et al. 1997). Follicular B cells are typically characterised by the expression of IgM, IgD, and high levels of CD21 and CD23.
As implied by their name, follicular B cells reside mainly in the primary follicles of the B cell zones around follicular dendritic cells in the spleen and other secondary lymphoid organs (Bajenoff et al. 2006, Cyster 2010, Wang et al. 2011). During an immune response, follicular B cells differentiate into antibody-producing cells and memory B cells, and engage with T follicular helper cells to generate effective antibody responses (Goodnow et al. 2010).

B-1 B cells are a unique mature B cell subpopulation distinguished by their phenotype, anatomic localization, self-renewing capacity and production of natural antibodies (Hayakawa et al. 1983, Hardy and Hayakawa 2001, Hardy 2006). B-1 cells typically express high levels of IgM, low levels of IgD and no CD23, and these cells are found in the peritoneal cavity and seem to develop from distinct progenitors mainly found in the fetal bone marrow (Montecino-Rodriguez et al. 2006, Dorshkind and Montecino-Rodriguez 2007). CD5+ B-1 cells produce natural antibodies against bacterial components, while CD5- B-1 cells respond to polysaccharides and other T cell-independent antigens during infections (Haas et al. 2005). Given that most of the characterisation of B-1 cells has been done in mice, it is currently unclear whether a similar subpopulation of B cells is functionally relevant in humans.

Akin to B-1 B cells, marginal zone (MZ) B cells are unique in their phenotypic characteristics, anatomical localization and function (Martin and Kearney 2002, Pillai et al. 2005). Marginal zone B cells reside in the outer white pulp of the spleen, between the marginal sinus and the red pulp, and express high levels of IgM and CD21, low IgD and no CD23 (Martin and Kearney 2002, Pillai et al. 2005). MZ B cells are thought to facilitate the transport of antigen-immune complexes from the circulation to the splenic follicles by capturing antigen-antibody complexes and physically shuttling them to the follicular areas (Guinamard et al. 2000, Cinamon et al. 2008, Arnon et al. 2013). Like B-1 B cells, MZ B cells seem to express a restricted BCR repertoire biased towards bacterial cell wall constituents and respond very rapidly to antigenic challenge (Chen et al. 1997, Martin and Kearney 2002, Pillai et al. 2005). Interestingly, MZ B cells uniquely depend on NOTCH2 signaling for their development and maturation (Carnoy et al. 2003, Kuroda et al. 2003, Pillai and Cariappa 2009). However, it is currently unclear whether the MZ B cells studied in
mice is equivalent to human MZ B cells given the differences in anatomical arrangement of the splenic marginal zone between mice and humans (Steiniger et al. 2006).

**B cell tolerance**

Studies with B cell lines suggested that immunoglobulin gene rearrangement could occur even after the assembly of functional V(D)Js (Feddersen and Van Ness 1985, Kleinfield et al. 1986, Levy et al. 1989). These secondary rearrangements were found to occur at both heavy and light chains, through cryptic recombination signal sequences that allow upstream $V_{H}$ genes to combine with pre-existing V(D)Js and by the replacement of pre-existing $V_{J_{k}}$ by recombination with downstream $J_{k}$s (Kleinfield et al. 1986, Reth et al. 1986, Harada and Yamagishi 1991). The physiological significance of these early observations became apparent from the independent studies of transgenic mice by Nemazee and Weigert, where mice were engineered to either express antibodies against the MHC I protein, H2-K$^{k}$ or double-stranded DNA (Gay et al. 1993, Tiegs et al. 1993). The self-reactive anti-MHC I B cells, which had low levels of surface IgM, expressed high levels of $Rag2$ mRNA and contained abundant circular DNA excision products from rearrangements of the endogenous lambda light chain (Tiegs et al. 1993). The experiments by Nemazee and Weigert indicated that strong binding to self-antigen induced ‘receptor editing’ to reduce or eliminate the self-reactivity of the antibody by rearranging the light chain (Gay et al. 1993, Tiegs et al. 1993). Thus, these observations provided evidence for an alternative mechanism of tolerance where B cells were rescued from clonal deletion and this represented a mechanism of receptor selection, instead of clonal selection as initially proposed by Burnet (Gay et al. 1993, Tiegs et al. 1993, Jankovic et al. 2004).

The B cells carrying self-reactive BCRs that fail to edit their receptors within 1-3 days undergo cell death in the bone marrow or the spleen (Hartley et al. 1993). Clonal deletion of B cells at the immature stage was first proposed by Lederberg as a mechanism for the suppression of self-reactive B cells during development (Lederberg 1959). *In vivo* experiments involving the treatment of mice with anti-
IgM antibodies indicated that deletion could be mediated by signals from the B cell receptor (Lawton et al. 1972). However, conclusive evidence for BCR-mediated deletion at the immature stage of B cell development was subsequently provided by a number of transgenic mouse models (Nemazee and Burki 1989, Hartley et al. 1991, Chen et al. 1995, Sandel and Monroe 1999). The process of clonal deletion was found to be dependent on the strength of BCR-antigen crosslinking and is mediated by the BCR-induced accumulation of the pro-apoptotic protein BIM (Hartley et al. 1991, Strasser and Bouillet 2003).

Tolerance to self-antigens can also be induced by intrinsic mechanisms of anergy, which render the B cells hyporesponsive to BCR-antigen interactions (Healy and Goodnow 1998). This phenomenon of anergy was initially observed in tissue culture experiments where developing B cells were exposed to varying amounts of anti-IgM, such that B cells exposed to high concentrations of anti-IgM were deleted, whereas cells interacting with intermediate levels of anti-IgM could develop further but displayed a functional handicap characterised by impaired proliferation and antibody production following stimulation (Pike et al. 1982). The anergic state was first demonstrated in vivo by Goodnow and colleagues by using sets of transgenic mouse strains in which B cells expressing a specific antibody (HyHEL10) against hen egg lysozyme (HEL) were engaged with the soluble HEL neo-self protein throughout development, resulting in B cells which were unable to produce antibodies when immunized with lysozyme (Goodnow et al. 1988). The anergic B cells in this model are characterised by selective downregulation of IgM and high expression of IgD on the B cell surface (Goodnow et al. 1988).

Similar observations of IgM\textsubscript{low}IgD\textsubscript{high} self-reactive B cells were also made in a number of transgenic mice models expressing BCRs that recognise DNA and other self-antigens, including insulin (Erikson et al. 1991, Nguyen et al. 1997, Borrero and Clarke 2002, Henry et al. 2009). Interestingly, characterisation of the IgM\textsubscript{low}IgD\textsubscript{high} B cell population in the human B cell repertoire indicated that a high proportion of B cells express antibodies that react with self-antigens to varying degrees (Duty et al. 2009, Quach et al. 2011). The VH4-34 heavy chain segment in humans is a striking example of an intrinsically autoreactive heavy chain gene, which confers reactivity against conserved I/i carbohydrates on the surface of red
blood cells and other cell types (Sanz et al. 1989, Pascual et al. 1992). Reactivity against I/i self-antigens is a key pathological component of cold-agglutinin disease and has been associated with lymphomas, systemic lupus erythematosus (SLE), Epstein-Barr virus and *Mycoplasma pneumoniae* infections (Pascual et al. 1991, Pascual et al. 1992, Chapman et al. 1993, Isenberg et al. 1993, Stevenson et al. 1993, Stevenson et al. 1993, Riboldi et al. 1994, van Vollenhoven et al. 1999). B cells bearing VH4-34 mature to become IgM\textsuperscript{low}IgD\textsuperscript{high} follicular B cells but are excluded from the memory B cell and plasma cell compartments (Pugh-Bernard et al. 2001, Duty et al. 2009).

The unique phenotype of IgM\textsuperscript{low}IgD\textsuperscript{high} expression on the surface of anergic B cells is contributed by the degradation of newly synthesized IgM in the endoplasmic reticulum, the rapid recycling of internalized IgD to the cell surface, and the increase in IgD mRNA in anergic B cells (Bell and Goodnow 1994, Glynne et al. 2000, Blery et al. 2006). Moreover, the constant release of calcium from the endoplasmic reticulum triggered by inositol-3-phosphate (IP3) following engagement of self-antigen and BCR may also contribute to reduced surface IgM expression (Miller et al. 2009). In addition to reduced surface IgM, BCR signaling in anergic cells seem to be tuned down at a proximal point in the signaling cascade. BCR signaling components that induce anergy in the MD4xML5 model have been characterised in mice carrying loss-of-function mutations in a number of signaling molecules (Cyster and Goodnow 1995, Cyster et al. 1996, Rathmell et al. 1996, Healy et al. 1997, Inaoki et al. 1997, Prodeus et al. 1998). The tuning down of antigen receptor signals in anergic B cells seem to depend on the tyrosine phosphatase SHP-1 and the tyrosine kinase LYN: the latter initiates antigen receptor signaling and activates SHP-1 as a negative feedback mechanism (Cyster et al. 1996, Cornall et al. 1998, Nishizumi et al. 1998).

Interestingly, antigen receptor signaling to the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) and c-JUN terminal kinase (JNK) are selectively crippled in anergic B cells (Healy et al. 1997). The continuous exposure to self-antigen induces low levels of calcium signaling which are insufficient to trigger activation of NF-κB transcription factors, but can trigger the calcineurin-mediated activation of the NFAT transcription factors (Healy et al. 1997, Glynne et
Moreover, gene expression profiling and functional studies in anergic B cells indicated that the MEK-ERK signaling axis is activated in anergic B cells, resulting in the expression of genes such as *Egr1* and a block in the plasma cell differentiation program (Healy et al. 1997, Glynne et al. 2000, Rui et al. 2003, Rui et al. 2006). The anergic state in self-reactive B cells appears to be regulated at different levels as the recovery of surface IgM levels occurs 4-10 days after withdrawal of the self-antigen from B cells, whereas T cell-dependent antibody responses remain crippled (Goodnow et al. 1991).

**The ‘common root’ hypothesis of autoimmunity and lymphoma**

Autoimmune diseases are a major contributor of the global disease burden (Lozano et al. 2012). Most autoimmune diseases can be managed by treatments, but they cannot be completely cured as one of the key factors stimulating an aberrant immune response are self-antigens, which cannot be eradicated from the body. The existence of autoimmune diseases in humans has been known for more than a century as the first human autoimmune disease, paroxysmal cold hemoglobinuria, was described by Donath and Landsteiner in 1904 (Von Herrath and Homann 2008). Since then, more than thirty autoimmune diseases have been characterised (Rose and Bona 1993, Cooper et al. 2009). The prevalence of autoimmune diseases was estimated to be 3.2% based on a study synthesizing data on 24 autoimmune diseases over a period of 30 years (Jacobson et al. 1997). However, the latter figures were likely to be an underestimate as more recent epidemiological studies suggested that the global prevalence of autoimmune disease was 7.6-9.4% (Cooper et al. 2009). The most prevalent autoimmune diseases are Hashimoto’s thyroiditis (1.6% of total population), Alopecia (0.9%), Celiac disease (0.8%), Sjögren’s syndrome (0.7%) and Psoriasis (0.7%) [Table 1-adapted from (Cooper and Stroehla 2003, Cooper et al. 2009). Interestingly, most autoimmune diseases seem to be diagnosed later in life, with the mean age at diagnosis being 47 years [Table 1-adapted from (Cooper and Stroehla 2003, Cooper et al. 2009)].
It is established that failure of self-tolerance is central to the pathogenesis of autoimmune diseases, but the mechanisms by which self-reactive lymphocytes escape from tolerance in human diseases are unclear. Autoimmunity is thought to result from a combination of environmental triggers, genetic variants and stochastic events (Davidson and Diamond 2001, Goodnow 2007). Viral infections have been suggested as a potential trigger of autoimmunity because of their capacity to trigger strong immune responses (Oldstone 1987). Experiments in mice have suggested that molecular mimicry and the bystander activation of antigen presenting cells and lymphocytes by cytokines following viral infection as potential mechanisms of virus-induced autoimmunity (Horwitz et al. 1998, Zhao et al. 1998). Although this proposed association between viral infection and autoimmune disease is an intriguing possibility, it has been challenging to demonstrate the causative role of specific viruses in human autoimmune disorders. For example, hepatitis C virus (HCV) which primarily causes chronic liver inflammation and organ damage, has also been associated with B cell lymphoproliferative disorders such as mixed cryoglobulinemia and non-Hodgkin’s lymphoma (Charles and Dustin 2009). However, how B cells become deregulated during HCV infection and gives rise to pathogenic B cell expansion is unclear.

In both humans and experimental animals, chronic exposure to certain chemicals has been associated with autoimmune responses (Bigazzi 1988). For instance, the exposure to subtoxic doses of mercury induces the production of autoantibodies in mice (Schiraldi and Monestier 2009). In addition, a number of clinical cases of drug-induced autoimmunity have also been reported (Dedeoglu 2009). Even though several hypothesis about the interaction of metal-ions and chemical compounds with lymphocytes and self-proteins have been suggested, the mechanisms by which autoimmunity is induced remain unclear as clinical studies are limited to case descriptions and few animal experiments have been performed (Griem and Gleichmann 1995, Olsen 2004).

Studies in both humans and experimental animals have indicated that defects in genes are linked to predisposition to autoimmune diseases (Goodnow et al. 2005, Rioux and Abbas 2005). Twin studies in humans have shown that several autoimmune diseases such as Celiac disease have a strong genetic component with
75% concordance rates among monozygotic twins (Hervonen et al. 2000, Greco et al. 2002, Bogdanos et al. 2012). However, similar studies have also indicated that other autoimmune diseases such as rheumatoid arthritis and multiple sclerosis potentially have a less prominent genetic component as the concordance rates in monozygotic twins is only about 15% and 30% respectively (Aho et al. 1986, Ebers et al. 1986, Sadovnick et al. 1993, Silman et al. 1993, Svendsen et al. 2002). The genetic basis of type 1 diabetes has been contradictory as findings of earlier twin studies suggesting that monozygotic siblings have a 22% higher progression risk to disease than dizygotic siblings could not reproduced in subsequent studies (Petersen et al. 1997, Redondo et al. 1999, Gale et al. 2001). More recently, a longitudinal study over a period of 44 years, which followed monozygotic twins that were initially discordant for type 1 diabetes, indicated that the incidence of islet autoimmunity was 65% (Redondo et al. 2008). Together, these human genetic studies indicate that common autoimmune diseases are likely to result from a complex interaction of defects in several genes and environmental factors.

Rare autoimmune diseases caused by or associated with a single genetic defect have highlighted a number of genes and pathways affected in disease and normal physiology (Cheng and Anderson 2012). For instance, the gene AIRE was initially found mutated in patients with autoimmune polyendocrine syndrome that manifests as autoimmune attack against endocrine organs, the skin and other tissues (Bjorses et al. 1998). This finding was subsequently confirmed in mice with Aire-deficiency where in the absence of thymic expression, T cells specific for self-antigens have been shown to escape negative selection, enter the periphery and attack the target tissues (Anderson et al. 2002, Liston et al. 2003). Autoimmune lymphoproliferative syndrome (ALPS) is another example of a rare disorder of immune tolerance breakdown, characterised by lymphadenopathy, splenomegaly, and autoimmune cytopenias (Canale and Smith 1967). Interestingly, the genetic defect contributing to this disease was first described in a mouse strain carrying the lymphoproliferation (lpr) mutation in the Fas gene as the mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease (Watanabe-Fukunaga et al. 1992). Mutations affecting the FAS gene was subsequently found in patients with a lymphoproliferative syndrome,
which closely resembled the disease studied in the lpr mice (Fisher et al. 1995, Rieux-Laucat et al. 1995).
<table>
<thead>
<tr>
<th>Rank</th>
<th>Disease</th>
<th>Average prevalence (per 100000)</th>
<th>Rate (%)</th>
<th>Mean age at diagnosis</th>
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Total=7.17
Mean=46.8

* compiled from (Cooper and Stroehla 2003, Cooper et al. 2009)
* not determined in (Cooper and Stroehla 2003)
Fig 1.1: Mean age at the time of diagnosis of autoimmune diseases and lymphoid malignancies. Each dot represents a specific type of disease. Autoimmune diseases and lymphoid cancers are represented by blue dots and red dots respectively. T1D: Type 1 diabetes, NHL: Non-Hodgkin's lymphoma, HL: Hodgkin's lymphoma.
Interestingly, three broad groups of autoimmune diseases emerge when the mean age at the time of diagnosis is considered (Table 1.1; Fig 1.1). For instance, autoimmune diseases such as Type 1 Diabetes are diagnosed early in life, whereas patients with Multiple sclerosis and Addison’s disease have a mean age of approximately 40 at the time of diagnosis, and autoimmune diseases such as Hashimoto’s thyroiditis, Sjögren’s syndrome, Rheumatoid arthritis and Systemic vasculitis are diagnosed later in life (Table 1.1; Fig 1.1). This pattern of age-related onset of autoimmune disease could be potentially explained by inherited genetic defects having a strong effect in autoimmune diseases diagnosed early in life, whereas diagnosis of autoimmune disease later in life could be associated with the accumulation of somatic mutations that deregulate critical regulatory circuits of the immune system (Goodnow 2007). The idea that germline and somatic mutations provide the inherited and stochastic mechanisms that disrupt tolerance mechanisms and result in autoimmunity was first proposed by Burnet in his 1972 monograph on autoimmunity (Burnet 1972). More recently, Goodnow independently derived and extended this idea using compelling examples from more than 30 years of molecular and cellular immunology research that has accumulated since Burnet’s proposal (Goodnow 2007). The main hypothesis put forward is that somatic mutations represent one of the major stochastic elements in the pathogenesis of autoimmune disease.

Somatic mutations occur frequently in lymphocytes. Each hematopoietic stem cell typically undergoes between an estimated 17 to 20 cell divisions to produce mature cells of the immune system (MacKey 2001). Given that the approximate mean somatic mutation rate of $10.6 \times 10^{-7}$ mutations per cell division (Araten et al. 2005) and that each of the $1.7 \times 10^4$ to $8.1 \times 10^4$ hematopoietic stem cells in humans produce about $1.1 \times 10^9$ to $5.2 \times 10^9$ mature cells (Abkowitz et al. 2002, Gordon et al. 2002), each mature cell would contain an average of $9 \times 10^4$ additional somatic mutations relative to the stem cell from which the mature cell was derived. Thus, the likelihood of a deleterious mutation affecting genes involved in the regulation of self-reactive lymphocytes is quite high, if we consider the billions of mature lymphocytes produced over a period of 60 years (the approximate age of diagnosis of autoimmune disease occurring later in life). Moreover, B lymphocytes recruited in a germinal centre reaction not only proliferate at a rapid rate of 8-12 hours per
cycle (Allen et al. 2007, Allen et al. 2007) but also express high levels of activation-induced cytidine deaminase (AID), which is crucial for somatic hypermutation and class switching of antibodies (Muramatsu et al. 2000). The activity of AID is not restricted to immunoglobulin genes as AID-mediated genetic lesions can be detected in genes such as BCL6, FAS, CD79A and CD79B (Pasqualucci et al. 1998, Shen et al. 1998, Muschen et al. 2000, Gordon et al. 2003, Liu et al. 2008). Thus, the rapidly dividing germinal centre B cells may accumulate sufficient unwanted genetic lesions that become pathologic in both lymphomas and autoimmune diseases.

The association of lymphoma and autoimmunity was recognised by clinical observations, which highlighted the co-occurrence of autoimmune diseases and haematological cancers and the study of mice that developed both types of disease (Dameshek and Schwartz 1959, Mellors 1966). Since these early observations, numerous population-based case-control and cohort studies have indicated that several autoimmune diseases including Rheumatoid arthritis, Sjogren’s syndrome and Systemic lupus erythematosus are associated with lymphoid malignancies (Engels et al. 2005, Zintzaras et al. 2005, Smedby et al. 2006, Soderberg et al. 2006, De Re et al. 2008, Ekstrom Smedby et al. 2008, Mellemkjaer et al. 2008, Anderson et al. 2009, Goldin and Landgren 2009, Fallah et al. 2014). Some of the proposed explanations for the association between autoimmune diseases and lymphomas include the role of chronic immune stimulation, treatment for autoimmune disease and shared genetic factors (Goldin and Landgren 2009).


Together, these studies provide additional support to the common root hypothesis of the overlapping pathogenesis of autoimmunity and lymphoma. Thus, somatic mutations that are critical for the pathogenesis of lymphomas may also occur in self-reactive clones or in stem cells. These somatic genetic lesions may cooperate with germline variants to trigger autoimmunity and may eventually lead to autoimmune disease. An interesting “experiment of nature” supporting the common root hypothesis comes from rare individuals who develop autoimmune lymphoproliferative syndrome (ALPS) due to heterozygous FAS somatic mutations in a fraction of their blood cells (Holzelova et al. 2004, Dowdell et al. 2010, Price et al. 2014). Moreover, a number of B cell lymphomas also contain heterozygous somatic mutations in FAS and individuals with germline FAS mutations have an increased risk of developing lymphomas (Gronbaek et al. 1998, Straus et al. 2001, Price et al. 2014). These findings suggest a strong link between FAS mutations, lymphoma and autoimmunity.

**Somatic genetic lesions in diffuse large B cell lymphoma**

B lymphocytes at different stages of differentiation give rise to approximately 90% of all lymphomas, with the remainder derived from T lymphocytes. Based on the reported incidence of lymphoid cancers in the USA and UK, it is estimated that
more than 500,000 humans are diagnosed with lymphomas around the world every year (Siegel et al. 2011, Smith et al. 2011, Siegel et al. 2013). Diffuse large B cell lymphoma (DLBCL) is the most common form of Non-Hodgkin’s lymphoma, accounting for approximately 40% of all newly diagnosed cases (Morton et al. 2006, Smith et al. 2011, Cultrera and Dalia 2012). DLBCL is also currently one of the least curable lymphomas, with about 50% success using a combination of chemotherapy and rituximab (Rosenwald et al. 2002). With the advent of genome-wide gene expression profiling, DLBCL has been sub-divided into three molecular subtypes (Alizadeh et al. 2000). The activated B cell (ABC), germinal centre B cell (GCB) and the primary mediastinal B cell lymphoma (PMBL) subtypes are histologically indistinguishable, but differ in the expression of hundreds of signature genes (Alizadeh et al. 2000). The subdivision of DLBCL holds promise for better diagnosis and improved treatment regimes, even though the use of gene expression profiling is yet to be translated into routine clinical practice.

The three DLBCL subtypes have been proposed to arise from different stages of normal B cell differentiation and use distinct oncogenic pathways (Staudt and Dave 2005, Lenz et al. 2008, Lenz and Staudt 2010, Rui et al. 2011). DLBCL subtypes have been found to respond differentially to standard chemo-immuno therapy and to regimens incorporating newly developed agents (Rosenwald et al. 2002, Dunleavy et al. 2009, Novero et al. 2014). Among the three subtypes of DLBCL, the ABC subtype has been associated with the lowest success rates following standard treatment regimes (Rosenwald et al. 2002). Interestingly, gene expression profiling and drug inhibition studies revealed that the ABC subtype has a striking dependence on signaling pathways activating the transcription factor NF-κB (Alizadeh et al. 2000, Ngo et al. 2006). The constitutive NF-κB activation in ABC DLBCL could contribute to the poor response following chemotherapy as the targets of this family of transcription factors prevent apoptosis (Baldwin 2001). Thus, these findings emphasized the need for the development of therapeutics targeting NF-κB signaling for the treatment of aggressive lymphomas, and triggered further investigation into the root cause of the increased NF-κB activity in ABC DLBCL.
The mechanisms responsible for constitutive NF-κB signaling in ABC DLBCL have been elucidated by a combination of RNA interference genetic screens and a recent wave of progress in cancer genomics with the advent of next-generation sequencing technologies (Orellana 1975, Ngo et al. 2006, Lenz et al. 2008, Davis et al. 2010, Morin et al. 2011, Pasqualucci et al. 2011, Pasqualucci et al. 2011, Lohr et al. 2012, Morin et al. 2013, Zhang et al. 2013, de Miranda et al. 2014). In the initial “Achilles heel” screens, shRNAs were screened to identify those that block lymphoma cell proliferation and survival in culture. This revealed toxicity of shRNAs targeting CARD11, MALT1, and BCL10 for ABC but not GCB DLBCL cell lines (Ngo et al. 2006). The survival of this aggressive lymphoma subtype relies on signaling from the antigen receptor to the NF-κB transcription factors, with CARD11, BCL10 and MALT1 being essential components of the signaling apparatus (Ngo et al. 2006, Thome et al. 2010). In approximately 10% of patients, gain-of-function mutations in the CARD11 oncogene was found to activate NF-κB and prolong cell survival (Lenz et al. 2008). In addition, about 20% of ABC lymphomas have mutations in CD79A or CD79B, which are rare or absent in GCB and other lymphoma subtypes (Davis et al. 2010). Loss-of-function mutations resulting in the inactivation of A20, a negative regulator of NF-κB signaling has been found to occur in 25% of ABC lymphomas (Compagno et al. 2009, Honma et al. 2009, Kato et al. 2009). Crippling the activity of A20 increases the activity of NF-κB signaling in malignant B cells (Wertz et al. 2004). More recently, high-throughput RNA resequencing of DLBCL has identified recurrent oncogenic mutations in MYD88 in 39% of ABC DLBCL tumors (Jeelall and Horikawa 2011, Ngo et al. 2011). Together, these genetic lesions contribute to the high NF-κB activity in ABC DLBCL.

**Role of NF-κB signaling in B cell biology**

The NF-κB family of transcription factors consists of five members: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA, c-Rel, and RelB (Gerondakis et al. 2006, Hayden and Ghosh 2008). These transcription factors are characterised by an N-terminal Rel homology domain that participates in the formation of homo- or heterodimers and for binding DNA (Hayden and Ghosh 2008). RelA, c-Rel, and RelB contain a C-terminal transcription activation domain,

A number of different stimuli activate NF-κB transcription factors to trigger their cytoplasmic to nuclear translocation (Bonizzi and Karin 2004, Vallabhapurapu and Karin 2009). Most stimuli use the well-characterised canonical NF-κB signaling pathway, which depends on RelA-p50 and c-Rel-p50 heterodimers (Bonizzi and Karin 2004, Vallabhapurapu and Karin 2009). This pathway depends on the activation of the trimeric IκB kinase (IKK) complex comprising the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ (also called NEMO: NF-κB essential modulator) (Hacker and Karin 2006).

Once activated, the IKK complex phosphorylates IκBα on Ser32 and Ser36, which tags the protein for Lys48-linked polyubiquitination by the Skp1, Cullin1 and F-box protein β transducin repeat-containing protein (βTRCP) SCF$IκB$ E3 ubiquitin ligase complex (Grimwood and Bines 2007). The ubiquitinated IκBα is subsequently degraded via the 26S proteasome, resulting in the exposure of the nuclear localisation signal on RelA and inducing nuclear translocation of RelA:p50 dimers (Salahuddin 1991, Grimwood and Bines 2007). Of the two catalytic IKK subunits, IKKβ is the main of IκB kinase activity in most cell types, but in the absence of IKKβ, IKKα can provide residual IκB kinase activity (Salahuddin 1991). Moreover, the deletion of IKKα does not affect classical IKK activity as the main function of IKKα is the activation of the alternative NF-κB pathway, which involves the processing of p100 and activation of RelB:p52 heterodimers (Olson et al. 1975, Hu et al. 1999, Bonizzi and Karin 2004). The alternative NF-κB pathway is activated in response to a subset of TNF family members, including CD40L, LTαβ and BAFF (Claudio et al. 2002, Coope et al. 2002, Yilmaz et al. 2003). Interestingly, the classical IKK signaling pathway feeds into the alternative pathway through the upregulation of NF-κB2 expression, but the processing of p100 seems to be dependent on the activation of NIK (NF-κB inducing kinase) and the phosphorylation by IKKα (Senftleben et al. 2001, Xiao et al. 2001).
NF-κB signaling is critical for the survival of developing B cells. Compound genetic deficiencies in NF-κB1/NF-κB2 and c-Rel/RelA result in arrested B-cell development at the transitional stages of development, and result in the absence of mature follicular and marginal zone B cells in mice (Franzoso et al. 1998, Grossmann et al. 2000, Claudio et al. 2006, Gerondakis et al. 2006, Gerondakis and Siebenlist 2010). The absence of signaling components of the alternative NF-κB pathway such as NF-κB2, NIK or IKKα result in partial reductions of mature B cell subsets due to the compounded defects in BAFF signaling and lymphoid organ architecture (Franzoso et al. 1998, Yamada et al. 2000, Kaisho et al. 2001, Stadanlick et al. 2008). Thus, in accordance with the role of the alternative NF-κB pathway in the development of marginal zone B cells, BAFF transgenic mice accumulate large numbers of splenic marginal zone B cells and develop autoimmunity (Lesley et al. 2004, Thien et al. 2004, Enzler et al. 2006).

Mature resting B cells constitutively express NF-κB1/c-Rel heterodimers and NF-κB1 homodimers, with lower levels of RelA and NF-κB2 (Grumont and Gerondakis 1994, Vallabhapurapu and Karin 2009). The expression of NF-κB factors downstream of both the classical and alternative pathways is thought to mainly contribute to the survival of mature B cells (Gerondakis and Strasser 2003). One of the first in vivo functions demonstrated for NF-κB was the requirement for c-Rel in B cell activation by ligands for the BCR or TLRs (Kontgen et al. 1995). Moreover, the fates of activated B cells such as proliferation, differentiation and survival during an immune response seems to be dependent the expression of genes regulated by NF-κB activity (Vallabhapurapu and Karin 2009). Signalling from BCR, CD40, and TLR receptors activate NF-κB during B-cell activation, such that strong receptor signaling leads to the activation of the IKK complex and the nuclear translocation of NF-κB1/c-Rel, NF-κB1/RelA, and NF-κB1 homodimers from cytoplasmic reserves (Kontgen et al. 1995, Grumont et al. 1998, Vallabhapurapu and Karin 2009). Signals from the B cell receptor that activate NF-κB transcription factors provide essential co-stimulatory signals that regulate the migration, survival and differentiation of mature B cells (Dong et al. 2010, Gerondakis and Siebenlist 2010).
Function of CARD11 in the immune system

CARD11, also known as CARMA1, was identified through bioinformatics approaches based on homology comparisons of the CARD domain (Bertin et al. 2001, Gaide et al. 2001). CARD11 consists of an amino-terminal CARD domain, a coiled-coil domain and carboxy-terminal PDZ (named after PSD-95, Dlg and ZO-1 proteins which have the same domain), SH3 (src homology 3), and GUK (guanylate kinase) domains (Bertin et al. 2001, Gaide et al. 2001, McAllister-Lucas et al. 2001, Thome 2004, Rawlings et al. 2006, Blonska and Lin 2009). CARD11 belongs to the MAGUK family of proteins that function as molecular scaffolds to assist recruitment and assembly of signaling molecules in the cytoplasmic membrane (Dimitratos et al. 1999, McAllister-Lucas et al. 2001). The CARD of CARD11 is involved in homotypic interaction with the adaptor BCL10, which contains an amino-terminal CARD motif (Costanzo et al. 1999, Koseki et al. 1999, Srinivasula et al. 1999, Yan et al. 1999, Bertin et al. 2001, Gaide et al. 2001). Recently, using an elegant combination of electron microscopy and nuclear magnetic resonance, Hao Wu's group showed that CARD11 nucleates a helical CARD filamentous structure by interacting with BCL10 (Qiao et al. 2013). The CARD, coiled coil, SH3 and GUK domains are all essential for the NF-κB activation by CARD11, whereas the PDZ domain appears to be dispensable (Pomerantz et al. 2002). The coiled coil domain of CARD11 appears to mediate self-oligomerisation as expression of the coiled coil domain of CARD11 inhibited NF-κB activation and IL-2 production in stimulated Jurkat T cells, and impaired the survival of ABC DLBCL cells lines (Tanner et al. 2007, Lenz et al. 2008). The SH3 domain of CARD11 seem to regulate membrane localisation and the recruitment of PKC, BCL10 and the IKK complex (Wang et al. 2004). The specific contributions of the SH3 and GUK domains to the function of CARD11 are currently unclear.

CARD11 is predominantly expressed in hematopoietic tissues such as spleen, thymus and circulating lymphocytes (Bertin et al. 2001). In unactivated mature lymphocytes, CARD11 is found in a latent form in the cytoplasm (Matsumoto et al. 2005, Sommer et al. 2005). Following antigen receptor engagement, CARD11 is phosphorylated by PKCβ in B cells and PKCθ in T cells in the linker region (also referred to as inhibitory domain), found between its coiled-coil and MAGUK

Following the assembly of the CBM complex, TRAF6 is recruited and its ubiquitin ligase domain is activated. TRAF6 attaches K63-linked polyubiquitin chains to itself and MALT1 to stabilise the protein-protein interactions and allow for the recruitment of the IKK proteins (Sun et al. 2004, Oeckinghaus et al. 2007). The role of MALT1 in the activation of NF-κB is still unclear (Thome et al. 2010). MALT1 was originally identified as a paracaspase and it was only recently found that MALT1 could cleave A20, which is a negative regulator of NF-κB signalling (Coornaert et al. 2008). Interestingly, the protease activity of MALT 1 was found to be essential for the survival of ABC DLBCL cell lines (Ferch et al. 2009, Hailfinger et al. 2009). Following the assembly of the IKK complex, TRAF6 ubiquitinates IKKγ, which is a required step in its activation as a kinase (Vallabhapurapu and Karin 2009). Ubiquitinated TRAF6 also interacts with TAB2, leading to activation of the associated kinase TAK1 (Sun et al. 2004, Shinohara et al. 2005, Vallabhapurapu and Karin 2009). TAK1 carries out the second required step in IKK activation, phosphorylation of IKKβ in its activation loop (Sun et al. 2004, Sato et al. 2005, Shinohara et al. 2005, Wan et al. 2006, Vallabhapurapu and Karin 2009). CARD11 also signals to the JNK signaling pathway as JNK2 activation following TCR stimulation was found to be selectively impaired in Card11-deficient mice (Blonska et al. 2007, Blonska and Lin 2009). Phosphorylated CARD11 is thought to recruit BCL10, which binds to TAK1 and MKK7 to activate JNK2 (Blonska et al. 2007, Blonska and Lin 2009). More recently, CARD11 was found to be essential for mTOR activation in T cells following TCR stimulation (Hamilton et al. 2014). This function of CARD11 was dependent on MALT1, but not BCL10 (Hamilton et al. 2014). However, it is not yet known whether CARD11 activity influences mTOR signaling in B cells.
Mice expressing loss-of-function CARD11 or deficient in CARD11 have severe defects in mounting humoral immune responses as B and T cell activation by antigen receptor signaling is defective (Egawa et al. 2003, Hara et al. 2003, Jun et al. 2003, Newton and Dixit 2003). CARD11 was thus found to be essential for antigen receptor-mediated proliferation and cytokine production in T and B cells, through the activation of JNK and NF-κB (Egawa et al. 2003, Hara et al. 2003, Jun et al. 2003, Newton and Dixit 2003). Moreover, lymphocyte development was normal in Card11-deficient mice, with the exception of the CD5+ peritoneal B cell subset and Foxp3+ regulatory T cell subset, which were severely compromised (Egawa et al. 2003, Newton and Dixit 2003, Molinero et al. 2009, Altin et al. 2011). B cells from ‘unmodulated’ mice, where only a single residue in the coiled-coil domain of CARD11 was changed, and B cells from knock-in mice with a ‘CARDless’ form of CARD11 were both defective in response to antigen receptor stimulation and failed to proliferate and differentiate into antibody producing cells, indicating that the coiled-coil and CARD domains of CARD11 were essential for the function of the protein in vivo (Egawa et al. 2003, Jun et al. 2003).

More recently, humans with loss-of-function mutations in CARD11 have been reported (Greil et al. 2013, Stepensky et al. 2013, Turvey et al. 2014). These rare individuals presented with a form of severe combined immunodeficiency characterised by profound hypogammaglobulinemia and Pneumocystis jirovecii pneumonia early during life (Greil et al. 2013, Stepensky et al. 2013). Similar to mice deficient in Card11, the numbers of B and T cells were normal in the affected individuals, with the selective absence of Foxp3+ regulatory T cells and differentiated B cell subsets (Molinero et al. 2009, Altin et al. 2011, Greil et al. 2013, Stepensky et al. 2013). Most naïve B cells displayed a CD10+CD38hi transitional phenotype (Molinero et al. 2009, Altin et al. 2011, Greil et al. 2013, Stepensky et al. 2013). Moreover, B and T cells from the CARD11-deficient patients did not respond to antigen stimulation and phorbol 12-myristate 13-acetate (PMA) induced canonical NF-κB activation (Greil et al. 2013, Stepensky et al. 2013). Interestingly, NF-κB signaling downstream of CD40 receptor was normal as CARD11-deficient B cells could form plasmablasts after CD40 and IL-21 stimulation in vitro, suggesting that the severe hypogammaglobulinemia
experienced by the patients was likely to be the consequence of disrupted T and B cell interactions during immune responses (Greil et al. 2013, Stepensky et al. 2013, Turvey et al. 2014).

Moreover, rare individuals with germline heterozygous gain-of-function mutations in \textit{CARD11} have also been recently described (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). These patients are characterised by a novel congenital B-cell lymphoproliferative disorder referred to as B-cell expansion with NF-κB and T-cell anergy (BENTA) (Snow et al. 2012). Patients with BENTA disease develop severe polyclonal B-cell lymphocytosis primarily of immature CD10+CD21\textsubscript{low} and mature follicular B cells, associated with splenomegaly and lymphadenopathy within the first year of life (Snow et al. 2012). Interestingly, despite excessive B cell accumulation, evidence of mild autoimmunity and B cell malignancy (CLL) has only been reported in two separate individuals of the 6 known patients with germline gain-of-function \textit{CARD11} mutations (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). Patients with gain-of-function mutations in \textit{CARD11} are also mildly immunodeficient as they experience recurrent sinopulmonary and viral infections with molluscum contagiosum and Epstein-Barr virus (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). 50% to 80% of circulating peripheral blood cells in patients with BENTA are CD19+CD20+CD5\textsubscript{intermediate}, which represents an expansion of approximately 10 fold compared to the number of B cells of the same phenotype in normal individuals (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). A significant increase in CD10\textsuperscript{hi}CD24\textsuperscript{hi}CD38\textsuperscript{hi} transitional B-cell numbers has also been reported in BENTA patients and T cell counts seem to be normal (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). Moreover, BENTA patients have low percentages of circulating memory and class-switched B cells, and poor immunoglobulin secretion and plasmablast differentiation \textit{in vitro} (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014).

The heterozygous missense mutations in patients with BENTA have been found predominantly within the coiled-coil or LATCH domain of \textit{CARD11}, with 1 mutation found in the CARD domain (Snow et al. 2012, Chan et al. 2013, Brohl et al. 2014, Turvey et al. 2014). These mutations result in spontaneous \textit{CARD11} aggregation, CBM complex formation and constitutive NF-κB activation (Snow et
al. 2012, Brohl et al. 2014, Turvey et al. 2014). Thus, B cells from BENTA patients are hyper-responsive to antigen receptor stimulation as they express higher levels of NF-κB target genes, proliferate more and accumulate to larger numbers in vitro compared to B cells from normal individuals (Snow et al. 2012, Brohl et al. 2014). Interestingly, CD4+ and CD8+ T cells from BENTA patients seem to be hyporesponsive, unless more robust co-stimulation is provided (Snow et al. 2012, Brohl et al. 2014). The poor response of T cells could contribute to defects in T cell help to B cells and the increased susceptibility to viral infections observed in BENTA patients.

**Oncogenic CARD11 mutations in diffuse large B cell lymphoma**

In normal antigen receptor signalling, signaling from the CARD11-BCL10-MALT1 complex is transient as it is limited by several negative feedback loops (Vallabhapurapu and Karin 2009, Moreno-Garcia et al. 2010, Thome et al. 2010). However, the activity of the CBM complex is constitutively required for the survival of ABC DLBCL cell lines (Ngo et al. 2006). Moreover, gain-of-function mutations in CARD11 were initially discovered in approximately 10% of ABC DLBCL cases (Lenz et al. 2008). Albeit at varying frequencies, CARD11 mutations have also been found in rare GCB DLBCL cases, which have paradoxically high levels of NF-κB target gene expression unlike most GCB DLBCL (Lenz et al. 2008). Since the initial discovery several other groups have reported CARD11 mutations in subtypes of DLBCL (Montesinos-Rongen et al. 2010, Dong et al. 2011, Morin et al. 2011, Pasqualucci et al. 2011, Lohr et al. 2012, Morin et al. 2013, Zhang et al. 2013).

The CARD11 mutants were found to constitutively activate NF-κB when introduced into cell lines (Lenz et al. 2008). The oncogenic activation of CARD11 by mutations found in DLBCL was explained by their effect on CARD11 subcellular localisation as CARD11 mutants form prominent cytoplasmic aggregates whereas wild-type CARD11 localises diffusely in the cytoplasm (Lenz et al. 2008). These macroscopic aggregates were found to co-localise with MALT1 and phosphorylated-IKKβ. These observations indicated that CARD11 aggregates were functional ‘platforms’ that constitutively signal to activate NF-κB (Lenz et al. 2008).
The lymphoma \textit{CARD11} mutations have been proposed to disrupt the folding of the \textit{CARD11} protein, exposing the 'buried' coiled-coil domain to allow formation of self-oligomers and facilitate binding to BCL10 and increase signalling to NF-κB transcription factors, through activation of the IKK complex (Lenz et al. 2008, Lamason et al. 2010).

Interestingly, most of the reported lymphoma mutations in \textit{CARD11} are found in exons 4 to 12, which encode for the coiled-coil domains of the protein (Lenz et al. 2008, Shaffer et al. 2012). The coiled-coil domain of \textit{CARD11} is crucial for the activation of the protein to function as a scaffold (Jun et al. 2003, Tanner et al. 2007, McCully and Pomerantz 2008). The coiled-coiled domain has also been proposed to form oligomers with several other \textit{CARD11} proteins binding to each other forming large membrane associated molecular platforms (Thome 2004, Rawlings et al. 2006). Thus, point mutations in the coiled-coil cripple the function of the protein in B cell activation following antigen receptor signaling (Jun et al. 2003). In addition, mutations in the coiled-coil domain have been found to impair its self-association or block membrane localisation of \textit{CARD11} (Tanner et al. 2007). The coiled-coil domain seems to be inhibited by the linker region (also referred to as the inhibitory domain by Pomerantz) as deletion of the linker region results in a constitutively active form of \textit{CARD11} (Sommer et al. 2005, McCully and Pomerantz 2008, Lamason et al. 2010). Together, these observations suggest a model whereby \textit{CARD11} mutations in DLBCL disrupt the inhibition of the coiled-coil domain by the linker region of \textit{CARD11}, giving rise to \textit{CARD11} proteins that constitutively bind to each other to form active aggregates which spontaneously nucleate the formation of BCL10 fibres (Qiao et al. 2013). Further analysis of \textit{CARD11} self-oligomerisation and the nucleation of BCL10 fibres will be required to enable the development of small molecule inhibitors to treat \textit{CARD11}-deopendent B cell malignancies and subsets of autoimmune diseases.

**Specific aims and questions addressed in this thesis**

(1) To what extent does \textit{CARD11} gain-of-function deregulate survival and growth of normal B cells? [Chapters 3 & 4]
The *CARD11* mutations found in DLBCL are considered ‘driver’ mutations without which the survival of the lymphomas is compromised. Thus, it has been proposed that *CARD11* mutations are acquired in pre-neoplastic B cell clones before accumulating and selecting for sufficient mutations to transform into clinically diagnosable lymphoma (Lenz and Staudt 2010, Rui et al. 2011). However, the consequence of acquiring these lymphoma-derived *CARD11* mutations in normal B cells, in isolation from the other ~30 mutations accumulated on average in lymphoma cells had not been addressed (Morin et al. 2011, Pasqualucci et al. 2011, Lohr et al. 2012, Morin et al. 2013, Zhang et al. 2013).

(2) Can gain-of-function *CARD11* mutations break B cell tolerance and trigger autoimmunity? [Chapter 5]

The association of lymphoma and autoimmunity was recognised by clinical observations, which highlighted the co-occurrence of autoimmune diseases and haematological cancers and the study of mice that developed both types of disease (Dameshek and Schwartz 1959, Mellors 1966). The common root hypothesis of the overlapping pathogenesis of autoimmunity and lymphoma suggests that somatic mutations that are critical for the pathogenesis of lymphomas may also occur in self-reactive B cell clones (Goodnow 2007). Thus, we specifically test effects of lymphoma *CARD11* mutations on the normal response of B cells to self-antigens.

(3) Can constitutively activated IKKβ mutations phenocopy effects of CARD11 gain-of-function mutations? [Results chapter 6]

IKKβ mediates activation of the canonical NF-κB pathway in response to pro-inflammatory stimuli, and protein kinase assays suggested that IKKβ accounts for nearly all of the catalytic activity of the IKK complex towards IκBα (Zandi et al. 1998, Scheidereit 2006). Activated IKKβ subsequently phosphorylates the inhibitory protein, IκBα, which is bound to NF-κB transcription factors to restrict them in the cytoplasm (Hacker and Karin 2006, Vallabhapurapu and Karin 2009, Gerondakis and Siebenlist 2010, Liu et al. 2012). Interestingly, naturally occurring gain-of-function mutations affecting the catalytic activity of IKKβ has been found in several cases of splenic marginal zone lymphomas (Rossi et al. 2011, Kai et al. 2014). Here, we focus the question on the extent to which constitutively active
IKKβ can phenocopy the effects of gain-of-function CARD11 mutants in normal B cells and in the context where the B cells encounter self-antigen.

(4) Can BENTA disease be further studied by using mice with a germline gain-of-function Card11 mutation? [Chapter 7]

Patients with germline heterozygous gain-of-function mutations in CARD11 have also been recently described (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). These patients are characterised by a novel congenital B-cell lymphoproliferative disorder referred to as B-cell expansion with NF-κB and T-cell anergy (BENTA) (Snow et al. 2012). Patients with BENTA disease develop severe B-cell lymphocytosis, associated with splenomegaly and lymphadenopathy within the first year of life (Snow et al. 2012). In this results chapter, we compare and contract the effects of two reported CARD11 germline mutations when studied in isolation from other genetic and environmental factors in mice. We also characterise a mouse strain with a germline Card11 mutation identified by exome sequencing of first generation offspring of C57BL/6 mice exposed to ENU (N-ethyl-N-Nitrosourea). Interestingly, the same CARD11 M365K mutation has been previously reported in several cases of ABC DLBCL.
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4.4 Sorting EGFP+ B cells by FACS ______________________________________ __52__
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(5) Flow cytometry _____________________________________________________ __54__
(6) Statistics __________________________________________________________ __55__
(1) Generation of \textit{Card11} and \textit{Ikbkb} mutations by site-directed mutagenesis

Mouse \textit{Card11} and \textit{Ikbkb} cDNAs were amplified by Platinum Pfx DNA polymerase (Invitrogen) from mouse spleen cDNA and cloned into pBluescript II SK(+) vector. PCR-based site-directed mutagenesis was used to introduce gain-of-function mutations into mouse \textit{Card11} and \textit{Ikbkb}. Amplified PCR products were purified and sequenced on an ABI 3730xl DNA Analyzer (at the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University) following the manufacturer's protocol (Applied Biosystems 2002). The mutations corresponded to those described by (Lenz et al. 2008) and the primers used are listed in Table 1. Wild-type and mutant \textit{Card11} and \textit{Ikbkb} genes were transferred into pMXs-IRES-GFP vector [provided by Dr. Kitamura; (Kitamura et al. 2003)]. The retroviral constructs were transfected into Phoenix ecotropic packaging cells (ATCC; CRL-3214) using calcium phosphate precipitation. The supernatants containing replication-defective retroviral particles were collected and were frozen at -80°C until used for transduction.

\textbf{Table 1: Primers used for site-directed mutagenesis of Card11 and Ikbkb}

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CARD11</td>
<td>5'TTTGCGGCGCCGCGCCGACCATTGCCAGAGAAGGAGGCCAGCTATG-3'</td>
<td>Initial cloning</td>
</tr>
<tr>
<td>CARD11</td>
<td>5'-AAAAGCTTCAGCTGGTCCTCGTCCACCCAGATG-3'</td>
<td>Initial cloning</td>
</tr>
<tr>
<td>CARD11 CS8</td>
<td>5'-TTTTAGATCTGCCACCATGCCAGGAGGGCCAG-3'</td>
<td>Retrovirus</td>
</tr>
<tr>
<td>CARD11 stop A2</td>
<td>5'-AAAAGCGGCCGCTCACAAGCTGGTCCTCGCC-3'</td>
<td>Retrovirus</td>
</tr>
<tr>
<td>CARD11 mt2 S</td>
<td>5'-GAGAGAACATCGTCCCCAAAGCTCAAGATG-3'</td>
<td>L251P</td>
</tr>
<tr>
<td>CARD11 mt2 A</td>
<td>5'-GCTATTCTTCAGTTGAGGGACTGATTCTTC-3'</td>
<td>L251P</td>
</tr>
<tr>
<td>CARD11 mt3 S</td>
<td>5'-TCCACATTGTGGAGGAAAGGCCATGAGGCCTCACAC-3'</td>
<td>G123S</td>
</tr>
<tr>
<td>CARD11 mt3 A</td>
<td>5'-AGTGTGTAGGGCTCTGTTTCTTCCACCAATGGT-3'</td>
<td>G123S</td>
</tr>
<tr>
<td>CARD11 mt6 S</td>
<td>5'-GCCCTTCCACTCCGAGTGGAGGACAGACACACAG-3'</td>
<td>D387V</td>
</tr>
<tr>
<td>CARD11 mt6 A</td>
<td>5'-CTGTGTGTGCTGCTGAGAACTGGGAGTGGAGGCC-3'</td>
<td>D387V</td>
</tr>
<tr>
<td>CARD11 mt10 S</td>
<td>5'-TCCACACTCGAGATCGACCGCTATTTAAACGCGACTGAAAG-3'</td>
<td>L227LI</td>
</tr>
<tr>
<td>IKKβ* S</td>
<td>5'-TGAATTCGCGACCATTGAGCTGCAGCTTCCACCAACCAACAAAG-3'</td>
<td>S177E, S181E</td>
</tr>
<tr>
<td>IKKβ* A</td>
<td>5'-AAAACCTCGAGTACGGGTTACCAGTAAAGCTTCTCT-3'</td>
<td>S177E, S181E</td>
</tr>
</tbody>
</table>
1.1 Site-directed PCR Mutagenesis

Site-directed PCR mediated mutagenesis was used to introduce the gain-of-function mutations in mouse *Card11* and *Ikbkb* cDNA. This was achieved using oligonucleotides listed in Table 1. The primers were designed using Primer3 (frodo.wi.mit.edu/primer3) and PrimerX (<www.bioinformatics.org/primerx/>), and ordered from GeneWorks Pty Ltd (Australia). PCR reactions were performed using Platinum Pfx DNA Polymerase (Invitrogen) in a total volume of 50μl containing the components shown in Table 2. The thermal program for the PCR reaction is shown in Table 3, and reactions were performed in a PTC-225 PCR machine (MJ Research). The products of the first PCR reactions were separated by electrophoresis on a 1% (w/v) agarose gel. DNA bands with size corresponding to the amplified fragments were excised from the agarose gel over the Safe Imager (Invitrogen). The DNA fragments were extracted from the agarose gel and purified using the QIAEX II Gel Extraction Kit (QIAGEN). The PCR products were used in a second PCR step to produce the mutated cDNA. The PCR products were purified using the PureLink PCR purification kit (Invitrogen).

### Table 2: Composition of site-directed mutagenesis PCR reactions

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Pfx Amplification Buffer</td>
<td>10</td>
</tr>
<tr>
<td>dNTP mixture (10mM dATP, dCTP, dGTP, dTTP)</td>
<td>1.5</td>
</tr>
<tr>
<td>Magnesium Sulphate, MgSO₄ (50mM)</td>
<td>1</td>
</tr>
<tr>
<td>Primer Mix (10μM each)</td>
<td>3</td>
</tr>
<tr>
<td>Template DNA (100ng/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Platinum Pfx DNA Polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>33</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50</td>
</tr>
</tbody>
</table>

*10xPfx Amplification Buffer, dNTP mixture, MgSO₄ and Platinum Pfx DNA Polymerase were all supplied in the Platinum® Pfx DNA Polymerase kit from Invitrogen.*
Table 3: Thermal program used for the site-directed mutagenesis PCR reactions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Denature</td>
<td>94</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denature</td>
<td>94</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Extension</td>
<td>68</td>
<td>4 x 60*</td>
<td></td>
</tr>
<tr>
<td>Post-Extension</td>
<td>68</td>
<td>180</td>
<td>1</td>
</tr>
</tbody>
</table>

*Extension time: 60s/kb DNA

1.2 Subcloning wild-type and mutant cDNA into pBluescript II SK (+) cloning vector

The mutated cDNA and a pBluescript II SK (+) cloning vector containing wild-type Card11 were digested with HindIII and NotI restriction endonucleases (NEB) at 37°C for 2 hours (Table 4).

Table 4: Typical composition of restriction enzyme reactions

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x NEB Buffer 3</td>
<td>5</td>
</tr>
<tr>
<td>10 x Bovine Serum Albumin (BSA)</td>
<td>5</td>
</tr>
<tr>
<td>DNA#</td>
<td>10-20</td>
</tr>
<tr>
<td>Restriction Enzyme 1</td>
<td>2</td>
</tr>
<tr>
<td>Restriction Enzyme 2</td>
<td>2</td>
</tr>
<tr>
<td>MilliQ water*</td>
<td>26-16</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50</td>
</tr>
</tbody>
</table>

*Volume of DNA used depended on the concentration; 500ng or more DNA was used so that a visible band was obtained when the digestion products are separated by agarose gel electrophoresis.

*Volume of MilliQ water added depended on the volume of DNA solution added, so that the total volume of the reaction was 50μl.
The digestion products were separated using 1% agarose gel electrophoresis and the DNA fragments corresponding to the size of the full-length cDNAs were excised and purified using the QIAEX II Gel Extraction kit (QIAGEN). The cDNA were ligated in to the pBluescript II SK (+) cloning vector using the Ligafast Rapid DNA Ligation System (Promega; Table 5). The ligation reaction mixtures were incubated with 50μl chemically competent E. coli (DH5α strain) for 10 minutes, on ice (Inoue et al. 1990). The mixtures were then incubated at 37°C for 30 seconds (heat shock), followed by 2 minutes incubation on ice. 300μl Luria Broth (LB, obtained from the Media Preparation Unit, JCSMR) supplemented with Ampicillin (100μg/ml) was added to the tubes and mixed rigorously. The samples were then centrifuged at 13000 rpm for 30 seconds. The volume of the supernatant was reduced to about 100 μl and the suspension was spread evenly over an LB/Ampicillin (100μg/ml) agar plate. The agar plates were incubated overnight at 37°C. Individual E. coli colonies were picked from the agar plate using a P20 pipette and cultured for 12-16 hours in 2ml LB/Ampicillin (100μg/ml) at 37°C, with vigorous shaking. The plasmids were isolated from the bacterial cultures using the Wizard Plus SV Minipreps DNA Purification System (Promega). The purified plasmids were digested with HindIII and NotI (NEB) to confirm the presence of the correct insert. The digested DNA fragments were then separated on a 1% agarose gel, and the DNA conjugated to the Gel Red dye (Biotium) were visualised using the Gene Genius Bio Imaging System (Syngene).

Table 5: Typical composition of ligation reactions using T4 DNA Ligase

<table>
<thead>
<tr>
<th>Components</th>
<th>Vector + Insert</th>
<th>Vector Only Control</th>
<th>Insert Only Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA Ligase</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2x Rapid Ligation Buffer</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Plasmid vector</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA insert</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>0</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
1.3 Sequencing of cDNA inserts

The cDNA inserts encoding IKKβ and CARD11 were sequenced using a combination of primers listed in Table 6, which allowed full sequence coverage of the cDNA insert. The sequencing PCR reactions were done in a total volume of 20μl (Table 7).

Table 6: List of primers used in the cycle sequencing PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKKβ Primers</td>
<td>(IKKBIntf) 5’-CGCTACCCTTCCCCAATAAT-3’&lt;br&gt;</td>
</tr>
<tr>
<td></td>
<td>(IKKBintr) 5’-TCTTCCCTCACGTCCTAG-3’</td>
</tr>
<tr>
<td>CARD11 Primers</td>
<td>(Carma1 CS4) 5’-AACTCGAGCTCGACGCTCAAC-3’</td>
</tr>
<tr>
<td></td>
<td>(Carma1 CS5) 5’-TGAACCTAAAGGCGATCCAG-3’</td>
</tr>
<tr>
<td></td>
<td>(Carma1 CA3) 5’-GTTCGCGTACAGCTTCTCAGG-3’</td>
</tr>
<tr>
<td></td>
<td>(Carma1 CA4) 5’-ACCAGGAGGCTCAGTGAGG-3’</td>
</tr>
<tr>
<td>T3 Primer*</td>
<td>5’-AATTAACCTCCTAAAGGG-3’</td>
</tr>
<tr>
<td>T7 Primer*</td>
<td>5’-TAATACGACTCAGTAGGGG-3’</td>
</tr>
</tbody>
</table>

*T3 and T7 primer binding regions are present on the pBlueScript II SK (+) vector.

Table 7: Thermal program used for the Cycle-Sequencing PCR reactions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Denature</td>
<td>96</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Denature</td>
<td>96</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>50</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>60</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were purified using 2μl EDTA (50mM), 2μl Sodium Acetate (1.5M) and 50μl 100% ethanol. The samples were vortexed briefly and centrifuged at 13000 rpm for 40 minutes at 4°C. The pellet was washed with 80% ethanol and air-dried for 20 minutes. The samples were submitted to the Biomolecular Resource Facility, JCSMR for analysis of the dye-tagged termination products on an ABI 3730xl DNA Analyzer. The sequence trace files were viewed using the
Chromas 2.33 Software (www.technelysium.com.au) and analysed using the CLC Sequence Viewer software. The sequence data was aligned with the reference cDNA sequence using the BL2SEQ online tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

1.4 Subcloning into the pMxs-IG retroviral vector

The cDNA inserts were subcloned into the pMxs-IG retroviral vector. The pMxs-IG vector can produce a single, bicistronic mRNA comprising the cDNA of interest, an Internal Ribosome Entry Site (IRES), and a second open reading frame encoding Enhanced Green Fluorescent Protein (Kitamura et al. 2003). cDNA inserts were also cloned into a different retroviral vector to produce EGFP-CARD11 fusion proteins. The plasmids were used to transform E.coli and colonies were selected and grown in 50ml LB/Ampicillin for 20 hours. The plasmids were then extracted from the bacteria by using the Midiprep procedure of the PureLink Hi-Pure Plasmid DNA Purification Kit (Invitrogen). The concentration of the purified DNA was measured on a Nanodrop 1000 (Thermo Scientific) at the Biomolecular Resource Facility, JCSMR, ANU.

1.5 Safety precautions and use of sterile microbiology techniques

The bacterial cultures and petri dishes that contained bacterial colonies were treated with general safety precautions, as the bacteria were considered pathogenic. Specifically, the following safety procedures were followed:

(i) All materials, media, tubes, plates, pipettes, and other items used for culturing microorganisms were appropriately discarded in biohazard bins that are sterilised by autoclaving.
(ii) A flame was used to produce a sterile working area on the bench.
(iii) All work areas were disinfected using 80% ethanol before and after working with cultures.
(iv) Gloves and protective eyewear were used when handling bacteria cultures, and disinfectant soap was used to wash hands after working with microorganisms.
(v) All cultures and plates were clearly labelled with their names and dates before incubation and storage. Plates and cultures were stored at 4°C for a maximum period of two weeks.
(2) Production of retrovirus using the Phoenix packaging cell line

The Phoenix cell line (ATCC; CRL-3214), an ecotropic helper-free retrovirus packaging cell line, was used to produce replication-defective retrovirus particles (Swift et al. 2001). Phoenix cells were cultured in complete DMEM medium (Table 8) in T75 flasks (Thermo Scientific) at 37°C, 5% CO₂ in a Biological Safety Cabinet II. The cells were maintained using the following protocol:

1. Gently remove and discard the culture media
2. Rinse cells with 1x Phosphate Buffered Saline (PBS; GIBCO)
3. Add 2ml Trypsin-EDTA (0.05% trypsin/0.53mM EDTA), and incubate for 2 minutes at 37°C.
4. Add 8ml complete DMEM medium and resuspend cells using an automatic pipette.
5. Transfer 2ml of the cell suspension to a new T75 flask containing 18ml of complete DMEM.
6. Incubate at 37°C, 5% CO₂.

Table 8: Composition of complete DMEM medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco's Modified Eagle's Medium (High Glucose)</td>
<td>GIBCO</td>
<td>500ml</td>
</tr>
<tr>
<td>Heat-Inactivated Fetal Calf Serum (HI-FCS); 10% v/v</td>
<td>Sigma</td>
<td>50ml</td>
</tr>
<tr>
<td>Penicillin (10,000 units), Streptomycin (10,000ug), Glutamine (29.2mg); 1% (v/v)</td>
<td>GIBCO</td>
<td>10ml</td>
</tr>
</tbody>
</table>

18-24 hours prior to transfection, Phoenix cells were seeded at 1.5-2 × 10⁶ cells per 60 mm tissue culture plate (BDFalcon) in complete DMEM medium. The plates were incubated at 37°C, 5% CO₂ to form a confluent, adherent monolayer. 5 minutes before transfection, 1.5μl 25μM chloroquine (SIGMA) was added to the culture and incubated at 37°C, 5% CO₂.
The following reagents were mixed in a 5 ml polystyrene tube (BD Falcon) at room temperature:

(i) X μl, 10μg purified DNA (*DNA was added in a drop to side of tube*).
(ii) Y μl double de-ionised H₂O (*DNA was washed to bottom of tube with water*).  
    *Note: X + Y = 439 μl DNA/H₂O mixture*
(iii) 61 μl 2M CaCl₂ (*mixed thoroughly with finger tapping*).
(iv) 0.5 ml 2xHBS [50mM HEPES (SIGMA), pH 7.05; 10mM KCl; 280mM NaCl; 1.5mM Na₂HPO₄] (*mixed for 20 seconds by bubbling using a pipette*).

The 1ml HBS/DNA/CaCl₂ suspension was transferred drop wise onto the 60mm dish containing Phoenix cells in 3ml of media, and the plate was rocked a few times to distribute DNA/CaPO₄ particles evenly. The plates were incubated at 37°C for 3 hours. The media was then replaced by fresh complete DMEM and incubated at 37°C, 5%CO₂. After 24 hours, the media was replaced by fresh complete RPMI media (Table 9), and the plates were incubated for another 24 hours at 37°C, 5%CO₂. The media (retrovirus supernatant) containing the retrovirus particles were collected in 15ml tubes and centrifuged at 2400rpm for 2 minutes to pellet down any Phoenix cells present. The retrovirus supernatant was then transferred to 5ml polypropylene tubes (BDFalcon) and stored at -80°C. The transfected Phoenix cells were collected and EGFP expression was measured by flow cytometry to determine efficiency of transfection.

**Table 9: Composition of complete RPMI medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roswell Park Memorial Institute (RPMI-1640)</td>
<td>GIBCO,</td>
<td>500ml</td>
</tr>
<tr>
<td>Heat-Inactivated Fetal Calf Serum (HI-FCS)</td>
<td>Sigma</td>
<td>50ml</td>
</tr>
<tr>
<td>Penicillin (10,000 units), Streptomycin (10,000ug), Glutamine (29.2mg)</td>
<td>GIBCO</td>
<td>10ml</td>
</tr>
<tr>
<td>50mM 2-Mercaptoethanol</td>
<td>SIGMA</td>
<td>560ul</td>
</tr>
</tbody>
</table>
(3) Retrovirus-mediated transduction of mouse primary B cells

IgHEL transgenic mice were injected intraperitoneally with 5mg hen egg lysozyme (HEL; Sigma) in 200ul phosphate buffered saline (PBS; GIBCO) to provide a pulse of antigen for activation in vivo (Goodnow et al. 1988, Reif et al. 2002). The spleens were yielded 6 hours after the injection, and splenocytes were cultured at a density of 4x10^6 cells/ml in complete RPMI containing 10μg/ml anti-CD40 antibody (FGK4.5 from BioXcell). For some experiments, B cells from IgHEL x ML5 HEL double transgenic mice or IgHEL controls were activated using 10μg/ml anti-CD40 (FGK4.5 from Bio X cell) and 10ng/ml IL4 (R&D systems). B cells from wild-type C57BL/6 mice were stimulated using 10μg/ml goat anti-mouse IgM (Jackson ImmunoResearch) and 10ug/ml anti-CD40 (FGK4.5 from Bio X cell). After 24 hours, the cells were spin-infected in 6-well plates at 920 x g for 90 min at room temperature with the retrovirus supernatant containing 10μl/ml DOTAP (Roche). The cells were then cultured in fresh RPMI containing 10μg/ml anti-CD40 for 36 hours. The cultured cells were washed with complete RPMI three times, and resuspended in cRPMI at a density of 1x10^6 cells/ml. The number of live EGFP+ cells was determined by hemocytometer counting of Trypan blue-negative cells in each culture combined with the results from flow cytometric analysis of the same cells.

3.1 Mouse strains used to generate results
Mice were maintained on a C57BL/6 background and were housed in specific pathogen-free conditions at the Australian Phenomics Facility and the John Curtin School of Medical Research. Prdm1^flx/flx^ mice crossed to mb1-Cre and littermate controls were provided by Dr Axel Kallies and Dr Steve Nutt (WEHI). The IL6-deficient mice were kindly provided by Dr Mark Febbraio (IDI Baker; with permission from Prof Manfred Kopf) and the Rag1^-/-^Yc^-/-^ mice were kindly provided by Dr Mark Smyth (PeterMac). All experimental mice were used between 8 and 16 weeks of age. All animals used in this study were cared for and used in accordance with protocols approved by the Australian National University Animal Experimentation Ethics Committee and the current guidelines from the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.
<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Notes</th>
<th>Supplier</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD4</td>
<td>Ig HEL transgenic</td>
<td>Australian Phenomics Facility (APF)</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>MD4 x CD45.1</td>
<td>Ig HEL transgenic</td>
<td>APF</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>MD4 x ML5</td>
<td>Ig HEL double transgenic</td>
<td>APF</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>Tnfaip3&lt;sup&gt;LasVegas&lt;/sup&gt;</td>
<td>ENU-induced Ile325Asn mutation; A20 partial loss of function</td>
<td>APF</td>
<td>Donor cells</td>
</tr>
<tr>
<td>Trp53&lt;sup&gt;Blast&lt;/sup&gt;</td>
<td>ENU-induced Ile195Asn mutation; p53 loss-of-function (10% of wildtype activity: IARC TP53 database)</td>
<td>APF</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>Vav-BCl2</td>
<td>Bcl2-transgenic, originally a gift of Dr S Cory, WEHI</td>
<td>APF</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>Prdm1&lt;sup&gt;b/x&lt;/sup&gt; mb1&lt;sup&gt;Cre&lt;/sup&gt;</td>
<td>Prdm1 deficiency in mature B cells</td>
<td>Dr S Nutt &amp; Dr A Kallies</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>IL6&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>IL6-deficient</td>
<td>Dr M Febrraio</td>
<td>Donor B cells</td>
</tr>
<tr>
<td>Rag1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Rag1 deficiency</td>
<td>APF</td>
<td>Recipients</td>
</tr>
<tr>
<td>Rag1&lt;sup&gt;-/-&lt;/sup&gt; x ML5</td>
<td>HEL-transgenic</td>
<td>APF</td>
<td>Recipients</td>
</tr>
<tr>
<td>Rag1&lt;sup&gt;-/-&lt;/sup&gt; GammaC&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>B cell, T cell and NK cell deficiency</td>
<td>Dr M Smith</td>
<td>Recipients</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Wildtype mice</td>
<td>APF</td>
<td>Donor B cells; controls</td>
</tr>
<tr>
<td>CARD11&lt;sup&gt;ENU 041:027&lt;/sup&gt;</td>
<td>ENU-induced Met365Lys mutation; gain-of-function CARD11</td>
<td>APF</td>
<td>Phenotyping and B cell assays</td>
</tr>
<tr>
<td>Card11&lt;sup&gt;ko/ko&lt;/sup&gt;</td>
<td>Naturally occurring mutation in the intronic region regulating expression of Card11 resulting in complete loss of CARD11 protein expression; Keisuke Horikawa; Unpublished data.</td>
<td>Dr Daniel Grey</td>
<td>Phenotyping and B cell assays</td>
</tr>
</tbody>
</table>
3.2 Culturing splenocytes and activation of primary B cells

Mice were sacrificed by cervical dislocation or carbon dioxide inhalation, and the spleens were collected and kept in 5ml cold RPMI media. Single cell suspensions of the spleens were prepared using a 70μm nylon mesh filter (BD Biosciences) with a 1ml syringe plunger (BD Tuberculin syringe). The cell suspensions were transferred to 15ml tubes and centrifuged for 7 minutes at 1200rpm/ 8°C. The cells were resuspended in ACK Red blood cell lysing solution (Table 11). The ACK solution is a hypertonic solution that causes lysis of the red blood cells present in the cell suspension. The red blood cells were removed from the cell suspension because they interfere with cell counts and also affect flow cytometry analysis as they non-specifically to antibodies.

Table 11: Composition of the ACK red blood cell lysis solution

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Chloride</td>
<td>0.15 M</td>
<td>SIGMA</td>
</tr>
<tr>
<td>Potassium Bicarbonate</td>
<td>10.0 mM</td>
<td>SIGMA</td>
</tr>
<tr>
<td>EDTA-diSodium</td>
<td>0.1 mM</td>
<td>Ajax Finechem (UNIVAR)</td>
</tr>
<tr>
<td>500 ml MilliQ water</td>
<td>Adjust pH to 7.2-7.4 with 1.0 M HCl</td>
<td></td>
</tr>
</tbody>
</table>

The mixture was filter sterlised using the Millipore Express Plus 73mm/0.22μm system.

After 3 minutes incubation in ACK solution at room temperature, 5mL complete RPMI media was added to the cell suspension to reduce the hypertonicity of the solution. The cells were then centrifuged at 1200 rpm/8°C, and the pellet was resuspended in 10ml RPMI media. The number of live cells in each cell suspension was counted using exclusion of the membrane-impermeable dye, 0.04% Trypan blue dye (SIGMA) in a haemocytometer.

The splenocytes were centrifuged at 1200rpm/8°C and resuspended in an appropriate volume RPMI media, to obtain a final concentration of 2x10⁶ cells per ml of media. B cells from wild-type C57BL/6 mice were stimulated using 10μg/ml anti-IgM (Jackson ImmunoResearch) and 10ug/ml anti-CD40 (FGK4.5 from BioXcell). B cells from IgHEL mice were activated in vivo with 5mg HEL injected
intraperitoneally, followed by anti-CD40 addition in culture. B cells from IgHEL x ML5 HEL double transgenic mice or IgHEL controls were activated using 10μg/ml anti-CD40 (FGK4.5 from Bio X cell) and 10ng/ml IL4 (R&D systems). After 24 hours, the cells were spin-infected in 6 well plates 5ml of the cell suspension (total of 10x10^6) was transferred into each well of a flat bottom 6 well culture plate (Costar). The plates were incubated at 37°C/ 5% CO₂ for 24 hours.

3.3 Transducing B cells with retrovirus particles

Approximately 24 hours after incubation with LPS, the cells were collected from the 6 well plates using a P1000 pipette. The collected cells were centrifuged at 1200rpm/20°C, and resuspended in 10ml complete RPMI media in a 50ml tube. The total number of live cells in the cell suspension was determined using 0.04% Trypan Blue dye (SIGMA) and a haemocytometer. The cells were then centrifuged at 1200rpm/20°C, and resuspended in an appropriate volume of complete RPMI media to obtain a concentrated cell suspension at 40x10^6 cells per ml.

Meanwhile, the frozen stocks of retrovirus particles from each transduction group were gently allowed to thaw at room temperature and 2ml of the retrovirus supernatant were transferred into each well of a 6 well culture plate. 100μl of the concentrated cell suspension (total of 4x10^6 cells) were transferred into the wells of the 6 well plates containing the retrovirus supernatant. 20μl DOTAP liposomal transfection reagent (Roche) was added to the cell/retrovirus supernatant suspension, and the plates were rocked gently for appropriate mixing. DOTAP improves transduction rates by promoting interaction between the retroviral particles and the cells in the suspension (DOTAP product manual, Roche). The retrovirus and cell mixture was centrifuged for 90 minutes at 2800 rpm at 30°C (spin-infection or spinoculation).

Upon completion of the spinoculation step, the cells were collected from the wells of the 6 well plate and transferred into 15ml tubes containing 5ml complete RPMI media at 37°C. The cell suspension was then centrifuged for 7 minutes at 1200rpm at 30°C, and resuspended in complete RPMI media supplemented with 10μg/ml anti-IgM (Jackson ImmunoResearch) and 10ug/ml anti-CD40 (FGK4.5 from Bio X cell). The resuspended cells were transferred into 6 well culture plates and
incubated at 37°C/ 5% CO₂ for about 36 hours. This allows sufficient time for the proviral RNA to enter B cells, where the RNA is reverse transcribed to proviral DNA (Lech and Somia 2008). The proviral DNA is unable to enter the cell nucleus and integrate into a host chromosome until the nuclear membrane breaks down during M-phase of cell cycle (Lech and Somia 2008).

3.4 Safety precautions when working with retrovirus particles
In addition to the guidelines for working in a Biosafety Level 2 lab, the following measures were taken:

(a) Retrovirus particles were produced and handled in designated biosafety tissue culture hoods and incubators.
(b) Retrovirus particles were produced using Phoenix packaging cells expressing an ecotropic mouse retrovirus receptor that is unable to transduce human cells (ATCC: CRL-3214).
(c) The production, storage and use of retrovirus particles were recorded on the lab spreadsheet.
(d) Contaminated wastes and cell culture flasks were incubated with 10% bleach solution for at least 30 minutes.

(4) Functional and molecular assays with transduced B cells

4.1 In vitro culture B cell survival and growth assays
36 hours following spinoculation, the cells were harvested from the culture plates into 15ml tubes and the cell suspensions were centrifuged for 7 minutes at 1200rpm/25°C. The cells were resuspended in 5ml fresh RPMI media, without anti-IgM and anti-CD40. This was the first wash step to remove the previously added stimuli. The total number of live cells in the cell suspension was determined using 0.04% Trypan Blue dye (SIGMA) and a haemocytometer. The cells from each transduction group were centrifuged for 7 minutes at 1200rpm/25°C, and the cells were resuspended in an appropriate volume of complete RPMI media to obtain a final concentration of 1x10⁶ cells per ml. This was the second wash step to remove any residual mitogens that could have remained in the cell suspension. 1x10⁶ cells
in 1ml of the cell suspension from each transduction group was transferred to each well of several 24 well plates, and incubated at 37°C/ 5% CO₂.

Each transduction group was cultured in at least triplicate wells. At several time points over a period of 3-5 days, the cultured B cells were collected in a 1.5ml microfuge tube, and centrifuged for 3 minutes at 1200rpm/25°C. The cells were then resuspended in 200μl FACS solution (10% Bovine Serum; 0.005% Sodium Azide in PBS). The total number of viable B cells in triplicate cultures was determined by using a hemocytometer and Trypan Blue dye exclusion counts. In addition, flow cytometry was used to measure the percentage of live transduced B cells present in the cultures.

### 4.2 Cell Trace Violet labeling of B cells for in vitro proliferation assays

Approximately 20x10⁶ cells were labeled with Cell Trace Violet (CTV; Invitrogen) using the following steps:

1. Prepare 5mM stock of CTV by adding 20ul DMSO to vial.
2. Resuspend 1x10⁷ to 5x10⁷ cells in 1ml complete RPMI in a 1.5ml tube.
3. Add 4ul 5mM CTV to the side of the 1.5ml tube containing cells.
4. Mix CTV and cell suspension and vortex immediately to ensure uniform distribution.
5. Incubate at room temperature for 5 minutes.
6. Wash cells three times using complete RPMI.
7. Culture cells at a density of 1x10⁶/ml complete RPMI (with or without proliferation stimuli).

Note that for proliferation assays with activated B cells that were retrovirally transduced, the cells were labeled with CTV 36 hours after the spinoculation step. For proliferation assays involving primary B cells, the cells were labeled with CTV prior to culture with mitogens.

### 4.3 Adoptive transfer into recipient mice

The transduced cells were recovered 36-40 hours after spin-infection with retroviral particles. 5x10⁶ total cultured B cells, consisting of a mixture of transduced and non-transduced B cells, were transferred into recipient mice.
through the lateral tail vein. The recipient mice were age-matched $Rag1^{-/-}$ mice (non-transgenic) and/or $Rag1^{-/-}$ mice expressing a soluble HEL transgene as self-antigen (HEL-transgenic). The spleens of the recipients were yielded for analysis of the donor cells. Except where noted otherwise, all mice used were 8-16 weeks old.

### 4.4 Sorting EGFP+ B cells by FACS

Retrovirus-transduced B cells were harvested in 15ml tubes 36 hours following spinoculation. The cell suspensions were centrifuged for 7 minutes at 1200rpm/25°C, and the cells were resuspended in an appropriate volume of PBS supplemented with 1% FCS (sorting media) to obtain a final concentration of 10x10^6 cells per ml. 7AAD- EGFP+ B cells were FACS sorted into RPMI media supplemented with 50% FCS using FACSARia I and II (BD Biosciences). Dr Harpreet Vohra and Mr Mick Devoy performed the FACS sorting.

### 4.5 Gene expression analysis

Retrovirus-transduced B cells were harvested 36 hours following spinoculation, FACS sorted and resuspended in Trizol reagent (Invitrogen). Phase separation was performed by the addition of chloroform and centrifugation at 12,000 g for 15 min, followed by isopropanol RNA precipitation. The air-dried RNA pellet was dissolved in RNase-free water, and mRNA expression was measured on Affymetrix mouse ST 1.0 arrays as per the manufacturer's instructions by the BRF staff, JCMSR, ANU.

### 4.6 Western blotting

Cultured B cells were washed twice with complete RPMI and cultured in the absence of anti-CD40 for 24 hours. Transduced EGFP positive B cells were sorted using FACSARia I and II (BD Biosciences), and washed with ice-cold PBS followed by lysis in SDS sample buffer (0.2M Tris-HCl pH 6.8, 10% SDS, 30% Glycerol, 10% β-mercaptoethanol, and Bromophenol blue). Lysates were sonicated for 15 min at 5 sec intervals (Branson Digital Sonifier), boiled in SDS sample buffer for 5 min at 95°C and centrifuged for 1 min at 13000 rpm. Proteins were resolved on 10% or 15% polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Non-specific binding to the membrane was blocked with 5% w/v bovine serum albumin in TBST (10mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% v/v Tween20) for 30 min followed by probing overnight at 4°C with antibody to
phospho-JNK (Cell Signaling), total JNK (Cell Signalling), phospho-NF-κB p65 (Cell Signaling), total NF-κB p65 (1:1000 dilution; #3034; Cell signaling), CARD11 (Cell Signaling), and IKKα/β (Santa Cruz). After incubation in primary antibody, membrane was washed three times in TBST and incubated with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (Cell Signaling) for 1 hour at room temperature. The membrane was then washed five times in TBST before detection using enhanced chemiluminescence (ECL) detection reagent (Perkin Elmer) and developed in dark room on to Kodak films. Membranes were reprobed with antibody to α/β tubulin (Cell Signaling) as a loading control. James Wang performed most of the Western blotting experiments.

4.7 Enzyme-linked immunosorbent assay
96-well plates were coated with HEL (10μg/ml; Sigma) for the anti-HEL ELISA, or rat anti-mouse IgM (0.5μg/ml; BD Pharmingen) and rat anti-mouse IgG1 (0.5μg/ml; BD Pharmingen) for the wild-type B cell transduction experiments in sodium carbonate buffer, pH 9.6, and blocked them with 1% BSA (Sigma). Serial dilutions of serum from recipient mice were transferred to the wells. After incubation and washing, plates were further incubated with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotechnology) for the anti-HEL IgM ELISA, or with biotin rat anti-mouse IgM (BD Pharmingen) followed by streptavidin alkaline phosphatase (Vector Labs) and alkaline phosphatase goat anti-mouse IgG1 (BD Pharmingen). After washing, the amount of enzyme bound to each well with the alkaline phosphatase substrate nitrophenyl phosphate was measured. The absorbance of the colored reaction product was read at 405nm by a 96-well plate reader (THERMOMax, Molecular Devices Corp.). Control sera were used to generate standard calibration curves and the relative units or amounts of test antibody present in sera were determined. IL6 ELISA was done using a detection kit from eBioscience.

4.8 Cell surface self-reactivity assay and Hep2 assay for the detection of autoantibodies
(i) Diluted sera (1:5 or 1:50) were incubated on HEp-2 substrate slides (INOVA), and bound antibodies were detected using goat anti-mouse IgG conjugated to
Alexa Fluor 488 (Invitrogen). Slides were viewed and imaged using an Olympus IX 71 microscope.

(ii) 3T3 cells were incubated with 1:10 diluted sera for 30 min on ice, washed twice and bound IgM or IgG antibodies detected by APC rat anti-mouse IgM (BD Pharmingen) and Alexa Fluor 488 goat anti-mouse IgG respectively (Molecular Probes). Cells were analyzed on an LSR II (BD Biosciences).

4.9 Transduction of bone marrow derived stem cells

GPE86+ producer cells were transduced with retrovirus particles containing genes encoding for EGFP, wild-type CARD11, CARD11Mut10 and CARD11Mut12 to establish stable retrovirus producing cells (Holst et al. 2006). The GPE86+ cells expressing high levels of EGFP were FACS sorted, expanded in culture and stored at -80°C in 10% DMSO in FCS. Bone marrow from wild-type mice were harvested from the femur and tibia, and cultured in complete DMEM supplemented with 20% FCS, 20ng/ml mouse IL3, 50ng/ml human IL6 and 50ng/ml mouse SCF (all from Peprotech) in 15cm plates (Thermo Scientific) at a density of 4x10^6 cells/ml. Stably transduced GPE86+ cells were irradiated at 1200 rads from an X-ray source (X-RAD 320, Precision X-ray, Inc) 24 hours after culturing bone marrow cells. The irradiated GPE86+ cells were plated onto 15cm plates at a density of 8x10^6 cells/plate in DMEM with 20% FCS. 24 hours after plating the GPE86+ cells, the cultured bone marrow cells were collected and 20x10^6 cells were co-cultured with the monolayer of the GPE86+ cells in complete DMEM supplemented with 20% FCS, 20ng/ml mouse IL3, 50ng/ml human IL6 and 50ng/ml mouse SCF for 48 hours. Rag1−/− recipients were irradiated at 450 rads and 2x10^6 cultured bone marrow cells were transferred by tail vein injection to reconstitute the recipients. Blood from recipients were obtained through orbital bleeds at 15 and 50 days post-reconstitution for flow cytometry analysis of blood cell populations.

(5) Flow cytometry

Single cell suspensions were prepared and then counted by Trypan blue exclusion. Equal numbers of cells were transferred in 96-well round-bottomed plates. Cells
were then incubated for 30 min at 4°C with an antibody ‘cocktail’ containing the appropriate combination of antibodies, each diluted to its optimal concentration in flow cytometry buffer (PBS containing 2% (vol/vol) bovine serum and 0.1% (wt/vol) Na\textsubscript{3}). Samples were washed twice, resuspended in flow cytometry buffer and were analyzed with an LSR II or LSR Fortessa (BD Biosciences). The following antibodies from BD Pharmingen were used: anti-B220 (RA3-6B2), CD25 (PC61), IgM\textsuperscript{a} (DS-1), CD19 (1D3), CD4 (RM4-5). 7-AAD and Qdot 605 streptavidin conjugate from Invitrogen, CD44 (IM7) from BioLegend, and IgM (II/41) from eBioscience were also used. HyHEL9 conjugated to Alexa Fluor 647 with a Monoclonal Antibody Labeling kit (Molecular Probes). Staining with HyHEL9 was done on cells pre-incubated with 50ng/ml HEL (SIGMA). Anti-BIM antibody (clone 3C5, a kind gift from Prof Andreas Strasser) was conjugated to Alexa Fluor 647 (Molecular Probes). For intracellular staining, a kit from BD was used to fix and permeabilise cultured B cells. Total IκB\textalpha and phospho-cJUN levels were measured using antibodies from Cell Signalling (kindly provided by Dr Ian Parish). FlowJo (Tree Star) was used for analysis of flow cytometry data.

(6) Statistics

In most cases, One-Way ANOVA followed by pairwise comparisons with Bonferroni post tests were performed. For situations where two groups were were analysed, the Mann-Whitney test was performed. GraphPad Prism 5 software was used for all statistical analysis, and differences were taken to be significant when \( P<0.05 \).
Chapter 3: From humans to mice - investigating the immediate consequences of lymphoma-associated CARD11 mutations

Introduction

CARD11 mutations investigated in this study

Experimental system used to introduce CARD11 mutations into mouse B cells

Lymphoma CARD11 mutants provide a growth advantage to activated B cells

B cells expressing CARD11 mutants increase in size and proliferate in culture

CARD11 mutants cause dose-dependent changes in expression of NF-κB responsive genes

Expression of CARD11 mutations results in lower IκB-α and increased phospho-cJUN levels

Combination of a gain-of-function CARD11 mutation with an ENU-induced loss-of-function Card11 mutation affects growth of activated B cells in vitro

Discussion

Fig 3.1. Characteristics of CARD11 mutations studied

Fig 3.2. Experimental system to study CARD11 mutations in vitro

Fig 3.3. Lymphoma CARD11 mutants provide a growth advantage to activated B cells

Fig 3.4. B cells expressing CARD11 mutants increase in size and proliferate in culture

Fig 3.5. CARD11 mutants cause dose-dependent changes in CD25, CD86 and B220

Fig 3.6. Expression of CARD11 mutations results in lower IκB-α and increased phospho-cJUN levels

Fig 3.7. Combination of a gain-of-function lymphoma-derived CARD11 mutation with an ENU-induced loss-of-function mouse Card11 mutation affects growth of activated B cells in vitro
Introduction

CARD11 is expressed in lymphocytes and functions as a scaffold for the recruitment of signalling partners BCL10 and MALT1 into a protein complex, which is essential for relaying signals from the B cell antigen receptor to NF-κB and AP-1/cJUN transcription factors (Bertin et al. 2001, Gaide et al. 2001, McAllister-Lucas et al. 2001, Blonska and Lin 2009, Thome et al. 2010, Blonska and Lin 2011, Qiao et al. 2013). Activation of the BCR-NF-κB signalling pathway is essential for B cells to respond to antigens (Thome et al. 2010, Blonska and Lin 2011). In mice expressing loss-of-function CARD11 or which do not express CARD11, B cells cannot mount protective responses as proliferation and differentiation following antigen-BCR engagement is severely impaired (Egawa et al. 2003, Hara et al. 2003, Jun et al. 2003, Newton and Dixit 2003).

In elegant studies by Staudt’s group, the CARD11-BCL10-MALT1 (CBM) complex was found to be essential for the survival in tissue culture of activated B cell-like diffuse large B cell lymphoma (DLBCL) cells, a group of aggressive lymphomas characterised by constitutive activation of NF-κB (Ngo et al. 2006). Gain-of-function mutations in CARD11 were subsequently found to be the driver of NF-κB activity in approximately 10% of the ABC DLBCL subtype of lymphoma (Lenz et al. 2008). The CARD11 mutations were not restricted to the ABC subtype as 3% of GC DLBCL cases were also found to have gain-of-function CARD11 mutations (Lenz et al. 2008). However, the frequencies of CARD11 mutations in these subtypes of DLBCL is likely to vary due to sampling effects as other groups reported different frequencies of CARD11 mutations in DLBCL (Morin et al. 2011, Pasqualucci et al. 2011, Lohr et al. 2012, Morin et al. 2013, Zhang et al. 2013). Interestingly, most of the lymphoma mutations in CARD11 were in exons 4 to 12, which encode for the coiled-coil domains of the protein (Lenz et al. 2008, Shaffer et al. 2012).

The coiled-coil domain of CARD11 is crucial for the activation of the protein to function as a scaffold (Jun et al. 2003, Tanner et al. 2007, McCully and Pomerantz 2008). The coiled-coiled domain has also been proposed to be form oligomers with other CARD11 proteins binding to give rise to membrane associated molecular platforms (Thome 2004, Rawlings et al. 2006). The lymphoma CARD11 mutations
have been proposed to disrupt the folding of the CARD11 protein, exposing the ‘hidden’ coiled-coil domain to allow formation of self-oligomers and facilitate binding to BCL10 (Lamason et al. 2010). Microscopy studies indicated that the mutant proteins formed macroscopic aggregates, when introduced in cell lines (Lenz et al. 2008). The mutant forms of the proteins also increased signalling to NF-κB transcription factors, through activation of the IKK complex (Lenz et al. 2008).

Moreover, expression of the mutant forms of CARD11 were found to be essential for the survival of several DLBCL cell lines, as shRNA-mediated knock-down of expression of the mutated form of CARD11, followed by enforced expression of the wild-type form of the protein, killed the cells in culture (Lenz et al. 2008). These CARD11 mutations are thus considered as ‘driver’ mutations, which have been hypothesised to arise in a pre-neoplastic B cell clone before accumulating and selecting for sufficient mutations to transform into bona fide lymphoma (Lenz and Staudt 2010, Rui et al. 2011). However, the consequence of acquiring these lymphoma-derived CARD11 mutations in normal B cells, in isolation from the other ~30 mutations accumulated on average in lymphoma cells (Morin et al. 2011, Pasqualucci et al. 2011, Lohr et al. 2012, Morin et al. 2013, Zhang et al. 2013) had not been addressed.

**CARD11 mutations investigated in this study**

In this results chapter, we investigate the immediate consequences of the acquisition of a range of CARD11 mutations in mouse primary mature activated B cells. The mutations studied in this chapter were chosen to represent regions of CARD11 that are frequently mutated in lymphoma, and specific amino acid residues that are conserved between human and mouse CARD11 [Figure 3.1a; (Shaffer et al. 2012)]. The numbers indicate the CARD11 mutations as originally described by (Lenz et al. 2008): mutations 2, 3, 6 and 10 (Table 3.1). Mutation 2 was found in the ABC DLBCL cell line OCI-LY3, whereas mutations 3 and 6 were found in primary ABC DLBCL samples (Lenz et al. 2008). Mutation 10 was chosen because it represented the addition of three nucleotides, resulting in the insertion
of an isoleucine at position 232 and was found in a case of GC DLBCL (Lenz et al. 2008). Mutation 13 was found in a study by (Compagno et al. 2009) in a case of ABC DLBCL, and it was of interest as it affected the CARD-domain of CARD11, but whether this mutation has similar gain-of-function effects as the more common coil-coiled domain mutants had not yet been determined. The mutation referred to as ‘Unmod’ is an ENU-induced mouse mutation, which was discovered by (Jun et al. 2003) in a study, which was one of the first to describe the function of CARD11 in B cells. The ‘Unmod’ allele results from a single amino acid substitution in the coiled-coil domain resulting in a partial loss-of-function CARD11 protein (hypomorphic allele): the mutation is hypothesised to stabilise the ‘inactive’ conformation of CARD11. All the lymphoma-acquired mutations resulted in amino acid changes, which either changed the charge or polarity of the residue. These charge or polarity changes in the coil-coiled domain of CARD11 have been hypothesised to affect folding of CARD11 such that a more ‘activated’ conformation is achieved (Lamason et al. 2010).

**Experimental system used to introduce CARD11 mutations into mouse B cells**

To introduce the human lymphoma-derived CARD11 mutations into mouse primary B cells, we developed a retrovirus gene transfer and culture strategy that allowed the tracking of transduced (EGFP+) and non-transduced (EGFP-) B cells present in the same culture (Figure 3.2a). IgHEL-transgenic B cells were initially activated *in vivo* through an intraperitoneal injection with 5mg hen egg lysozyme (HEL) dissolved in 200 μl of PBS. The spleen was prepared into a single cell suspension of lymphocytes and these were cultured with 10μg/ml anti-CD40 monoclonal antibody to provide a T cell-help ‘mimic’ signal for the antigen-activated B cells [Methods Section 3- Note that previous experiments established that 6 hours was sufficient to activate Ig^HEL^-B cells *in vivo*; (Reif et al. 2002)]. Using a plate centrifugation method called “spinoculation” [Methods Section 3, (Reif et al. 2002)], the dividing B cells were transduced with ecotropic retroviral particles containing replication-defective proviral RNA encoding for CARD11 mutant proteins. Two types of retroviral vectors were used in the experiments: (i) *CARD11-IRES-EGFP* vector which produced CARD11 and EGFP as two separate
proteins from a single, bicistronic mRNA; or (ii) *EGFP-CARD11* vector which produced a CARD11 fusion protein with EGFP added at the N-terminus of the CARD11 protein sequence. The products of these two vectors produced comparable effects when introduced in B cells (Fig 3.1 b). B cells retrovirally transduced with wild-type CARD11 or an empty vector expressing EGFP only were used as controls. After spinoculation and further culture with the retroviral particles and anti-CD40 for 24 hours, the cells were washed and cultured in fresh medium without anti-CD40 at a density of 1x10^6/ml in media only in triplicate wells. The time of placing the cells in fresh medium, removed from exogenous anti-CD40 growth stimulation, is described as Day 0. The cells were collected at 24 hours intervals from Day 0 to Day 4 for the analysis of numbers of EGFP+ cells and expression of cell-surface markers. Flow cytometry analysis was done on 7AAD-B220+ activated B cells using the gating strategy illustrated in Figure 3.1c.

**Lymphoma CARD11 mutants provide a growth advantage to activated B cells**

Upon culture without an exogenous growth stimulus (no anti-CD40), the numbers of viable 7AAD- B220+EGFP+ activated B cells expressing EGFP only (empty vector) or wild-type CARD11 (CARD11\textsuperscript{WT}) decreased gradually over 4 days relative to the starting numbers at Day 0. By contrast, EGFP+ B cells expressing the lymphoma *CARD11* mutations either increased in number or remained steady over time (Fig 3.3a, b).

The EGFP+ subset exhibited much higher EGFP fluorescence per cell when transduced with empty IRES-EGFP vector compared to those transduced with CARD11-IRES-EGFP vectors. This presumably reflects lower rates of EGFP-translation initiation from the IRES downstream from the CARD11 coding region, compared to when EGFP is the only coding region and does not require translation-reinitiation from the IRES. By contrast, no appreciably difference in the distribution of EGFP per cell was discernable between vectors encoding CARD11\textsuperscript{WT} or CARD11\textsuperscript{Mut} (Fig 3.3a).
When the proteins were expressed as N-terminal EGFP-CARD11 fusion proteins, this also resulted in a much lower range of EGFP-fluorescence per cell compared to transduction with a control vector encoding unmodified EGFP (Fig 3.3 a, d). This may reflect less efficient folding, fluorescence, or stability of the EGFP-CARD11 fusion protein. Nevertheless, in cells with EGFP-CARD11 fusion proteins that differed only by presence or absence of the various point mutations, the level of EGFP expressed in each B cell should provide a direct measure of the relative amount of EGFP-CARD11 protein in that cell compared to any other cell in the same or parallel samples. On Day 0, the distribution of EGFP-CARD11 fluorescence in B cells transduced with the wild-type CARD11 fusion protein vector was not discernably different from those transduced with CARD11 mutant fusion protein vectors (Fig 3.3d). Moreover, when cells with matched levels of EGFP-CARD11 fluorescence were selectively gated, many of the CARD11 mutations caused a significant increase in mean cell size as measured by forward scatter (FSC) of laser light, both at Day 0 and at Day 1 (Fig 3.3e). Hence the early effects on B cell population size are unlikely to be due to differences in the relative expression of CARD11 caused by the lymphoma mutations, but instead appear to reflect differences in the activity of the expressed wild-type and mutant proteins.

The effects of CARD11 mutants on the number of EGFP+B220+7AAD- activated B cells could be grouped into three categories: (1) mutants which caused a 3-fold or greater increase in cell numbers (e.g. Mut10, Mut3); (2) mutants which caused an increase of approximately 2-fold in cell numbers (e.g. Mut 2, Mut13), and (3) mutants which either maintained B cell numbers or did not provide an apparent survival advantage to activated B cells in the absence of external stimuli (Mut 6). The numbers of B cells expressing CARD11 mutations 2 and 10 increased by 3- and 3.5-fold over 4 days, indicating that CARD11Mut12 and CARD11Mut10 provided the B cell with the strongest survival and growth advantage (Fig 3.3b). Interestingly, the effects of CARD11Mut13, altering the CARD-domain, were comparable to the coil-coiled domain mutant CARD11Mut3, as the expression of both mutants resulted in an increase in the B cell numbers by approximately 2-fold over the time course (Fig 3.3b). Based on the population cell count analysis, CARD11Mut6 seemed to provide a survival advantage to the activated B cells as the numbers were maintained just above the relative number of B cells expressing wild-type CARD11.
at Days 3 and 4 (Fig 3.3b). The non-transduced EGFP-B220+7AAD- present in the same cultures decreased in numbers over the 4-day time-period. The absence of an effect on EGFP- cells indicated that the growth and survival observed in the EGFP+ B cells was a cell-autonomous effect of CARD11 mutant proteins within individual transduced B cells, and not secondary to production of a growth factor capable of acting on non-transduced cells in the same culture (Fig 3.3c).

**B cells expressing CARD11 mutants increase in size and proliferate in culture**

To determine the effects of CARD11 mutations on cell growth, we compared the mean fluorescence intensity (MFI) FSC-A at Day 1. At this time-point, the cultured B were withdrawn from anti-CD40 stimulation for 24 hours. On average, B cell expressing CARD11\textsuperscript{Mut2} and CARD11\textsuperscript{Mut10} had the highest FSC-A MFI, indicating that the greatest increase in cell size (Fig 3.4a). B cells expressing the other CARD11 mutants were not different in FSC-A MFI compared to B cells expressing wild-type CARD11 at this time-point. This result suggest that the increase in cell size with only CARD11\textsuperscript{Mut2} and CARD11\textsuperscript{Mut10} at Day 1 correlates with the increase in B cell numbers over the 4 day period (Fig 3.3). Interestingly, enforced expression of wild-type CARD11 caused B cells to increase in FSC-A MFI compared to B cell expressing EGFP only, indicating that the signalling events leading to increase in cell size may be sensitive to changes in the levels of CARD11 protein.

To further explore this possibility, we subdivided CARD11\textsuperscript{Mut10} and CARD11\textsuperscript{WT} EGFP+ expressing B cells into ‘low’, ‘medium’ and ‘high’ categories, with the gates selected such that the same number of events are analysed in each group (Fig 3.4b). Note that these experiments were done with EGFP-CARD11 fusion proteins such that EGFP+ ‘low’, ‘medium’ and ‘high’ categories would correspond to B cells expressing ‘low’, ‘medium’ and ‘high’ amounts of CARD11 protein. The expression level of gain-of-function CARD11\textsuperscript{Mut10} influenced FSC-A MFI, as cell size increased gradually from the EGFP ‘low’ to the EGFP ‘high’ group. These results suggest that there is a dose-dependent relationship between cell size and the level of CARD11\textsuperscript{Mut10} expressed. This dose-dependent effect, however, did not extend to CARD11\textsuperscript{WT}-expressing B cells (Fig 3.4b).
Next, we asked whether the CARD11-expressing B cells divide in culture. To track cell division, the B cells were loaded with the Cell Trace Violet (CTV) reagent at the time of withdrawal of anti-CD40 (Day 0) and dilution of the dye analysed by flow cytometry after 3 days in culture with media only. A broad range of CTV fluorescence was observed (e.g. 10<sup>2</sup> to 10<sup>4</sup> units for CARD11<sup>Mut10</sup>) and it was not possible to resolve discrete populations with half the amounts of CTV fluorescence. This probably resulted from heterogeneity in cell cycle status and cell volume in the activated B cells at the time of CTV loading (Fig 3.4c). Nevertheless, B cells expressing EGFP only (EV) or wild-type CARD11 (WT) had the same distribution and average fluorescence intensity for CTV, indicating that the CARD11 WT expression did not induce activated B cells to divide. Using the average CTV MFI values from the EV and WT control groups, a model for the number theoretical cell divisions was generated, with the assumption that at each cell division B cells will contain approximately half the amount of CTV they contained prior to division (Fig 3.4c). The average CTV MFI from B cells expressing the mutant versions of CARD11 were interpolated from the model curve to determine the approximate number of cell divisions the cells have gone through over a period of 3 days. On average, CARD11<sup>Mut10</sup>-expressing B cells divided 3 times, CARD11<sup>Mut2</sup> and CARD11<sup>Mut13</sup>-B cells divided 2 times, while the expression of CARD11<sup>Mut3</sup> and CARD11<sup>Mut6</sup> resulted in a single round of cell division (Fig 3.4c). Moreover, there was no evidence that the B cells which express high levels of CARD11<sup>Mut10</sup> protein divided more, despite a slight reduction in the CTV MFI in the EGFP ‘high’ expressing B cells compared to the EGFP medium’ and EGFP ‘low’ populations (Fig 3.4d). These results indicate that all CARD11 mutants are able to induce at least one round of proliferation over a 3-day period. The ability to induce more rounds of proliferation correlated with the EGFP+ B cell count results, as the mutants inducing more rounds of proliferation accumulated larger numbers of B cells over time (Fig 3.3).
**CARD11 mutants cause dose-dependent changes in expression of NF-κB responsive genes**

To measure the consequences of NF-κB activation in the CARD11 mutant-expressing B cells, we analysed the expression levels of CD25 and CD86 24 hours after withdrawal of the transduced B cells from anti-CD40 (Day 1; Fig 3.1a). Both CD25 and CD86 are regulated by the NF-κB transcription factors (Ballard et al. 1988, Zou and Hu 2005). CD25 is the high-affinity receptor for the pro-survival cytokine IL2, and CD86 is a cell surface protein expressed on activated B cells, which is involved in interactions with T cells (Leonard et al. 1983, Leonard et al. 1984, Jeannin et al. 1997). CARD11<sup>Mut2</sup>- and CARD11<sup>Mut10</sup>-B cells expressed an average of 2 fold and 3-fold more CD25 compared to B cells transduced with wild-type CARD11 (Fig 3.5a). B cells expressing other CARD11 mutants had comparable levels of CD25 as the wild-type control cells. However, the levels of CD86 were increased on the surface of B cells expressing the CARD11 gain-of-function mutants, except for CARD11<sup>Mut6</sup>-B cells (Fig 3.5b). CARD11<sup>Mut3</sup>- and CARD11<sup>Mut13</sup>-B cells expressed 2-fold more CD86, while CARD11<sup>Mut2</sup>- and CARD11<sup>Mut10</sup>-B cells expressed 3 to 4-fold more CD86 compared to B cells transduced with wild-type CARD11 (Fig 3.5b). We further examined the phenotypic changes induced by expression of CARD11 mutations in activated B cells by measuring the levels of B220, a cell surface protein expressed on B cells, which is down-regulated as B cells differentiate into plasmablasts (Fairfax et al. 2008). NF-κB transcription factors also regulate the expression of IRF4 and BLIMP1, which are required for plasma cell differentiation (Turner et al. 1994, Shapiro-Shelef et al. 2003, Fairfax et al. 2008, Martins and Calame 2008). The expression of B220 was reduced on the surface of B cells by approximately 1.5-fold by Mut3 and Mut13 and 4-fold by Mut2 and Mut10, compared to the wild-type control cells (Fig 3.5c). Interestingly, the level of B220 on B cells expressing CARD11<sup>Mut6</sup> was not reduced (Fig 3.5c).

B cells expressing wild-type CARD11 expressed slightly increased levels of CD25 and CD86, and lower levels of B220 compared to the Empty vector control B cells, indicating that NF-κB activation may be sensitive to changes in the levels of CARD11 protein expressed (Fig 3.5a, b, c). We, thus, subdivided CARD11<sup>Mut10</sup> EGFP+ expressing B cells into ‘low’, ‘medium’ and ‘high’ categories, and measured
the levels of CD25 and B220 (Fig 3.5d, e). The expression of CD25 increased gradually from the EGFP ‘low’ to the EGFP ‘high’ group, whereas the expression of B220 decreased from the EGFP ‘low’ to the EGFP ‘high’ group (Fig 3.5d, e). These results suggest that, akin to the relationship observed with cell size (Fig 3.4b), there is a dose-dependent relationship between the levels of CD25 and B220, and the level of CARD11Mut10 expressed in activated B cells. These findings indicate that gain-of-function CARD11 mutants cause dose-dependent changes in the levels of CD25, CD86 and B220.

Expression of CARD11 mutations results in lower IκB-alpha and increased phospho-cJUN levels

These phenotypic changes in CD25, CD86 and B220 expression may arise as a consequence of transcription factor activation in the transduced B cells, given that CARD11 is known to positively regulate signalling to the NF-κB and cJUN transcription factors (Bertin et al. 2001, Gaide et al. 2001, McAllister-Lucas et al. 2001, Thome et al. 2010, Blonska and Lin 2011). Once activated, CARD11 recruits BCL10 and MALT1 to form helical fibers that activate the IKK kinase complex and MKK7 (Bertin et al. 2001, Gaide et al. 2001, McAllister-Lucas et al. 2001, Blonska and Lin 2009, Thome et al. 2010, Blonska and Lin 2011, Qiao et al. 2013). IKK activates the NF-κB family of transcription factors by phosphorylating the inhibitory protein, IkappaB alpha (IκBα), which holds NF-κB proteins in the cytoplasm (Scheidereit 2006). Once phosphorylated, IκBα proteins are tagged for proteosomal degradation by the addition of K-48 linked polyubiquitin chains. IκBα also forms part of a negative feedback regulating NF-κB signalling, as increased NF-κB activity results in increased transcription of mRNA encoding IκBα (Scheidereit 2006). In lymphocytes, mitogen-activated kinase 7 regulates the CARD11-mediated activation of JNK, which in turn phosphorylates and activates the cJUN transcription factor (Blonska et al. 2007, Blonska and Lin 2009). To directly measure the consequences of the gain-of-function lymphoma CARD11 mutants on NF-κB and JNK signalling, we measured the total levels of IκBα and the levels of phospho-cJUN by intracellular staining and flow cytometry analysis of transduced activated B cells at Day 1 (Fig 3.1a ; Fig 3.6).
Intracellular fluorescent staining with an antibody to IκBα was reduced in B cells expressing all the CARD11 mutants compared to cells expressing the CARD11 wild-type control (Fig 3.6a, b). On average, CARD11Mut10 B cells had 1.5-fold lower IκBα, whereas the other mutants had a more modest reduction in IκBα levels compared to the wild-type control B cells (Fig 3.6b). Interestingly, B cells expressing wild-type CARD11 contained approximately 1.2-fold higher levels of IκBα compared to the empty vector controls. While it would be important to verify these findings by Western blotting, one possible explanation for the apparent difference between CARD11 mutants and the wildtype CARD11 is that increased expression of wild-type CARD11 was sufficient to activate some increase in NF-κB-induced IκBα (Nfkbia) mRNA but was insufficient to induce phosphorylation and degradation of the resulting IκBα protein. The increase in NF-κB activity promoted by enforced CARD11WT may thus be counter-acted by increased expression of the IκBα.

Compared to the control B cells expressing wild-type CARD11, intracellular fluorescent staining for phospho-cJUN was increased in B cells expressing all the CARD11 mutants, except for CARD11Mut6 (Fig 3.6c, d). In B cells expressing CARD11Mut10, for instance, the levels of p-cJUN were approximately 1.5-fold higher compared to the wild-type control (Fig 3.6d). While lower than cells with mutant CARD11, staining for phospho-cJUN was nevertheless increased in B cells expressing wild-type CARD11 compared to the empty vector controls (Fig 3.6d). This suggests that increased CARD11 expression may be sufficient to increase JNK activity. However the overall shifts in fluorescence in these experiments are subtle, and lack specificity controls to establish the origin of the increased fluorescent signals with certainty. Western blotting of sorted EGFP+ cells would be important to verify these conclusions. Western blotting experiments conducted by James Wang, a PhD candidate in our lab, confirmed the findings that signaling to NF-κB and JNK pathways are increased in B cells expressing CARD11Mut10 (Fig 3.6f).

To determine whether varying the levels of mutant CARD11 protein would influence signalling to NF-κB and JNK, we measured the levels of IκBα and p-cJUN in CARD11Mut10 EGFP+ expressing B cells subdivided into ‘low’, ‘medium’ and ‘high’ categories. The levels of IκBα decreased gradually from the EGFP ‘low’ to the EGFP...
‘high’ group, whereas the levels of p-cJUN did not change from the EGFP ‘low’ to the EGFP ‘high’ group (Fig 3.6e). These findings indicate that signalling to NF-κB may be more sensitive than JNK signalling to changes in the levels of CARD11\textsuperscript{Mut10} protein. Taken together, these results suggest that the gain-of-function CARD11 mutants activate NF-κB and JNK signalling.

**Combination of a gain-of-function CARD11 mutation with an ENU-induced loss-of-function Card11 mutation affects growth of activated B cells in vitro**

The increase in signalling through the IKK complex and JNK kinase indicate the potential for the use of small molecule inhibitors to inhibit the growth and survival of B cell lymphomas with CARD11 gain-of-function mutations (Manning and Davis 2003, Karin et al. 2004). However, given that IKK and JNK signalling are important in almost all cell types, we sought to gain insight into whether developing CARD11-specific inhibitors could be useful in CARD11-driven disease. We used a genetic approach to combine a lymphoma-derived gain-of-function CARD11 mutation (Mut10) with a partial loss-of-function ENU-induced Card11 mutation (Unmod) discovered in mice (Jun et al. 2003). The CARD11\textsuperscript{Unmod} variant is encoded by a T to A substitution, which results in the change from a leucine to a lysine at position 305 of the protein. This change from a nonpolar to a basic residue did not alter the accumulation of CARD11 protein in B cells (Jun et al. 2003) and has been hypothesized to alter the folding of the coiled coil domain such that it may not adopt a ‘fully’ active conformation in response to phosphorylation. From previous experiments, we have seen that CARD11\textsuperscript{Mut10} is one of the strongest, if not, the strongest gain-of-function variant of CARD11 as B cells which express CARD11\textsuperscript{Mut10} expand in culture, have higher levels of CD25 and CD86 expression, and have increased signalling to NF-κB and JNK pathways. Here, we specifically asked whether superimposition of the Unmod mutation would cancel out the growth-inducing effects of CARD11\textsuperscript{Mut10}. Note that these experiments were done using CARD11-IRES-EGFP bicistronic vectors.

Over a period of four days in culture, the number of B cells expressing CARD11\textsuperscript{Mut10} increased by approximately 8-fold, while the number of non transduced (EGFP-)
and B cells expressing wild-type or Unmod CARD11, EGFP only decreased in comparison to the starting numbers (Fig 3.7b, c). Interestingly, the combination of the Unmod variant with CARD11\textsuperscript{Mut10} variant resulted in a 3.8-fold decrease in the relative numbers of B cells (Fig 3.7a, b). B cells expressing the Unmod+Mut10 version of CARD11 had a reduced FSC-A mean fluorescence intensity, indicating that these B cells have a reduced size compared to B cells expressing CARD11\textsuperscript{Mut10} (Fig 3.7d). In addition, the expression of CARD11\textsuperscript{Unmod+Mut10} induced less surface CD25 expression compared to CARD11\textsuperscript{Mut10}. B cells, suggesting that NF-κB signalling may be affected in B cells expressing the double mutant (Fig 3.7e). These results indicate that the growth-inducing effects of a gain-of-function CARD11 variant remain susceptible to an inhibitory modifier of the coiled-coil domain. If small molecules could be identified with similar inhibitory activity to the Unmod Leu294Lys substitution, these might be particularly suited to suppress lymphoma growth.

Discussion

From the results presented in this chapter, we have seen that enforced expression of CARD11 bearing various lymphoma point mutations is sufficient on its own to provide a growth advantage to activated B cells, which increase in size and proliferate in culture. How these CARD11 mutant proteins give rise to these growth-inducing effects is an interesting question that is yet to be addressed. The expression of NF-κB-responsive regulators of cell division such as c-MYC and cyclin D could be measured in B cells expressing the CARD11 mutants (Duyao et al. 1990, Wang et al. 1996, Guttridge et al. 1999). In addition, given that the CARD11 mutants seemed to provide a survival advantage to the activated B cells, the expression of NF-κB-regulated pro-survival factors such as A1, Bcl-xL and Bcl2 could also be measured (Grumont et al. 1999, Lee et al. 1999, Zong et al. 1999, Catz and Johnson 2001).

Even though we have observed increase signaling to JNK and NF-κB pathways in activated B cells expressing mutant CARD11, the range of detection of total IκB-α and phospho-cJUN by flow cytometry was quite narrow (Fig 3.6). These small
changes may represent the actual change in the levels of signaling occurring in the cells, but are also likely to reflect the limitations of the sensitivity of the detection method and may also be influenced by the cell-to-cell variation in levels of the proteins measured in activated B cells. Thus, to control the levels and kinetics of CARD11 expression, an inducible and reversible expression system could be used. For instance, CARD11 could be fused to a destabilizing domain (DD), which targets it for degradation (Banaszynski and Wandless 2006). The protein could be then stabilised by the addition of a ligand that binds the DD and inactivates it (Banaszynski and Wandless 2006). One recently engineered system makes use of a DD from *E. coli* dihydrofolate reductase and is stabilized by the DHFR inhibitor trimethoprim (Iwamoto et al. 2010).

In resting B cells, CARD11 resides in a latent form in the cytoplasm, and upon antigen receptor engagement, CARD11 is phosphorylated by PKCβ in a “linker” region residing between its coiled-coil and MAGUK domains (Matsumoto et al. 2005, Sommer et al. 2005). Phosphorylated CARD11 relocalises to the plasma membrane, where it recruits a multiprotein complex consisting of MALT1, BCL10, TRAF6, TAK1, caspase8, and c-Flip (McCully and Pomerantz 2008). The coiled-coil domain mediates several essential functions in CARD11 (Tanner et al. 2007). We have seen that point mutations in the coiled-coil can enhance or reduce the function of the protein (Jun et al. 2003, Lenz et al. 2008). Moreover, certain mutations in the coiled-coil domain impair its self-association, whereas others block membrane localization of CARD11 (Tanner et al. 2007).

The coiled-coil domain appears to be negatively regulated by the linker/inhibition domain, as deletion of this region results in a constitutively active form of CARD11 (Matsumoto et al. 2005, Sommer et al. 2005). Functional studies indicated that the linker/inhibitory domain may fold back on the coiled-coil domain, inhibiting its function (Lamason et al. 2010). Thus, oncogenic CARD11 mutations were found to disrupt the interactions of the coil-coiled domain and linker/inhibitory domain, thus bypassing the requirement for phosphorylation of CARD11 (Lamason et al. 2010). The gain-of-function mutations were also found to bind to more BCL10, but whether this is due to the formation of larger CARD11 scaffolds or an increase in the stability of CARD11-BCL10 interactions remains unclear (Lamason et al. 2010).
In addition, we found that a gain-of-function CARD11 CARD-domain mutant (Mut13) had comparable growth-inducing effects as CARD11 coil-coiled domain mutants, suggesting that the activity of mutant CARD11 can be enhanced by mutations in both the coil-coiled and CARD domains. Consistent with these findings, it was found that an isolated linker peptide can associate with a peptide consisting of the amino-terminal CARD and coiled-coil domains (McCully and Pomerantz 2008).

Interestingly, we observed that the combination of CARD11Mut10 with an ENU-induced loss-of-function mouse Card11 mutation reduced the growth of activated B cells in vitro (Fig 3.7). Future experiments could investigate how the gain-of-function CARD11 mutations disrupt the normal folding, activation, localisation and recruitment of signaling molecules by CARD11. Addressing how the ‘Unmod’ mutation interferes with the function of CARD11 would also provide valuable insights into the properties of CARD11-specific small molecule inhibitors, which could be developed for the treatment of CARD11-driven disease. The importance of therapeutic targeting of CARD11 was also proposed by (Lenz et al. 2008), as it was found that overexpression of an isolated coiled-coil domain killed ABC DLBCL cell lines with either wild-type or mutant CARD11 (Lenz et al. 2008). To complement these functional studies, a structural analysis of CARD11 will be required to determine how a small molecule might interfere with the function of the protein.
**Fig 3.1.** Characteristics of *CARD11* mutations studied. Schematic of CARD11 protein illustrating the CARD, coil-coiled, PDZ, SH3 and GUK domains. The amino acid sequence of the first 480 residues of wild-type CARD11 is given in the box, with the CARD domain sequences in blue and coil-coiled domain sequences in green. The amino acids highlighted in red are mutated in cases of Diffuse Large B cell lymphoma, while the residue in green (bold; L305) was mutated creating a partial loss of function in the ENU-mutant *unmodulated* mouse strain.

Table 3.1 describes the CARD11 mutations investigated in this result chapter.
Table 3.1 Characteristics of mutations studied

<table>
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<th>CARD11 mutation</th>
<th>Δ nucleotide</th>
<th>Δ amino acid</th>
<th>Δ hydrophobicity side chain</th>
<th>Reference</th>
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<tr>
<td>13</td>
<td>G to A</td>
<td>C49Y</td>
<td>Nonpolar to uncharged polar</td>
<td>Compagno et al Nature 2009</td>
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<tr>
<td>3</td>
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<td>G123S</td>
<td>Nonpolar to uncharged polar</td>
<td>Lenz et al Science 2008</td>
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<tr>
<td>2</td>
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<td>Nonpolar to nonpolar</td>
<td>Lenz et al Science 2008</td>
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<tr>
<td>Unmod</td>
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<td>L305Q</td>
<td>Nonpolar to basic</td>
<td>Jun et al Immunity 2003</td>
</tr>
<tr>
<td>6</td>
<td>A to T</td>
<td>D387V</td>
<td>Acidic to nonpolar</td>
<td>Lenz et al Science 2008</td>
</tr>
</tbody>
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Fig 3.2. Experimental system to study CARD11 mutations in vitro. (a) Outline of the experimental strategy to examine the consequences of acquiring different CARD11 mutations in normal antigen-activated B cells. (b) Comparison of the effects on fold population expansion at Day 3, FSC-A and CD25 expression at Day 1 of expressing CARD11Mut10 from two types of retroviral vectors that were used in the experiments: (i) CARD11-IRES-EGFP vector (labeled 10*) which produced CARD11 and EGFP as two separate proteins from a single, bicistronic mRNA; (ii) EGFP-CARD11 vector (labeled 10) which produced a CARD11 fusion protein with EGFP added at the N-terminus of the CARD11 protein sequence. (c) Gating strategy used to analyse CARD11-transduced live B cells at each time point in the in vitro growth assay. The numbers on the plots indicate the percentage of the population of interest.
(a) 

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<table>
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<tr>
<th>LTR</th>
<th>EGFP</th>
<th>CARD11</th>
<th>LTR</th>
</tr>
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</table>
and/or

<table>
<thead>
<tr>
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<th>CARD11</th>
<th>IRES</th>
<th>EGFP</th>
<th>LTR</th>
</tr>
</thead>
</table>

```

- Acute pulse HEL antigen (~ 6 hours)
- Ig^HEL^ transgenic
- Culture splenocytes (Day -3)
  - + anti-CD40
- Activated B cells (Day -2)
  - + anti-CD40
- Mixture EGFP+ & EGFP- (Day 0)
- Culture without antigen or anti-CD40
- Cell counts and flow cytometry analysis (Days 0-4)

(b) 

- #EGFP+ cells at Day 3 (relative to starting at Day 0)
  - EV WT 10^* 10

- FSC-A MFI (x10^6)
  - EV WT 10^* 10

- CD25 MFI (x10^6)
  - EV WT 10^* 10

(c) 

- SSC-A
  - FSC-H
  - SSC-H

- 7AAD
  - FSC-A

- B220
  - EGFP
Fig 3.3. Lymphoma CARD11 mutants provide a growth advantage to activated B cells. (a) Flow cytometry plots analyzing the EGFP+ B cells transduced with the indicated retroviral vectors at different time-points. The numbers on the plots indicate the percentage of the EGFP+ population. Relative number of live (b) EGFP+ and (c) EGFP- B cells in tissue culture without antigen or anti-CD40 for five days, expressed as percentage of the starting number on Day 0. (d) Representative histogram overlay and corresponding scatter plot of the EGFP MFI in B cells transduced with CARD11 fusion protein vector at Day 0. (f) FSC-A MFI measured on a slice of narrow expression of EGFP (indicated in red on the dot plots) measured at Day 0 and Day 1. One-way ANOVA followed by Bonferroni post-test was used for statistical analysis. Numbers are mean ± SEM of 3 independent cultures, and data are representative of 3 independent experiments. EGFP-CARD11 vectors, which produced a CARD11 fusion protein with EGFP added at the N-terminus of the CARD11 protein sequence, were used for these experiments.
**Fig 3.4.** B cells expressing CARD11 mutants increase in size and proliferate in culture. (a) Representative histogram overlay of forward scatter area (FSC-A) and corresponding column graph of the geometric mean fluorescence intensity (MFI) of FSC-A of cultured B cells expressing the indicated vectors at Day 1 (n=3 separate cultures of transduced cells with each vector; experiment repeated 3 times). (b) FSC-A profiles and MFI of CARD11\textsuperscript{Mut10} and CARD11\textsuperscript{WT} B cell populations expressing low, medium and high levels of EGFP at Day 1 (n=3 separate cultures of transduced cells with each vector; experiment repeated 3 times). (c) Representative histogram overlay of fluorescence from Cell Trace Violet (CTV), and corresponding graph of a model constructed based on the theoretical CTV MFI per cell division, with the average CTV MFI of cultured B cells expressing the indicated vectors at Day 3 (n=3; experiment repeated 2 times). (d) CTV profiles of CARD11\textsuperscript{Mut10} B cell populations expressing low, medium and high levels of EGFP-CARD fusion protein, and corresponding column graph of the CTV MFI at Day 3 (n=3; experiment repeated 2 times). *EGFP-CARD11* vectors, which produced a CARD11 fusion protein with EGFP added at the N-terminus of the CARD11 protein sequence, were used for these experiments.
**Fig 3.5.** CARD11 mutants cause dose-dependent changes in CD25, CD86 and B220. Representative histogram overlay of (a) CD25, (CD86) and (c) B220, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of EGFP+ B cells expressing the indicated vectors in individual cultures at Day 1 (n=3 independent cultures of cells transduced with each vector; experiment repeated 3 times). (d) CD25 and (e) B220 profiles of CARD11\textsuperscript{Mut10} B cell populations expressing low, medium and high levels of EGFP, and corresponding column graph of the average MFI in each culture at Day 1 (n=3; experiment repeated 3 times). EGFP-CARD11 vectors, which produced a CARD11 fusion protein with EGFP added at the N-terminus of the CARD11 protein sequence, were used for these experiments.
**Fig 3.6.** Expression of *CARD11* mutations results in lower IκB-α and increased phospho-cJUN levels. (a) Representative flow cytometry plots of total IκB-α levels in B cells expressing the indicated vectors at Day 1. (b) Representative histogram overlay of IκB-α, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of cultured B cells expressing the indicated vectors at Day 1 (n=3; experiment repeated 3 times). (c) Representative flow cytometry plots of phospho-cJUN levels in B cells expressing the indicated vectors at Day 1. (d) Representative histogram overlay of phospho-cJUN, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of cultured B cells expressing the indicated vectors at Day 1 (n=3; experiment repeated 3 times). (e) IκB-α and phospho-cJUN profiles of *CARD11*<sup>Mut10</sup> B cell populations expressing low, medium and high levels of EGFP, and corresponding column graph of the average MFI at Day 1 (n=3; experiment repeated 3 times). (f) Western blot for phospho/total p65 and phospho/total JNK in cell lysates from EV, WT, *CARD11*<sup>Mut10</sup>, and constitutively active IKK-β (IKK-β*)–expressing B cells. Cells cultured in media without anti-CD40 for 24 h before EGFP+ cells were sorted [Note that this figure was contributed by James Wang]. *EGFP-CARD11* vectors, which produced a *CARD11* fusion protein with EGFP added at the N-terminus of the *CARD11* protein sequence, were used for these experiments.
[Contributed by James Wang]
**Fig 3.7.** Combination of a gain-of-function lymphoma-derived *CARD11* mutation with an ENU-induced loss-of-function mouse *Card11* mutation affects growth of activated B cells *in vitro*. (a) Flow cytometry plots analyzing the EGFP+ population B cells expressing the indicated vectors at Day 0 and Day 4. The numbers on the plots indicate the percentage of the EGFP+ population. Relative number of live (b) EGFP+ and (c) EGFP- B cells in tissue culture without antigen or anti-CD40 for five days, expressed as percentage of the starting number on Day 0. (d) FSC-A MFI and CD25 MFI of B cells expressing the indicated vectors at Day 1. Numbers are mean ± SEM of 3 independent cultures, and data are representative of 2 independent experiments. *CARD11-IRES-EGFP* vector which produced CARD11 and EGFP as two separate proteins from a single, bicistronic mRNA were used for these experiments.
Chapter 4: Gain-of-function CARD11 induce self-limiting 
expansion and plasmablast differentiation in vivo

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Introduction

Non-Hodgkin's B cell lymphomas highjack the features of normal B cells for their survival, suggesting that the function of such neoplasms depends on the differentiation stage of the B cells from which they originate (Kuppers 2005, Rui et al. 2011). Germinal center B cells can give rise to many types of lymphoma, including diffuse large B cell lymphoma, follicular lymphoma, and Burkitt's lymphoma (Klein and Dalla-Favera 2008). These types of lymphoma seem to bear the imprints of the differentiation program of the normal B cell from which they arise, with additional genetic disruptions in the malignant cells, which overcome normal regulatory programs (Alizadeh et al. 2000, Ranganath and D'Esposito 2001, Klein et al. 2003). ABC DLBCL, for instance, have the plasma cell expression program, with high expression of the transcription factors IRF4 and XBP1 (Shaffer et al. 2004, Klein et al. 2006, Sciammas et al. 2006). However, ABC lymphomas appear to select for genetic lesions that interfere with *Blimp1*, thus preventing terminal differentiation into plasma cells (Shaffer et al. 2000, Pasqualucci et al. 2006, Tam et al. 2006).

A block in terminal differentiation seems to be an important step in the pathogenesis of the ABC DLBCL subtype (Shaffer et al. 2000, Pasqualucci et al. 2006, Tam et al. 2006). However, the nature of the precursor cell from which the lymphoma B cells originate is unclear. ABC DLBCL cells express high levels of AID and the IgH genes expressed have been mutated, but they have not undergone class switch recombination, expressing IgM (Lossos et al. 2000, Pasqualucci et al. 2004, Lenz et al. 2007). Thus, it has been proposed that ABC DLBCL could either originate from IgM positive post-germinal-center memory cells or from a pre-germinal-center activated B cell that expresses AID (Cattoretti et al. 2006, Seifert and Kuppers 2009, Shaffer et al. 2012).

In the previous results chapter, we have seen that gain-of-function *CARD11* mutations result in proliferation, growth and survival when introduced in activated B cells in vitro. The CARD11 mutants also increased signalling to NF-κB and JNK pathways to varying degrees. Here, we use a retrovirus-mediated gene transfer strategy to introduce the strongest gain-of-function, lymphoma-derived *CARD11*Mut10 mutant in pre-germinal centre activated B cells. The genetically
modified B cells were then transferred \textit{in vivo} to investigate the consequences of the acquisition of the CARD11$^{\text{Mut10}}$ version of CARD11 in activated B cells.

\textbf{Gain-of-function CARD11 promotes self-limiting B cell expansion}

We used the retrovirus-mediated gene transfer system described in Chapter 3 to introduce CARD11$^{\text{Mut10}}$ into activated B cells. However, instead of culturing the mixture of transduced and non-transduced B cells, we transferred the B cells into \textit{Rag1}^{-/-} recipients (Fig 4.1a). B cells retrovirally transduced with wild-type CARD11 or an empty vector expressing EGFP only were used as controls. The transferred B cells could be more easily tracked in the \textit{Rag1}^{-/-} recipients as they lacked endogenous mature B cells (Mombaerts et al. 1992). The lack of B cells in these recipients is also likely to increase by more than 10-fold the circulating levels of the B cell survival factor, BAFF (Lesley et al. 2004), with possible functional effects that are discussed later. After transfer of the cultured cells at Day 0, the spleens of the recipients were analysed at different time intervals up to Day 60 (Fig 4.1a). Flow cytometry analysis was done on IgM$^+$ CD4-HEL-binding EGFP$^+$ cells using the gating strategy illustrated in Figure 4.1b.

At the time of transfer (Day 0), there were approximately 3-fold fewer EGFP$^+$ cells in the CARD11$^{\text{WT}}$ and CARD11$^{\text{Mut10}}$ groups compared to the empty vector transduction group (Fig 4.2a). The reduced numbers of CARD11$^{\text{WT}}$ and CARD11$^{\text{Mut10}}$ expressing B cells was a result of lower transduction rates, which are likely to reflect lower titres of transduction-competent retroviral particles because the much larger \textit{Card11}-bearing retroviral genome is close to the packaging size limit for ecotropic type C retroviral particles. However, a comparable frequency and number of cells were transduced with CARD11$^{\text{WT}}$ or CARD11$^{\text{Mut10}}$ at the time of injection into recipient mice. Despite this, 10 days after transfer into \textit{Rag1}^{-/-} recipients there were on average 100-fold more EGFP$^+$ B cells expressing CARD11$^{\text{Mut10}}$ than expressing CARD11$^{\text{WT}}$ B cells in the spleens of the recipients (Fig 4.2b). As a percentage of transferred B cells, EGFP$^+$ CARD11$^{\text{Mut10}}$ B cells increased from 4% at Day 0 to 80% at Day 10, while the percentage of EGFP$^+$ cells in the empty vector or CARD11$^{\text{WT}}$ control groups remained unchanged or decreased slightly over the same time period (Fig 4.2a, b), indicating that B cells expressing the mutant CARD11 selectively proliferated.
Analysis of recipients at an earlier timepoint, 5 days after transfer, there were 10-fold more EGFP+ CARD11$^{\text{Mut10}}$ B cells than EGFP+ CARD11$^{\text{WT}}$ controls, indicating that the CARD11$^{\text{Mut10}}$ expressing B cells had already begun proliferating rapidly in vivo (Fig 4.2c). However, the numbers of B cells expressing control EGFP only and CARD11$^{\text{WT}}$ in the spleens of the recipients also increased between Day 5 and Day 20, although there was little or no accompanying increase in the percentage of these control EGFP+ cells as a fraction of all the transferred B cells (Fig 4.2c). The slow increase in all the transferred B cells, whether transduced with control vectors or not, may result from activated B cells gradually homing to the spleen or from low-level homeostatic proliferation of all activated B cells in response to B cell lymphopenia and high levels of BAFF.

Interestingly, the dramatic proliferation of B cells expressing CARD11$^{\text{Mut10}}$ between days 5 and 10 was rapidly corrected by a decrease in their numbers by a mean of 40-fold (Fig 4.2c). Similarly, the percentage EGFP+ cells decreased from 80% to 25% from Day 10 to Day 21, indicating that the CARD11$^{\text{Mut10}}$ B cells numbers are preferentially culled by a negative feedback mechanism. The numbers and frequency of CARD11$^{\text{Mut10}}$ B cells then remained relatively static from Day 21 to Day 60 (Fig 4.2c). These results indicate that activated B cells expressing CARD11$^{\text{Mut10}}$ undergo an initial burst of proliferation, but this increase in cell numbers is somehow self-correcting either by cell death, migration or differentiation. These findings indicate that enforced expression of a gain-of-function CARD11 mutant protein is not sufficient to give rise to unchecked proliferation and B cell lymphoma-like disease in mice.

**B cells expressing CARD11$^{\text{Mut10}}$ adopt a Blimp1-dependent FSC-A high, B220 low and CD19 low phenotype**

To gain insight into the potential mechanisms underlying the initial expansion and subsequent decrease in CARD11$^{\text{Mut10}}$ B cell numbers, we examined the phenotype of the B cells at Day 10 in vivo. More than 70% of the CARD11$^{\text{Mut10}}$ expressing B cells were larger than the EGFP only control cells, with a 1.5-fold increase in FSC-A
MFI (Fig 4.3a). In addition, the levels of B220 and CD19 were 10-fold and 5-fold lower in CARD11Mut10 B cells compared to the EGFP only control cells (Fig 4.3a). This high FSC-A, low B220 and low CD19 phenotype adopted by the CARD11Mut10 B cells was consistent with differentiation into plasmablasts [Fig 2.3a; (Fairfax et al. 2008)]. At the time of transfer (Day 0), the CARD11Mut10 B cells were already phenotypically different from B cells expressing CARD11WT or EGFP only. B cells transduced with the vector encoding for CARD11Mut10 had higher FSC-A MFI, and expressed lower levels of B220 (Fig 4.3b). Also, as observed in the previous results chapter, the B cells expressing CARD11Mut10 also expressed 1.7-fold more CD25 (IL2RA), an NF-κB-responsive cell surface marker, compared to the control B cells at Day 0 (Fig 4.3b). Interestingly, studies in human B cells indicated that increased IL2-IL2RA signalling may contribute to plasma cell differentiation (Waldmann et al. 1984, Mittler et al. 1985); 22634617). Thus, it is likely that the increase expression of CD25 in this context of CARD11Mut10 B cells also contributes to differentiation of the B cells into plasmablasts, although this remains to be experimentally tested. Moreover, the CARD11Mut10 B cells also expressed higher mRNA levels of the regulators of plasma cell differentiation Prdm1, Irf4 and Xpb1 (Fig 4.3c), supporting the conclusion that the CARD11Mut10 B cells differentiated into plasmablasts in vivo.

Next, we asked whether the FSC-A\textsuperscript{high}B220\textsuperscript{low}CD19\textsuperscript{low} phenotype adopted by CARD11Mut10 B cells depend on the expression of the transcription factor BLIMP1 (encoded by Prdm1), which is essential for the terminal differentiation of B cells into plasma cells (Turner et al. 1994, Shapiro-Shelef et al. 2003, Fairfax et al. 2008, Martins and Calame 2008). Because germline Prdm1 deficiency causes embryonic lethality, we used mice with a conditional B cell-specific Prdm1-deficiency (mb1-\textit{cre} \times Prdm1\textsuperscript{fl/fl}) and littermate controls (Prdm1\textsuperscript{fl/fl}) provided by Dr Axel Kallies and Dr Steve Nutt (WEHI, Melbourne). Since the mice were not Ig-transgenic and the B cells had a polyclonal BCR repertoire, the activation and flow cytometric tracking strategy had to be modified (Fig 4.4a). Spleen-derived B cells were activated using anti-CD40 and anti-IgM, and transduced with retroviral vectors encoding for CARD11Mut10 and EGFP only. The transduced cells were transferred into \textit{Rag1}\textsuperscript{−/−} recipients at Day 0 and the donor cells were analysed in the spleens of the
recipients at Day 10 after transfer. Flow cytometry analysis was done on IgM+/−CD19+/−CD4−EGFP+ cells using the gating strategy illustrated in Figure 4.4b.

At Day 10, CARD11Mut10-expressing polyclonal B cells with wildtype Prdm1 exhibited the same increase in FSC-A and profound decrease in B220 and CD19 that was observed in HEL-specific Ig-transgenic B cells (green histograms and columns in Fig 4.5a). However in the absence of Prdm1, the FSC-A MFI of CARD11Mut10 B cells (red histograms and columns) was reduced to levels comparable to EGFP-only control cells (grey histograms and columns). There was no difference in FSC-A MFI between Prdm1-sufficient (Prdm1fl/fl) or Prdm1-deficient (mb1-cre x Prdm1fl/fl) empty vector transduced B cells. Interestingly, the levels of B220 and CD19 were increased in mb1-cre x Prdm1fl/fl B cells expressing EGFP only compared Prdm1fl/fl B cells transduced with the same vector, indicating that a small fraction of the EGFP only activated B cells expressing BLIMP1 also downregulated B220 and CD19 in vivo (note ‘shoulders’ of low B220 and CD19 expressing B cell populations in histograms; Fig 4.5a). The levels of B220 on Prdm1-deficient B cells were markedly increased on CARD11Mut10-transduced B cells compared to cells in which Prdm1 was expressed, although not to levels of EGFP-only transduced B cells indicating that CARD11Mut10 promotes B220 downregulation by BLIMP1-dependent and independent mechanisms (Fig 4.5a). By contrast, the CD19 levels on CARD11Mut10 Prdm1-deficient B cells were increased to levels comparable to the EGFP-only controls of the same genotype (Fig 4.5a), indicating that BLIMP1 is essential for activated CARD11 to downregulate CD19 expression.

Given that the B220 levels measured on the B cells represent the combined expression of the CD45RA, CA45RB, and CD45RC exons to produce a particular glycosylated isoform of CD45RABC, the decrease in total B220 detected by the CD45R antibody could indicate the disruption of expression of one particular CD45/Ptprc exon. To test for this possibility, we specifically measured the levels of CD45RA and CD45RB epitopes on the surface of the transferred B cells. In B cells with normal Prdm1, CARD11Mut10 decreased the mean CD45RA expression by an average of 34-fold but only decreased CD45RB expression by 2.7-fold (Fig 4.5b). Thus the loss of B220 appears to reflect mostly a change in CD45 splicing,
apparently silencing of the CD45RA-encoding *Ptprc* exon 4 but not exon 5 encoding the CD45RB epitope. In T cells, *Ptprc* exon 4 is preferentially silenced by the activation induced RNA-binding protein, HNRNPLL (Wu et al. 2008). Since Hnrnpll mRNA encoding this protein is greatly increased in plasma cells (Benson et al. 2012), increased expression of this silencing factor as part of the plasma cell differentiation program is likely to explain the selective loss of CD45RA and B220 epitopes on B cells expressing CARD11_mut10.

Like the downregulation of the B220 epitope, the downregulation of CD45RA was markedly attenuated but not abolished in Prmd1-deficient B cells expressing CARD11_mut10 (green histograms and columns in Fig 4.5b). Moreover, in cells expressing EGFP-only vector CD45RA levels were homogeneously increased by Prmd1-deficiency (compare blue with grey histograms). By contrast, the smaller downregulation of CD45RB promoted by CARD11_mut10 was fully abolished in Prmd1-deficient B cells (Fig 4.5b). Thus BLIMP1 mediates the change in CD45 isoform expression brought about by CARD11_mut10, although the silencing of CD45RA and B220 epitopes reflects both BLIMP1-dependent and -independent mechanisms.

**Expression of CARD11_mut10 in BLIMP1-deficient B cells does not result in lymphoma-like disease**

The results above indicated that many of the changes in cellular phenotype that occur in B cells expressing activated CARD11 represent a plasma cell differentiation program largely dependent upon the plasma cell transcription factor BLIMP1. In ~70% of DLBCL, expression or function of BLIMP1 is disrupted by loss-of-function Prdm1 missense mutations, truncations, or gene loss, or BLIMP1 expression is inhibited by increased expression of the transcriptional repressor of Prmd1, BCL6 (Pasqualucci et al. 2006, Tam et al. 2006, Mandelbaum et al. 2010). BLIMP1 represses proliferation of plasmablasts by inhibiting the expression of the NF-κB-regulated proliferation-inducing factor MYC, such that B cells stop dividing and undergo terminal differentiation into plasma cells (Turner et al. 1994, Shaffer et al. 2000, Shaffer et al. 2002). Thus, we investigated whether
the combination of the loss of Prdm1 along with the expression of a gain-of-function CARD11 mutation would be sufficient to allow B cells to expand and proliferate continuously. More specifically, we hypothesized that the decline in CARD11\textsuperscript{Mut10} B cell numbers observed between Day 10 and Day 21 in the spleen of \textit{Rag1}\textsuperscript{-/-} recipients is a result of BLIMP1-mediated suppression of MYC. To test this hypothesis, equal numbers of CARD11\textsuperscript{Mut10} Prmd1-deficient and Prmd1-sufficient polyclonal B cells were transferred into \textit{Rag1}\textsuperscript{-/-} recipients and the numbers of the transferred B cells were analysed over a period of 60 days (Fig 4.6a).

While comparable percentages and numbers of EGFP+ CARD11\textsuperscript{Mut10}-expressing B cells with Prmd1-deficiency or wild-type Prdm1 were transferred on Day 0 (red and green columns in Fig 4.6a), by Day 10 following transfer the numbers of CARD11\textsuperscript{Mut10} Prdm1\textsuperscript{+/} with intact BLIMP1 were on average 10-fold higher than CARD11\textsuperscript{Mut10} Prdm1-deficient cells, and their frequency was twice as high among transferred B cells (Fig 4.6b). CARD11\textsuperscript{Mut10} Prdm1-deficient cells still increased dramatically relative to EGFP-only control B cells, reaching 10-times higher mean numbers on Day 10 despite one tenth as many cells being transferred. Thus BLIMP1 is not required for CARD11\textsuperscript{Mut10} to induce proliferation \textit{in vivo}, but BLIMP1-mediated plasmablast differentiation apparently further promotes accumulation of CARD11\textsuperscript{Mut10} B cells instead of curtailing it as had been hypothesised.

Also contrary to the hypothesis of Prdm1 acting to curtail proliferation was the finding that from Day 18 to Day 58 after transfer the numbers of CARD11\textsuperscript{Mut10} Prmd1-deficient B cells did not continue to increase in the spleens of the recipients, but instead followed a similarly dramatic decline in percentage and total number to the CARD11\textsuperscript{Mut10} Prmd1-sufficient B cells (Fig 4.6c). These results suggest that the combination of a gain-of-function CARD11 mutant with absence of Prdm1 in activated B cells are not sufficient, own their own, to result in lymphoma-like disease in mice, and that other mechanisms exist to self-correct the CARD11-driven burst of proliferation.
**IL6 production by CARD11^{Mut10} B cells contributes to plasmablast differentiation**

IL6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis (Kishimoto 2005, Kishimoto 2010). In B cells, IL6-STAT3 signalling plays a role in B cell activation and plasma cell differentiation, and interestingly this cytokine was initially isolated as 'B cell stimulatory factor 2' (Kishimoto 2005, Kishimoto 2010). In addition to its role in normal B cell responses, IL6 can also contribute to the formation of B cell malignancies (Kishimoto 2010, Shaffer et al. 2012). Mice engineered to express high levels of IL6 develop plasmacytomas and by the age of 18 months about 29% of the transgenic mice also develop follicular lymphomas and DLBCL (Suematsu et al. 1989, Kovalchuk et al. 2002). In humans, high levels of IL6 production and active STAT3 signalling were found to be a feature of a subset of cases of ABC DLBCL (Ding et al. 2008, Lam et al. 2008). Production of a viral form of IL6 by Human Herpesvirus 8 is responsible for the plasma cell-type multicentric Castleman's Disease (Suthaus et al. 2012). Thus, autocrine IL6-STAT3 may provide survival signals to lymphoma cells (Hirano et al. 2000, Ding et al. 2008, Lam et al. 2008, Shaffer et al. 2012, Zhang et al. 2012).

From an analysis of transcriptome changes induced by CARD11^{Mut10} in activated B cells, *Il6* was found to be the most highly upregulated gene in CARD11^{Mut10} B cells compared to EGFP only control B cells (Fig 4.7a). To validate the microarray results and verify whether B cells transduced with CARD11^{Mut10} were producing more IL6, EGFP+ B cells were sorted and cultured in media only. Equal numbers of CARD11^{Mut10} and EGFP only controls were cultured, and the culture supernatants were collected 24 hours later. On average, 7 times more IL6 were detected in the culture supernatants of CARD11^{Mut10} B cells, compared to the EGFP only control culture supernatants (Fig 4.7a).

Next, we investigated whether the production of IL6 by CARD11^{Mut10} B cells transferred *in vivo* affected the plasmablast differentiation of the transferred cells. B cells from the spleens of *Il6*-deficient mice [(Kopf et al. 1994); Obtained from Dr Marc Febbraio, Baker IDI, Melbourne; with the permission from Dr Manfred Kopf]
and wild-type control mice were stimulated with anti-IgM and anti-CD40 in culture, and the activated B cells were transduced with vectors encoding for CARD11\textsuperscript{Mut10} and EGFP only (Empty vector). The B cells were transferred into \textit{Rag1}^{-/-} recipients and analysed 10 days after transfer. Irrespective of the presence or absence of IL6, B cells expressing CARD11\textsuperscript{Mut10} accumulated to similar numbers by Day 10 (Fig 4.7b). However, the \textit{Il6}-deficient CARD11\textsuperscript{Mut10} B cells had a different phenotype compared to the \textit{Il6}-sufficient B cells expressing CARD11\textsuperscript{Mut10}, as approximately 50% of the CARD11\textsuperscript{Mut10} \textit{Il6}^{-/-} B cells had lower FSC-A MFI, and expressed more B220 and CD19 compared to the wild-type cells expressing the same vector (Fig 4.7c). These results indicated that autocrine IL6 production promotes the plasmablast-differentiated phenotype acquired by CARD11\textsuperscript{Mut10} B cells \textit{in vivo}. When the EGFP+ \textit{Il6}-deficient and \textit{Il6}-sufficient populations were further subdivided into three groups based on CD19 and B220 expression, on average 13% of CARD11\textsuperscript{Mut10} \textit{Il6}^{-/-} B cells adopted a B220^{+/high} CD19^{+/high} undifferentiated B cell phenotype compared to a mean of 5% of CARD11\textsuperscript{Mut10} IL6-wild-type B cells (Fig 4.7d). The mean proportion of partially differentiated B cells expressing intermediate levels of B220 and CD19 was also significantly increased in the absence of autocrine IL6 production (21% \textit{Il6}-deficient v/s 3.5% \textit{Il6}-sufficient CARD11\textsuperscript{Mut10} B cells; Fig 4.7d). Reciprocally, the mean percentage of fully differentiated plasmablasts with the phenotype B220^{low/-} CD19^{low/-} was significantly reduced from 77% in B cells with intact IL6 to 49% in the absence of IL6 (Fig 4.7d). These results indicate that autocrine IL6 production by CARD11\textsuperscript{Mut10} B cells promotes their differentiation into plasmablasts, but does not discernably affect the number of B cells accumulating in the spleens at Day 10.

\textbf{CARD11\textsuperscript{Mut10} cooperates with loss-of-function A20}

Elevated NF-κB signalling as the result of enforced expression of CARD11 in activated B cells increases the expression of negative feedback regulators such as IκB-α and A20, which function to dampen activation of the NF-κB transcription factors (Vallabhapurapu and Karin 2009). The loss of \textit{TNFAIP3}, which encodes the A20 protein, commonly occurs through loss-of-function mutations or deletions in approximately 20% of MALT lymphomas, 30% of Hodgkin’s lymphoma and 25% of
ABC DLBCL cases (Compagno et al. 2009, Kato et al. 2009). Here, we examined the impact of the combination of a partial loss-of-function A20 mutation with gain-of-function CARD11^{Mut10} on activation and expansion of B cells, specifically testing whether the decline in numbers observed between Day 10 and Day 21 is affected. *Tnfaip3* mutant B cells were isolated from the *lasvegas* mouse strain, which carries an ENU-induced partial loss-of-function allele resulting from a single T>A mutation in *Tnfaip3* exon 6 that substitutes asparagine at residue 325 in place of an isoleucine found in the OTU domain of A20 (Horikawa, K et al. manuscript in preparation). Unlike mice homozygous for a *Tnfaip3* null allele, which die shortly after weaning from a lethal MyD88 and macrophage-mediated inflammatory syndrome (Lee et al. 2000, Turer et al. 2008), homozygotes for the *lasvegas* mutation are healthy as adults so that they provide a source of resting spleen B cells that appear phenotypically normal.

At the time of transfer (Day 0), the average FSC-A of CARD11^{Mut10} *Tnfaip3^{LasV/LasV}* B cells was slightly increased compared to wild-type B cells transduced with the same vector. Interestingly, the EGFP- non-transduced *Tnfaip3^{LasV/LasV}* B cells were significantly larger than their EGFP- wild-type counterparts, and also expressed higher levels of CD25, indicating that the partial loss-of-function A20 resulted in hyper-responsiveness to anti-CD40 (Fig 4.8a). Above the effect of the *Tnfaip3* mutation seen in EGFP-negative cells, still higher levels of CD25 were expressed by the EGFP+ CARD11^{Mut10} *Tnfaip3^{LasV/LasV}* B cells, and these levels were also higher than CD25 on wild-type B cells expressing the CARD11^{Mut10} vector (Fig 4.8a). These results suggest that the *Tnfaip3^{LasV/LasV}* B cells expressing CARD11^{Mut10} were highly activated at the time of transfer from additive effects of the *Tnfaip3* mutation and CARD11^{Mut10}.

At Day 12 after transfer, the numbers of CARD11^{Mut10} *Tnfaip3^{LasV/LasV}* B cells accumulated to a mean of 3.3 times more than the wild-type B cells expressing CARD11^{Mut10} (Fig 4.8b). The difference between *Tnfaip3* mutant and wildtype B cells became larger by day 21, because the wild-type B cells numbers decreased by 84% whereas the mean number of *Tnfaip3* mutant cells had decreased by only 33% (Fig 4.8b, c). The number of CARD11^{Mut10} *Tnfaip3^{LasV/LasV} B cells in the spleen nevertheless declined gradually from Day 21 to Day 60 (Fig 4.8c). These results
indicate that \textit{Tnfaip3/A20} contributes to the rapid loss of cells after the initial \textit{CARD11}-induced burst of proliferation \textit{in vivo}. While the loss-of-function \textit{A20} mutation cooperated with gain-of-function \textit{CARD11}^{\text{Mut10}} to maintain B cell numbers in the spleen at Day 21, it was not sufficient to allow sustained proliferation or produce lymphomas by Day 60 \textit{in vivo}.

\textbf{Discussion}

\textbf{NF-κB} activation through the classical pathway is a hallmark of the ABC DLBCL subtype (Alizadeh et al. 2000). The survival of most ABC DLBCL cell lines depends on the CBM complex, a signaling hub consisting of \textit{CARD11}, BCL10, MALT1, and other proteins (Ngo et al. 2006). The CBM complex is required for the activation of IKKβ and the classical NF-κB pathway downstream of the antigen receptors in B cells (Thome et al. 2010). We have previously seen that the lymphoma-derived \textit{CARD11} mutants have the ability to activate NF-κB and JNK signalling, and promote proliferation of activated B cells \textit{in vitro} (Chapter 3). Here, we have shown that activated B cells, which acquire the gain-of-function \textit{CARD11}^{\text{Mut10}}, proliferate rapidly and differentiate into plasmablasts when transferred in recipient mice. However, this initial rapid population expansion is self-limiting: it peaked at day 10 and by 20-days \textit{in vivo} the numbers of \textit{CARD11}^{\text{Mut10}} expressing B cells decreased drastically in the recipients. The experiments here test some of the possible mechanisms that drive plasmablast differentiation and cause their accumulation to be so dramatically self-limiting.

\textbf{BLIMP1} is specifically induced during plasma cell differentiation and essential for that process. Three lines of evidence indicate that \textbf{BLIMP1/PRDM1} is also a tumour suppressor in B cell malignancy. First, BLIMP1 represses expression of MYC, which itself is essential for B cell proliferation (Lin et al. 1997). Second, in a substantial fraction of ABC DLBCL tumors the \textit{PRDM1} gene is disrupted by truncation, deletion, and mutation, or it is epigenetically silenced (Pasqualucci et al. 2006, Tam et al. 2006, Mandelbaum et al. 2010). Third, deletion of \textit{Prdm1} in mouse germinal center B cells results in a lymphoproliferative disease with marginal zone hyperplasia that gives rise to DLBCL-like disease (Calado et al. 2010, Mandelbaum et al. 2010). Moreover, when \textit{Prdm1}-deficient mice are crossed to transgenic mice expressing a
constitutively active form of IκB kinase (IKKβ), ABC DLBCL-like lymphomas are observed (Calado et al. 2010). However, clonal B cell lymphomas are observed only after a long latency, suggesting that accumulation of other cooperating genetic lesions is required (Calado et al. 2010).

Together these findings made it very attractive to hypothesise that the cessation of CARD11Mut10-driven B cell accumulation and the dramatic loss of progeny after day 10 was due to induction of BLIMP1 and differentiation into short-lived plasma cells. In a simple tumour suppressor model, it was expected that combining increased NF-κB activity and a block in terminal plasma cell differentiation would be sufficient on its own to produce ABC DLBCL. We tested this simple model by expressing CARD11Mut10 in Prdm1-deficient activated B cells. The experiments showed that CARD11Mut10 does induce BLIMP1-mediated plasmablast differentiation in most of the progenies that accumulate by day 10. But contrary to the hypothesis, the Prdm1-deficient B cells accumulated to lower numbers than Prdm1-wildtype B cells and their decline between days 10 and 20 was just as steep. No outgrowth of continuously dividing cells was observed by Day 60 following transfer of the cells in recipient mice. These findings indicate that induction of BLIMP1 and plasmablast differentiation is not the primary mechanism that curtails the proliferation of B cells with activated CARD11 in vivo. Additional regulatory mechanisms must be at play, which need to be disrupted to give rise to transformed, continuously proliferating B cells.

While BLIMP1 deficiency did not prevent the sudden disappearance of CARD11Mut10 B cells after day 10, the lasvegas partial loss-of-function mutation in Tnfaip3/A20 did delay the loss of cells on day 21 as well as increasing the numbers at the peak on day 10. Moreover, TNFAIP3 is more frequently inactivated in ABC-DLBCL by mutations in one or both alleles than PRDM1 (Compagno et al. 2009). Taken together, these results favour the conclusion that a primary self-limiting circuit in CARD11Mut10 – induced proliferation is induction of the NF-κB negative regulator Tnfaip3/A20. We reached a similar conclusion in parallel studies of the transient and much more rapidly self-termination burst of B cell proliferation induced by the oncogenically activated allele of MyD88L265P (Wang et al. 2014). In those experiments, the Tnfaip3LasV mutation delayed the sudden decrease in dividing progeny apparently by preventing Bim-mediated apoptosis (Wang et al.
For proliferation induced by oncogenic CARD11 or MyD88, the mutant \textit{Tnfaip3}^\text{LatV} allele only delayed the loss of progeny. Since it is only a partial loss of function, and in many lymphomas there is homozygous complete loss of the TNFAIP3 gene, it is possible that the residual A20 function mediates the eventual loss of dividing B cells. This could be addressed by repeating the experiments in B cells from mice with a conditional deletion of \textit{Tnfaip3} in B cells. Alternatively, it is possible that \textit{Prdm1}-induced differentiation represents a secondary self-correcting mechanism when \textit{Tnfaip3} is no longer sufficient. Testing this would require complex breeding experiments to produce B cells with conditional inactivation of both \textit{Tnfaip3} and \textit{Prdm1}.

The experiments in this chapter also reveal that autocrine production of IL-6 plays a significant role in driving plasmablast differentiation in CARD11$^{\text{Mut10}}$-expressing B cells, but not in the accumulation of cells. Interestingly, IL-6 was originally identified as B cell differentiation-inducing factor 2 as it was involved in mediating the terminal differentiation of B cells (Hirano et al. 1986, Muraguchi et al. 1988). Early experiments by Kishimoto’s group showed that addition of IL-6 to cultured activated B cells induced immunoglobulin secretion, but did not affect the growth of the B cell population (Muraguchi et al. 1988). The promoter region of the \textit{Il6} gene in B cells contains binding sites for the NF-κB, AP-1 and C/EBP transcription factors, among which the NF-κB binding region apparently play the dominant role in CD40-mediated expression of IL-6 (Libermann and Baltimore 1990, Shimizu et al. 1990, Vanden Berghe et al. 1999, Baccam et al. 2003). Moreover, the NF-κB activation of IL-6 expression seems to occur in cooperation with c-Jun (Xiao et al. 2004). Thus, the activation of both NF-κB and c-Jun in the CARD11$^{\text{Mut10}}$-expressing B cells may explain the increased expression of \textit{Il6} mRNA (Chapter 3- Fig 3.6; Chapter 4- Fig 4.7a).

Once IL-6 is produced by the CARD11$^{\text{Mut10}}$ transduced B cells, it may act as an autocrine signalling factor in a similar fashion to multiple myeloma cells (Kawano et al. 1988). The binding of IL-6 to its receptors IL-6R and gp130 triggers signaling through the recruitment of the JAK kinase, which binds to and activates the transcription factor STAT3 (Yamasaki et al. 1988, Murakami et al. 1993, Lutticken et al. 1994). IL-6 through STAT3 is sufficient to induce the production of mRNA encoding for the transcription factor BLIMP1 (Piskurich et al. 2000, Fearon et al.
Interestingly, the absence of BLIMP1 expression in CARD11\textsuperscript{Mut10} B cells cased a reduction in the accumulation of B cells, whereas disruption of autocrine IL-6 production did not affect the B cell numbers at Day 10 (Fig 4.6 and 4.7). The expression of CARD11\textsuperscript{Mut10} in activated B cells causes increased NF-κB activation, which leads to high expression of Irf4 and Prdm1 mRNA as IRF4 is a direct target of the NF-κB transcription factors and given that IRF4 induces expression of Prdm1 (Mittrucker et al. 1997, Sciammas et al. 2006). Thus, the lack of an effect on the accumulation of B cells at Day 10 in the absence of autocrine IL-6 signaling could be explained by the NF-κB/IRF4-mediated induction of sufficient amounts of BLIMP1, which enabled the threshold of growth-inducing effects to be achieved. However, the disruption of plasma cell differentiation in a fraction of CARD11\textsuperscript{Mut10} B cells in the absence of IL-6 signaling suggests that IL-6 and STAT3 may be required for the sustained expression of Prdm1. It would thus be informative to measure the levels of Irf4 and Prdm1 in the fraction of Il6-deficient cells that did not differentiate into plasmablasts as it has been previously reported that graded expression of IRF4 coordinates the plasma cell differentiation program (Sciammas et al. 2006).

What other genes and pathways need to be deregulated to overcome the block in further growth of the CARD11\textsuperscript{Mut10} B cell population after 10 days \textit{in vivo}? Clues from whole genome human DLBCL sequencing studies may help to address this key question in the future as sufficient CARD11-driven lymphomas should be sequenced to analyze the genes and pathways which are disrupted in the malignant cells. Recently, Marra and colleagues have found a possible trend of high co-occurrence of \textit{CARD11} mutations with mutations in \textit{BTG2} and, to lower extents, with \textit{BCL6} and \textit{BCL2} translocations (Morin et al. 2011). \textit{BTG2} is a member of the anti-proliferative gene family known as B-cell translocation gene/ transducers of ErbB2 (BTG/TOB) (Matsuda et al. 2001, Duriez et al. 2004). BTG2 has been implicated in regulation of cell cycle progression, cellular growth and differentiation, DNA repair, apoptosis, and senescence (Rouault et al. 1996, Matsuda et al. 2001, Duriez et al. 2004). BTG2 is mainly expressed in non-cycling cells and its forced over-expression causes growth arrest by repressing cyclin D1 (Farioli-Vechioli et al. 2007). Thus, BTG2 expression was reduced in a large proportion of human kidney and breast carcinomas, suggesting that it is a tumor
suppressor (Kawakubo et al. 2004, Boiko et al. 2006, Takahashi et al. 2011). Moreover, given that the expression of BTG2 is regulated by NF-κB suggest for the potential of a negative feedback mechanism, which could block proliferation in CARD11$^{\text{Mut10}}$ expressing B cells (Duriez et al. 2002). Thus, the expression of BTG2 in B cells expressing CARD11$^{\text{Mut10}}$ could be measured, and the consequence of BTG2 knockdown in CARD11$^{\text{Mut10}}$ B cells could be experimentally tested in future experiments.
Fig 4.1 Experimental system to study CARD11 mutations *in vivo*

(a) Outline of the experimental strategy to examine the consequences of a CARD11 gain-of-function mutation in Ig^HEL^-transgenic antigen-activated B cells. (b) Gating strategy used to analyse CARD11-transduced live B cells at each time point in the *in vivo* cell transfer assay. The numbers on the plots indicate the percentage of the population of interest.
(a) Mut10: L232LI

Controls:
(i) Empty vector
(ii) Wild-type CARD11

(b) Flow cytometry analysis

- i.p. 5mg HEL (6 hours)
- Ig^HEL^ transgenic mice
- Culture splenocytes + anti-CD40 (Day 0)
- Spinculation + anti-CD40 (Day -2)
- Mixture EGFP+ & EGFP- (Day 0)
- Transfer into Rag1^- recipients
- Collect spleens for flow cytometry analysis (Days 5-60)
Fig 4.2 CARD11^{Mut10} promotes self-limiting B cell proliferation in vivo

Flow cytometry plots analyzing the EGFP+ B cells transduced with the indicated retroviral vectors at (a) the time of transfer Day 0 and (b) Day 10 post-transfer. The bar graphs indicate the numbers and percentages of EGFP+ B cells. (c) Relative number of EGFP+ and percentage of EGFP+ B cells detected in Rag1^{-/-} recipients at the indicated time points. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 3 independent experiments.
Fig 4.3 Expanded B cells adopt a FSC-A high, B220 low and CD19 low phenotype. (a) Representative histogram overlays and corresponding column graphs of the geometric mean fluorescence intensity (MFI) of FSC-A, B220 and CD19 on EGFP+ B cells expressing the indicated vectors at Day 10 post-transfer. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 3 independent experiments. (b) Representative histogram overlay and corresponding column graph of the geometric mean fluorescence intensity (MFI) of FSC-A, B220 and CD25 on EGFP+ B cells expressing the indicated vectors at the time of transfer (n=3; data are representative of 3 independent experiments). (c) Expression of Prdm1, Irf4 and Xbp1 in EGFP+ B cells transduced with the indicated vectors at the time of transfer (n=3; data are representative of 2 independent microarray experiments).
Fig 4.4 Experimental system to study effects of gain-of-function CARD11 in polyclonal B cells in vivo. (a) Outline of the experimental strategy to examine the consequences of a CARD11 gain-of-function mutation in polyclonal anti-IgM and anti-CD40 activated B cells. (b) Gating strategy used to analyse CARD11-transduced live B cells at each time point in the *in vivo* cell transfer assay. The numbers on the plots indicate the percentage of the population of interest.
(a) Mut10: L232LI

**Control:** Empty vector

- Culture splenocytes
- Spinoculation
- Mixtured
- Transfer into Rag1^- recipients
- Collect spleens for flow cytometry analysis

(b) Flow cytometry analysis:

- **SSC-A**
- **FSC-H**
- **SSC-H**
- **FSC-A**
- **B220**
- **CD19**
- **CD4**
- **IgM**
- **EGFP**
Fig 4.5 FSC-A high, B220 low and CD19 low phenotype of CARD11$^{\text{Mut10}}$ B cells is partially Blimp1-dependent. Representative histogram overlay and corresponding column graph of the geometric mean fluorescence intensity (MFI) of (a) FSC-A, B220 and CD19, or (b) CD45RA and CD45RB on wild-type and Prdm1-deficient B cells expressing the indicated vectors at Day 10 post-transfer in $\textit{Rag1}^{-/-}$: Grey: EV/wild-type, Green: CARD11$^{\text{Mut10}}$/wild-type, Blue: EV/Prdm1-deficient, Red: CARD11$^{\text{Mut10}}$/Prdm1-deficient. (n=3-4; data are representative of 2 independent experiments).
(a) [Histograms showing FSC-A, B220, and CD19 distributions with corresponding bar graphs for FSC-AMFI, B220 MFI, and CD19 MFI.]

(b) [Histograms showing CD45RA and CD45RB distributions with corresponding bar graphs for CD45RA MFI and CD45RB MFI.]

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<tr>
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<tr>
<td>CARD11&lt;sup&gt;Mut10&lt;/sup&gt;</td>
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Fig 4.6 Proliferation of CARD11\textsuperscript{Mut10} B cells remains self-limiting in the absence of Blimp1. Flow cytometry plots of the EGFP+ wild-type and Prdm1-deficient B cell populations transduced with the indicated retroviral vectors at (a) the time of transfer Day 0 and (b) Day 10 post-transfer. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. (c) Percentage of EGFP+ and relative number of EGFP+ B cells detected in Rag1\textsuperscript{+/} recipients at the indicated time points. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 2 independent experiments.
Fig 4.7 Autocrine IL6 production by CARD11\textsuperscript{Mut10} B cells contributes to plasmablast differentiation. (a) Expression of \textit{Il6} mRNA and IL6 production by EGFP+ in B cells expressing empty vector and CARD11\textsuperscript{Mut10} at the time of transfer (n=3; data is representative of 2 independent experiments). (b) Relative number and percentage of EGFP+ B cells detected in the spleens of \textit{Rag1\textsuperscript{-/-}} recipients at Day 0 (time of transfer) and Day 10. (c) Flow cytometry plots of the EGFP+ wild-type and \textit{Il6}-deficient B cell populations transduced with the indicated retroviral vectors at Day 10 post-transfer. The corresponding histogram overlays are representative of FSC-A, B220 and CD19 expression on CARD11\textsuperscript{Mut10}, expressing wild-type (red) and \textit{Il6}-deficient (green) B cells. (d) Flow cytometry analysis of EGFP+ B cells at Day 10 post-transfer based on CD19 and B220 expression. Column graphs indicate the percentage of CARD11\textsuperscript{Mut10}-expressing wild-type and \textit{Il6}-deficient B cell populations. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 2 independent experiments.
(a) IL6 mRNA expression

(b) [Data contributed by James Wang]

Day 0

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Day 10

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<td>Empty vector, CARD11^{M110}</td>
</tr>
<tr>
<td>II6^{ko}</td>
<td>Empty vector, CARD11^{M110}</td>
</tr>
</tbody>
</table>

(c) Day 10

| Empty vector | CARD11^{M110} |

[Flow cytometry images]
Fig 4.8 CARD11$^{\text{Mut10}}$ cooperates with loss-of-function A20 to maintain B cell numbers in the spleen at Day 21

(a) Representative flow cytometry plots, histogram overlay and corresponding column graph of the geometric mean fluorescence intensity (MFI) of FSC-A and CD25 expression of wild-type and Tnfaip3$^{\text{LasV/LasV}}$ B cells expressing CARD11$^{\text{Mut10}}$ at Day 0. (b) Flow cytometry plots of wild-type and Tnfaip3$^{\text{LasV/LasV}}$ B cells expressing CARD11$^{\text{Mut10}}$ at Day12 and Day21 post-transfer in Rag1$^{-/-}$ recipients. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells at the two time points. (c) Percentage of EGFP+ and relative number of wild-type and Tnfaip3$^{\text{LasV/LasV}}$ B cells expressing CARD11$^{\text{Mut10}}$ detected in Rag1$^{-/-}$ recipients at the indicated time points. Numbers are mean ± SEM of 3 recipients, and data are representative of 2 independent experiments.
Chapter 5: Lymphoma-associated *CARD11* mutations prevents self-antigen induced death

Introduction

Experimental system to study B cell response to self-antigen *in vivo*

CARD11<sup>Mut10</sup> prevents self-antigen induced death, drives expansion, plasmablast differentiation and autoantibody production

A range of lymphoma *CARD11* mutations switch self-antigen stimulation from death to proliferation and autoantibody secretion

*CARD11* mutations promote production of IgG-switched antibodies from polyclonal B cells

*CARD11* mutation can break tolerance in anergic B cells

Gain-of-function CARD11 mutants promote BIM degradation

Discussion

Fig 5.1 Experimental system to study B cell response to self-antigen *in vivo*

Fig 5.2 *CARD11* Mut10 prevents self-antigen induced death, drives population expansion, plasmablast differentiation and autoantibody production

Fig 5.3 A range of lymphoma *CARD11* mutations switch self-antigen stimulation from death to proliferation and autoantibody secretion

Fig 5.4 *CARD11<sup>Mut6</sup>* cooperates with self-antigen to enhance division of B cells

Fig 5.5 *CARD11* mutations promote production of IgG-switched antibodies from polyclonal B cells

Fig 5.6 *CARD11* mutation can break tolerance in anergic B cells

Fig 5.7 Gain-of-function CARD11 mutants decrease BIM protein levels
Introduction

Actively acquired self-tolerance and immunity are opposite processes that pose a central conundrum: how do antigen receptors switch between signalling lymphocyte death or proliferation when engaged by the same antigen in different contexts? This problem is exemplified by the surface immunoglobulin antigen receptors (BCRs) on mature, recirculating B-lymphocytes. Upon binding antigen, these receptors trigger multiple rounds of B cell clonal proliferation in the context of infection or immunization but induce anergy and apoptosis when they bind continuously to antigens that are part of the body (self-antigens). Differential amplitude and duration of signalling to intracellular calcium, PI3 kinase, MAP kinases, and the transcription factors NFAT, Jun/Fos and NF-κB correlates with differential signaling of growth or death by the B cell antigen receptor (Healy and Goodnow 1998). However, as with most tolerance/immunity checkpoints, the nature of the switch in mature B cells remains unresolved because no individual genetic alteration of these pathways has yet been found to be sufficient to switch self-antigen induced B cell death into proliferation.

An experiment of nature that provides clues to this problem comes from a group of common human cancers that includes non-Hodgkin’s lymphoma and chronic lymphocytic leukaemia, where a clone of B cells that has accumulated 30 damaging somatic mutations on average becomes locked in an endless growth cycle (Morin et al. 2011, Pasqualucci et al. 2011). It is not yet known how these mutations individually or collectively affects the normal response to self-antigens, although multiple lines of evidence suggest that the growth of human B cell malignancies is promoted by continuous BCR stimulation by self-antigens (Cleary et al. 1986, Borche et al. 1990, Friedman et al. 1991, Kobayashi et al. 1993, Chiorazzi and Ferrarini 2003, Rui et al. 2011, Stevenson et al. 2011). RNAi screens in tissue culture revealed that cells from activated B cell sub-type of diffuse large B cell lymphoma (ABC-DLBCL) died when components of the antigen receptor signal-transduction cascade were depleted (Ngo et al. 2006, Lenz et al. 2008, Davis et al. 2010).
One important branch of the BCR signalling cascade terminates on the transcription factor, NF-κB, which is activated by acute antigen receptor engagement when an antigen is foreign but not chronic receptor engagement by self-antigens (Healy and Goodnow 1998). NF-κB is activated by the BCR through a pathway whereby PKCb phosphorylates and activates CARD11 (also called Carma1) to form a CARD11-BCL10-MALT1 (CBM) complex (Jun et al. 2003, Thome 2004, Rawlings et al. 2006). The CBM complex in turn activates IκB kinase (IKK) to phosphorylate and degrade the inhibitor of NF-κB, IκBα, allowing NF-κB to move to the nucleus and cooperate with other transcription factors in promoting B cell survival, proliferation and differentiation (Jun et al. 2003, Thome 2004, Rawlings et al. 2006, Gerondakis and Siebenlist 2010).

As previously introduced, thirteen percent of DLBCL cases, including ABC- and germinal centre (GC)-types, have acquired activating somatic point mutations of various amino acids in the coiled-coil regulatory domain of CARD11 that are required for the proliferation of an ABC-DLBCL lymphoma cell line in culture (Lenz et al. 2008). However, many other mutations also recur in B cell malignancy that may be needed as an ensemble to switch the way B cells respond to chronic stimulation (Rui et al. 2011), and indeed dependence on the CARD11 branch of the cascade did not extend to the GC subtype of diffuse large B cell lymphoma (Ngo et al. 2006, Lenz et al. 2008). Likewise, dysregulated NF-κB signaling on its own was insufficient to initiate B cell growth in mature, circulating B cells in transgenic mice expressing a constitutively active allele of IKKβ and dysregulated expression of the NF-κB target gene, Bcl2, delayed self-antigen induced death but did not result in a switch into proliferation (Sasaki et al. 2006). Collectively, these results focus the question on what effects on the normal response to self-antigens, if any, do lymphoma CARD11 mutations have on their own?

Here we examine this question using a retroviral gene transfer strategy to genetically manipulate a cohort of self-antigen-binding mature B cells in vivo. Using this system, all lymphoma-derived CARD11 mutations tested conferred upon CARD11 a potent ability to switch the effect of chronic binding of self-antigen from death into inducing B cell proliferation, and autoantibody production. We conclude that regulation of CARD11 is a central switch governing the decision between
antigen-induced death and growth in mature B cells. Mutations in this switch, when paired with somatically acquired antigen receptors against self, represent a powerful initiator of aberrant B cell growth and differentiation.

**Experimental system to study B cell response to self-antigen in vivo**

We used a retroviral gene transfer approach to introduce the gain-of-function \textit{CARD11} mutations in activated immunoglobulin transgenic (MD4) B cells as described in the previous results chapters (Goodnow et al. 1988). The mixture of transduced (EGFP+) and non-transduced (EGFP-) Ig\textsuperscript{HEL} B cells was transferred into syngeneic recipients with or without circulating hen egg lysozyme (HEL) antigen [Fig 5.1a; (Goodnow et al. 1988)]. In the experiments described in this chapter, we compared the effects of the following four CARD11 mutants: Mut3 (G123S), Mut10 (L232LI), Mut2 (L251P), and Mut6 (D387V). Vectors encoding \textit{CARD11}\textsuperscript{WT} and EGFP only (Empty vector) were used to control for the effects of transduction and overexpression of \textit{CARD11} and EGFP respectively. At the time of transfer, the transduced B cells expressed twice the amount of endogenous \textit{CARD11} protein present in activated B cells (Fig 5.1b). There was no difference between the level of mutated \textit{CARD11} protein compared to cells transduced with \textit{CARD11}\textsuperscript{WT} vector, indicating that the mutations have minimal or negligible effects on accumulation of the protein (Fig 5.1b).

To test and validate the adoptive transfer approach, equal numbers of B cells expressing EGFP only (Empty vector) were transferred into non-transgenic and HEL-transgenic recipients and the frequency of B cells was analysed in the spleens after 24 hours. The two types of recipients were both on the \textit{Rag1}\textsuperscript{−/−} background, with the only difference between the recipients being the presence of soluble monomeric hen egg lysozyme (HEL) in the circulation of the HEL-transgenic mice. There was a 5-fold reduction in the frequency of EGFP+ B cells in the spleens of HEL-transgenic B cells compared to the non-transgenic recipients (Fig 5.1c) and the remaining cells had increased intracellular levels of the pro-apoptotic protein BIM (Fig 5.1d). These results confirmed previous findings and established that self-
reactive HEL-binding activated B cells are deleted in the periphery when engaged with HEL self-antigen.

**CARD11\textsuperscript{Mut10} prevents self-antigen induced death, drives expansion, plasmablast differentiation and autoantibody production**

Next, we asked whether the enforced expression of wild-type CARD11 or CARD11\textsuperscript{Mut10} could affect self-antigen mediated deletion. Consistent with the loss of EGFP+ cells expressing empty vector after 1 day, at longer times of 5 or 12 days after transfer the mean numbers of empty vector transduced cells were decreased 54-fold and a 146-fold, respectively, in HEL-transgenic recipients relative to non-transgenic recipients (Fig 5.2a, b). The number of EGFP+ B cells expressing CARD11\textsuperscript{WT} was also reduced in the HEL-transgenic recipients relative to the non-transgenic recipients by 17-fold and 3-fold at Day 5 and Day 12, respectively (Fig 5.2a, b). The fold reduction in EGFP+ B cell numbers in the HEL-transgenic recipients appeared less when expressing CARD11\textsuperscript{WT} compared to empty vector, raising the possibility of a subtle inhibition of deletion by CARD11\textsuperscript{WT} over-expression. However, this could simply reflect the fact that there were fewer CARD11\textsuperscript{WT}-B cells in non-transgenic recipients and close to background numbers in HEL-transgenic recipients. By contrast, B cells expressing CARD11\textsuperscript{Mut10} were not deleted in the presence of self-antigen in the HEL-transgenic recipients, and instead there were 4.7 and 6.4-fold more EGFP+ B cells accumulating on Days 5 and 12, respectively, in the HEL-transgenic recipients compared to the number of cells in the non-transgenic spleens (Fig 5.2a, b). This result indicates that CARD11\textsuperscript{Mut10}-expressing B cells not only avoided BIM-mediated deletion in response to self-antigen, but instead they proliferated and/or survived better when stimulated by self-antigen.

The B cells expressing CARD11\textsuperscript{Mut10} had comparable FSC-A profiles and CD19 levels in the non-transgenic and the HEL-transgenic recipients (Fig 5.2c). The levels of B220 were lower on about 50\% of CARD11\textsuperscript{Mut10}-B cells in HEL-transgenic recipients compared to the levels on cells in non-transgenic recipients (Fig 5.2c).
The high FSC-A, low B220 and CD19 expression suggested that the \( \text{CARD11}^{\text{Mut10}} \)-expressing B cells engaging with self-antigen also differentiated into plasmablasts.

To determine whether the transferred B cells could also produce anti-HEL antibodies, the serum from the recipient mice was collected at Day 12 following transfer. An ELISA was performed to determine the relative amounts of HEL-specific antibodies present in the serum of the recipients. There were slightly higher amounts of anti-HEL antibodies detected in non-transgenic recipients, compared to HEL-transgenic recipients in which empty vector and \( \text{CARD11}^{\text{WT}} \) control B cells were transferred (Fig 5.2d). This result is consistent with the deletion of most control B cells in the presence of self-antigens in the HEL-transgenic recipients. Interestingly, approximately 100-fold more anti-HEL antibodies were detected in the serum of both non-transgenic and HEL-transgenic recipients in which \( \text{CARD11}^{\text{Mut10}} \)-B cells were transferred, compared to the relative levels of anti-HEL antibodies in the non-transgenic recipients receiving the control B cells (Fig 5.2d). These results indicate that the expression of \( \text{CARD11}^{\text{Mut10}} \) prevents self-antigen induced BIM-mediated deletion, and drives expansion, differentiation and autoantibody production by self-reactive B cells.

**A range of lymphoma \textit{CARD11} mutations switch self-antigen stimulation from death to proliferation and autoantibody secretion**

Mutations from DLBCL cases predominantly occur in the coiled-coil domain of CARD11 (Shaffer et al. 2012). The lymphoma-derived mutations affect multiple residues of the domain, suggesting that different mutations may confer similar gain-of-function properties to CARD11. However we have previously seen that the CARD11 mutants activate NF-\( \kappa B \) and JNK signalling to different extents (Chapter 3). Thus, we asked whether other lymphoma CARD11 mutants would also be able to oppose self-antigen mediated deletion in HEL-transgenic recipients, or whether this phenomenon was a unique property of \( \text{CARD11}^{\text{Mut10}} \).

Activated immunoglobulin-transgenic B cells were transduced with the following CARD11 mutants: Mut6, Mut3, Mut2 and Mut10, and approximately equal numbers
of transduced EGFP+ B cells were transferred into non-transgenic and HEL-transgenic recipients (Fig 5.3a, b). There was about 4-fold less EGFP only (empty vector) and CARD11WT-B cells in the spleens of HEL-transgenic recipients compared to non-transgenic recipients 12 days after transfer (Fig 5.3a, c). By contrast, the number of B cells expressing all the CARD11 mutants accumulated to higher numbers in the spleens of HEL-transgenic mice compared to non-transgenic mice (Fig 5.3a, c). B cells expressing CARD11Mut6, which was found to be the least activated mutant form of CARD11 from in vitro growth studies (Chapter 3), accumulated to 20-times higher mean number in the presence of self-antigen in HEL-transgenic recipients compared to non-transgenic recipients (Fig 5.3a, c). There were approximately 40-fold more B cells expressing CARD11Mut3, 13-fold more CARD11Mut2 B cells and 5-fold more CARD11Mut10 B cells in HEL-transgenic recipients compared to non-transgenic recipients (Fig 5.3a, c). Moreover, B cells expressing the mutant gain-of-function versions of CARD11 adopted a FSC-A high, B220low and CD19low phenotype, indicating the B cells expanded, differentiated and accumulated as plasmablasts in the spleens of the recipients (Fig 5.3a). The levels of anti-HEL antibodies were, however, very low or not detectable in non-transgenic and HEL-transgenic recipients with cells expressing EGFP only (EV), wild-type CARD11, and CARD11Mut6 and CARD11Mut3, whereas relatively high levels of anti-HEL antibodies were detected in both types of recipients with CARD11Mut2 and CARD11Mut10 B cells (Fig 5.3d). These results indicate that gain-of-function CARD11 mutants can switch the normal B cell response to self-antigen from BIM-mediated death to expansion and autoantibody secretion. The absence of measurable anti-HEL antibodies in the recipients with B cells expressing CARD11Mut6 and CARD11Mut3 may reflect the relatively low numbers of B cells present in the recipients at the time of analysis and the inhibition of detection of small amounts of antibody due to blocking by an excess of antigen in the serum (Fig 5.3c).

The finding above that B cells expressing the 'weakest' lymphoma allele, CARD11Mut6, accumulated 20-times more in the presence of self-antigen (Fig 5.3a, c) suggested that this mutant version of CARD11 synergised with signals downstream of antigen-BCR to promote proliferation of the B cells. To examine this possibility, we set up an in vitro assay where we could track the response of
cell trace violet (CTV) labeled B cells in the presence of self-antigen. Immunoglobulin transgenic B cells were transduced with wild-type CARD11, CARD11\textsuperscript{Mut10} and CARD11\textsuperscript{Mut6}, and cultured in media only or media with 100ng/ml HEL for a period of 4 days. The percentage of EGFP+ B cells was higher in all the cultures with HEL, compared to the media only controls (Fig 5.4a). However, there was a 4-fold reduction in the number of CARD11\textsuperscript{WT} expressing B cells cultured in the presence of HEL compared to the number of CARD11\textsuperscript{WT}-B cells in the absence of HEL (Fig 5.4b). By contrast, the number of B cells expressing CARD11\textsuperscript{Mut10} and CARD11\textsuperscript{Mut6} increased in the presence of HEL, consistent with the \textit{in vivo} transfer results (Fig 5.3, Fig 5.4a, b). The FSC-A of more than 80% of the CARD11Mut10 and CARD11Mut6 expressing B cells was increased in the presence of HEL, indicating that the B cells were larger (Fig 5.4b). The CARD11\textsuperscript{Mut6} expressing B cells diluted CTV further in the presence of HEL, compared to the same B cells cultured in media only, indicating that there was cooperation between self-antigen-BCR signalling and CARD11\textsuperscript{Mut6} to promote cell division (Fig 5.4c). Interestingly, this difference in the level of CTV-dilution did not extend to the CARD11\textsuperscript{Mut10} B cells, as there was little difference in the level of CTV present in the EGFP+ cells in the presence or absence of HEL, despite the number of EGFP+ cells had increased in the presence of self-antigen (Fig 5.4c). Based on a model of the theoretical levels of CTV present in the cultured B cells at each cell division, CARD11\textsuperscript{Mut6} B cells divided a mean of seven times in the presence of HEL compared to once in the absence of HEL (Fig 5.4d). The remaining control CARD11\textsuperscript{WT} B cells seem to undergo one additional round of proliferation in the presence of HEL, whereas CARD11\textsuperscript{Mut10} paradoxically divide one round less in the presence of HEL (4 cell divisions) compared the absence of HEL (5 cell divisions; Fig 5.4d). These findings indicate that the weakly activated CARD11\textsuperscript{Mut6} allele synergises with self-antigen to enhance B cell division, and suggest that some CARD11 mutants may depend on signals from the antigen receptor for their gain-of-function phenotype to become apparent.
CARD11 mutations promote production of IgG-switched antibodies from polyclonal B cells

We next investigated if the various CARD11 mutations could provoke IgG autoantibody secretion by normal B cells with a diverse repertoire of specificities. B cells from non-transgenic mice were retrovirally transduced with vectors encoding mutant CARD11 or controls encoding wild-type CARD11, mutant IKKβ*, or empty vector. The B cells were transferred into Rag1−/− recipients and spleen cells and serum collected 12 days later. Compared to the controls, recipients of normal B cells expressing mutant CARD11 had higher concentrations of IgM and IgG1 in their sera (Fig 5.5a, b). The induction of serum IgG by the different CARD11 mutants correlated with their potency to induce plasmablast accumulation in the same animals, with CARD11Mut10 being the most potent and CARD11Mut6 the least potent (Fig 5.4b). IgG antibodies with a diffuse pattern of binding to the cytoplasm and nucleus of HEp-2 cells (Fig 5.4c), and IgM and IgG antibodies binding to the surface of 3T3 mouse fibroblast cells (Fig 5.4d, e) were also selectively increased in serum of recipients of B cells expressing mutant CARD11. Interestingly, low levels of IgM and IgG1 antibodies were detected in the recipients with B cells expressing a gain-of-function IKKβ mutant. This result will be further examined in Chapter 6 of this thesis.

CARD11 mutation can break tolerance in anergic B cells

To test the ability of CARD11 mutations to break an established state of tolerance in self-reactive B cells and induce proliferation and autoantibody secretion, we used anergic B cells harvested from IgHEL x HEL double-transgenic mice (Goodnow et al. 1988). The self-reactive B cells were activated with anti-CD40 and anti-IgM, and maintained in the constant presence of HEL antigen during retroviral transduction. The transduced B cells were then transferred into HEL-transgenic recipients or non-transgenic recipients and the B cells analysed in the spleen of the recipients at Day 12 (Fig 5.6a). The state of anergy established in IgHEL x HEL B cells is representative of anergic B cells with many different self-specificities and affinities analysed in various Ig-transgenic mice, and of polyclonal anergic B cells
in human peripheral blood (Healy and Goodnow 1998, Goodnow and Ohashi 2012). The Ig\textsuperscript{HEL}x HEL anergic B cells are characterised by an approximate 10-fold down-regulation of surface IgM (Fig 5.6b), the selective uncoupling of NF-κB signalling and the inability to differentiate into plasmablasts (Healy and Goodnow 1998, Glynne et al. 2000).

At the time of transfer, a fraction of the activated Ig\textsuperscript{HEL}x HEL anergic B cells transduced with CARD11\textsuperscript{Mut10} expressed lower levels of CD25, an NF-κB-regulated cell surface protein, compared to Ig\textsuperscript{HEL} control B cells transduced with the same vector (Fig 5.6c). This result indicated that NF-κB signalling in the anergic B cells was partly restored with the expression of gain-of-function CARD11\textsuperscript{Mut10} (Fig 5.6c). Moreover, approximately 15% less activated Ig\textsuperscript{HEL}x HEL anergic B cells were recovered compared to non-anergic B cells from Day -2 to Day 0 of tissue culture, indicating that the activated anergic B cells may have a survival defect (Fig 5.6d).

CARD11\textsuperscript{Mut10} nevertheless broke the established state of tolerance in the Ig\textsuperscript{HEL}x HEL anergic B cells, preventing their elimination in HEL-transgenic recipients (Fig 5.6e). Approximately 5-fold more CARD11\textsuperscript{Mut10}-expressing anergic B cells accumulated in the spleens of HEL-transgenic recipients compared to the number of the same cells in the non-transgenic recipients, indicating that the additional growth-promoting signals provided by the self-antigen were retained in the anergic B cells (Fig 5.6g, h). Moreover, the CARD11\textsuperscript{Mut10}-expressing anergic and non-anergic B cells were phenotypically identical as they differentiated and accumulated as FSC-A high, CD19low B220low plasmablasts (Fig 5.6f), and produced relatively large amounts of anti-HEL autoantibodies (Fig 5.6h).

**Gain-of-function CARD11 mutants promote BIM degradation**

Given that self-antigen-BCR engagement increased levels of BIM in empty vector transduced B cells, we examined whether the CARD11 mutants were able to modulate the levels of BIM. Activated B cells were transduced with wild-type or various CARD11 mutants, and cultured in the absence of anti-CD40 and HEL antigen for 48 hours before analysis of intracellular levels of BIM by flow
cytometry or by sorting EGFP+ cells and Western blotting. B cells expressing the CARD11 mutants had lower BIM levels than B cells expressing CARD11 wild-type or empty vector controls (Fig 5.7a, b). The magnitude of Bim downregulation appeared to be inversely proportional to the capacity of the CARD11 mutants to induce growth signals (Chapter 3). Moreover, Western blotting for BIM confirmed the lower levels present in CARD11_{Mut10}-B cells, and showed that the band corresponding to the extra-long isoform of BIM had a mobility shift, indicative of phosphorylation of BIM (Fig 5.7c). Interestingly, BIM is known to be phosphorylated by ERK and phosphorylated BIM is subsequently degraded (O’Reilly et al. 2009). Thus, modulation of BIM protein could be one mechanism through which B cells expressing CARD11 mutant proteins avoid self-antigen induced apoptosis.

Discussion

The findings presented in this chapter demonstrate that a range of CARD11 mutations acquired in different human lymphomas share the property of switching the mature B cell response to continuous stimulation by self-antigens from cell death into B cell proliferation, differentiation and autoantibody secretion. In normal mature B cells, continuous self-antigen exposure desensitizes antigen receptor signaling to NF-κB and JNK while preserving signaling for calcium oscillations, NFAT, ERK and Bim induction, and this is thought to explain the absence of proliferation and induction of apoptosis in self-reactive B cells (Healy et al. 1997, Healy and Goodnow 1998, Enders et al. 2003). At the opposite extreme, optimal BCR crosslinking by T-cell independent type 2 antigens is sufficient to induce proliferation and plasmablast differentiation. It is not known whether a global re-wiring or a single pivotal switch explains how the mature B cell response to antigen ranges from proliferation and plasma cell differentiation on the one hand to anergy and death on the other. The data here establish that regulation of a single molecule, CARD11, serves as the switch between antigen-induced signaling of death or proliferation and plasma cell differentiation.
What lies downstream of the CARD11 switch is an intriguing question for the future. Activation of NF-κB by antigen receptor signaling through CARD11 might have been predicted to be the mechanism for switching the response to self antigen from death to growth, given that NF-κB is essential for activation of numerous B cell survival genes (e.g. Bcl2, BclxL, Bfl1/A1), growth genes (e.g. Irf4, Myc, Cyclin D), and differentiation genes (Irf4) (Gerondakis and Siebenlist 2010). Enforced expression of Bcl-2 nevertheless only slows the rate of self-antigen induced death in mature B cells, but does not switch them into proliferation (Cyster et al. 1994). Likewise, enforced expression of Myc using the same retroviral-transduction assay used here does not inhibit self-antigen induced death nor induce proliferation even when combined with p53 inactivation (Rui, L & Goodnow, C, unpublished data). The unique ability of CARD11 to switch self-reactive B cells from death into growth and plasma cell differentiation may stem from CARD11 binding to many different signaling proteins (McCully and Pomerantz 2008), which may change the dynamics or balance of NF-κB subunits that are activated or enlist additional signaling pathways such as JNK or p38 (Thome 2004). It will be interesting to determine whether overexpression of the other CBM complex components, BCL10 and MALT1, and enforced expression of gain-of-function versions of IKKβ and MKK7 can phenocopy the effects of lymphoma-CARD11 mutations.

Our findings also provide experimental evidence for the long-standing hypothesis that self-reactive antigen receptors, which arise frequently by VDJ rearrangements in immature B cells (Wardemann et al. 2003) and by V-region hypermutation in antigen-activated B cells (Shlomchik et al. 1990), can cooperate with somatic mutations in signaling molecules to initiate neoplastic proliferation and production of anti-self antibodies (Cleary et al. 1986, Borche et al. 1990, Friedman et al. 1991, Kobayashi et al. 1993, Chiorazzi and Ferrarini 2003, Rui et al. 2011, Stevenson et al. 2011). As opposed to binding microbial antigens, antigen receptors that bind to self-antigens provide B cells with an endless source of receptor stimulation. Given the high rate of somatic point mutation in antigen-activated B cells (Liu et al. 2008), the finding that single somatic mutations at various sites in the coiled-coil domain of CARD11 were sufficient to switch antigen receptor-induced death into growth, without requiring accumulation of numerous
other mutations, identifies CARD11 as an especially weak link in the normal control of B cell malignancy and autoimmunity. The occurrence of CARD11 mutations together with self-reactive Ig V-regions can be expected to have an equally potent influence on therapeautic responses, and hence should be tested in a broad range of B cell malignancies and autoimmune diseases.
**Fig 5.1 Experimental system to study B cell response to self-antigen *in vivo.***

(a) Outline of the experimental strategy to examine the consequences of acquiring different CARD11 CARD or coil-coiled domain mutations in Ig^HEL^ B cells. (b) Immunoblot for CARD11 on cell lysates from sorted EGFP+ cells expressing control empty vector (EV), wild-type CARD11 (WT), and lymphoma CARD11 mutants Mut2, Mut3, Mut6, Mut10. Transduced B cells were cultured in media without anti-CD40 for 24 hours before EGFP+ cells were sorted. (c) Analysis of empty vector transduced live B cells at 24 hours following transfer into non-transgenic or HEL-transgenic recipients on a Rag1^−/−^ background. The numbers on the plots indicate the percentage of EGFP+ cells. (d) BIM expression measured by flow cytometry on EGFP+ cells gated in (c) (BIM-A647 antibody provided by Dr. Steve Daley). Anti-CD40 and anti-IgM activated spleen B cells from a homozygous Bim knockout mouse were used as a negative staining control. Numbers on the bar graph are MFI ± SEM of 3 independent recipients, and data is representative of 2 independent experiments.
**Fig 5.2** *CARD11* Mut10 prevents self-antigen induced death, drives population expansion, plasmablast differentiation and autoantibody production. (a) Flow cytometry plots analyzing EGFP+ B cells transduced with the indicated retroviral vectors at Day 10 post-transfer in non-transgenic and HEL-transgenic recipients on a *Rag1*−/− background. (b) Number of EGFP+ B cells expressing the indicated vectors at the time of transfer and detected in *Rag1*−/− recipients at the indicated time points. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 5 independent experiments. Numbers in parentheses are the fold change in mean number of B cells expressing the indicated vector in HEL-transgenic recipients relative to non-transgenic recipients. (c) FSC-A, B220 and CD19 expression on *CARD11*Mut10-transduced Ig^HEL transgenic^ B cells transferred in non-transgenic and HEL-transgenic recipients. In the histogram overlays, the grey histograms are empty-vector transduced B cells in non-transgenic recipients on Day 10. (d) Relative units of anti-HEL IgM^a^ measured by ELISA on serum collected from non-transgenic and HEL-transgenic mice 10 days after transfer of B cells expressing the indicated vectors (n=3 mice per group; representative of 3 independent experiments).
Fig 5.3 A range of lymphoma *CARD11* mutations switch self-antigen stimulation from death to proliferation and autoantibody secretion. (a) Representative flow cytometry plots analyzing the EGFP+ B cells transduced with the indicated retroviral vectors at Day 12 post-transfer in non-transgenic and HEL-transgenic recipients on a *Rag1*<sup>-/-</sup> background. (b) Number of EGFP+ B cells from each transduction group transferred into each recipient at Day 0 (input). (c) Column graph summarising flow cytometric analysis of splenocytes 12 days after transplantation of B cells, transduced with vectors encoding the indicated *Card11* mutations, into HEL-transgenic or non-transgenic recipients. Numbers represent mean ± SEM number of EGFP+ B cells in the spleen 12 days after transplantation (n=3-6 recipient mice per group). B cells were gated from HEL-binding IgM<sup>a+</sup> B cells in the spleen. (d) Relative units of anti-HEL IgM measured by ELISA on serum collected from non-transgenic or HEL-transgenic mice 11 days after transplantation (n=3-9 mice per group). Data are representative of 4 independent experiments.
Fig 5.4 CARD11**Mut6** cooperates with self-antigen to enhance division of B cells.

(a) Flow cytometry plots analyzing the EGFP+ Ig**HEL** B cells transduced with the indicated retroviral vectors at Day 3 of tissue culture in media only or in the presence of 100ng/ml HEL. The numbers on the plots indicate the percentage of EGFP+ cells among viable B cells. Representative histogram overlays of (b) FSC-A and (c) Cell Trace Violet (CTV) comparing transduced B cells expressing the indicated vectors in the presence or absence of HEL. (d) Representative graph of a model constructed based on the theoretical CTV MFI per cell division, with the average CTV MFI of B cells expressing the indicated vectors cultured in the presence or absence of HEL antigen for 3 days (n=3; experiment repeated 2 times).
Fig 5.5 CARD11 mutations promote production of IgG-switched antibodies from polyclonal B cells. Normal polyclonal B cells were transduced with vectors encoding the indicated CARD11 wild-type (WT) or mutant proteins, constitutively active IKKβ*, or empty vector (EV) control. B cells were transferred into Rag1-deficient hosts and sera were collected from the recipients after 12 days. (a, b) ELISA results showing the concentration of serum IgM (a) or IgG1 (b) in individual recipients and the mean concentrations. (c) Representative HEp-2 staining by IgG in serum from recipients of cells expressing the indicated proteins (n=4 recipients per transfer group). Each image was captured using an identical exposure. Representative histogram overlays indicating binding of serum (d) IgM and (e) IgG bound to the surface of mouse 3T3 cells. Corresponding column graphs indicate the mean fluorescence intensity (MFI). n=3-4; experiment repeated 2 times.
**Fig 5.6 CARD11 mutation can break tolerance in anergic B cells.** (a) Outline of the experimental strategy to examine the consequences of acquiring CARD11 Mut10 in Ig-transgenic B cells and Ig^HEL-X^ HEL-double transgenic B cells. Ig^HEL-X^ HEL-double transgenic B cells were continuously exposed to 500ng HEL in the tissue culture media throughout the culture and transduction period. (b) Relative expression of IgM^a^ on the surface of cultured non-transduced Ig-transgenic B cells and Ig^HEL-X^ HEL-double transgenic B cells at Day 0 (time of cell transfer). (c) Representative flow cytometry profiles of the CARD11^Mut10^-transduced Ig-transgenic B cells and Ig^HEL-X^ HEL-double transgenic B cells at Day 0. Corresponding histogram overlays of FSC-A and CD25 expression on the EGFP+ cells compared to empty vector expressing Ig-transgenic B cells. (d) Percentage recovery of Ig-transgenic B cells and Ig^HEL-X^ HEL-double transgenic B cells at Day 0 relative to Day -2. (e) Representative flow cytometry plots analyzing Ig-transgenic B cells and Ig^HEL-X^ HEL-double transgenic B cells transduced with CARD11 Mut10 at Day 12 post-transfer in non-transgenic and HEL-transgenic recipients on a Rag1^-/-^ background. B cells were gated from HEL-binding IgM^a+^ B cells in the spleen. (f) Representative histogram overlays of FSC-A, B220, CD19 and IgM^a^ expression on EGFP+ populations gated on the flow cytometry plots in (e). (g) Number of EGFP+ B cells from each transduction group and genotype transferred into each non-transgenic and HEL-transgenic recipient at Day 0 (input). (h) Column graph summarising flow cytometric analysis of splenocytes 12 days after transplantation of B cells, transduced with the indicated vectors, into HEL-transgenic or non-transgenic recipients. Numbers represent mean ± SEM number of EGFP+ B cells in the spleen 12 days after transplantation (n=3-6 recipient mice per group). (i) Relative units of anti-HEL IgM^a^ measured by ELISA on serum collected from non-transgenic or HEL-transgenic mice 11 days after transplantation (n=3-6 mice per group). Data are representative of 3 independent experiments.
(a) 

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Controls:
(i) Empty vector
(ii) Wild-type CARD11

(b) 

Non-transgenic  Ig-transgenic  IgxHEL-double transgenic

(c) 

CARD11

(d) 

% Recovery

Vector: EV  WT  Mut10  EV  WT  Mut10

Donor: Ig-transgenic  IgxHEL-transgenic
Fig 5.7 Gain-of-function CARD11 mutants decrease BIM protein levels

(a) Representative flow cytometry plots show intracellular staining for BIM and EGFP in B cells transduced with the indicated vectors at Day 0 relative to cell transfer. Corresponding histogram overlay comparing BIM levels in B cells transduced with wild-type CARD11 (blue) and CARD11 Mut10 (red). (b) BIM levels expressed as mean fluorescence intensity (MFI) in independent cultures (dots) measured as above. (c) Immunoblot analysis of B cell lysates from cells expressing control empty vector (EV) and wild-type CARD11 (WT), and lymphoma CARD11 Mut10, probed with an antibody to Bim and re-probed with an antibody to tubulin. The different Bim isoforms are labelled. Transduced B cells were cultured in media without anti-CD40 for an additional day (Day 0+24 hours) before EGFP + cells were sorted.
Chapter 6: Overexpression of gain-of-function IKKβ* triggers 
B cell proliferation and growth in vitro, but death in vivo

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Introduction

NF-κB transcription factors are master regulators of inflammatory, immune and apoptotic responses (Hayden and Ghosh 2008, Vallabhapurapu and Karin 2009). In the canonical pathway, NF-κB dimers are held in the cytoplasm via binding to IκB proteins, which mask their nuclear localization signals (Hayden and Ghosh 2008, Vallabhapurapu and Karin 2009). When cells are stimulated by NF-κB inducers, Ser/Thr-specific IKKs phosphorylate IκBs, which are then polyubiquitinated with Lys48-linked ubiquitin chains and degraded (Scheidereit 2006). Freed NF-κB dimers subsequently enter the nucleus to promote gene transcription (Hayden and Ghosh 2008, Vallabhapurapu and Karin 2009). In the non-canonical pathway, activated IKK phosphorylates the IκB-like C-terminal domain in the NF-κB family member p100 triggering ubiquitination but only partial proteolytic degradation, preserving the active N-terminal fragment, NF-κB2, which is then free to enter the nucleus (Scheidereit 2006). NF-κB signaling pathways are associated with a vast number of human diseases including inflammation and cancer, which renders IKK a potentially important therapeutic target (Karin 2006).

IKK was originally purified from HeLa cells as a multi-protein complex containing the kinase subunits IKKα and IKKβ, and the regulatory protein NEMO (also known as IKKy) (Chen et al. 1996, DiDonato et al. 1997, Mercurio et al. 1997, Woronicz et al. 1997, Zandi et al. 1997, Rothwarf et al. 1998, Yamaoka et al. 1998). IKKβ consists of an N-terminal kinase domain (KD), a ubiquitin-like domain (ULD), a scaffold/dimerization domain (SDD) and a C-terminal NEMO binding domain (NBD) (Figure 6.1a) (Xu et al. 2011). IKKβ is a ubiquitously expressed kinase, which is essential for the activation of classical NF-κB signalling (Li et al. 1999, Hacker and Karin 2006). IKKβ mediates activation of the canonical NF-κB pathway in response to pro-inflammatory stimuli, and protein kinase assays suggested that IKKβ accounts for nearly all of the catalytic activity of the IKK complex towards IκBα (Zandi et al. 1998, Scheidereit 2006).

IKKβ forms part of a cytoplasmic kinase complex, along with IKKα and IKKY, which is assembled after the activation of several cell surface receptors (Hayden and Ghosh 2008). In B cells, the activation of Toll-like receptors, antigen receptors, TNF
receptors and CD40 receptor can signal for the assembly of the IKK complex (Vallabhapurapu and Karin 2009, Gerondakis and Siebenlist 2010). The activation of the IKK complex is achieved through the action of several kinases such as TAK1 and ubiquitin E3 ligases such as TRAF6 (Hacker and Karin 2006, Vallabhapurapu and Karin 2009, Gerondakis and Siebenlist 2010, Liu et al. 2012). The kinase activity IKKβ is specifically activated by the phosphorylation of two specific serine residues at position 177 and 181 in the kinase activation loop motif (Hacker and Karin 2006, Vallabhapurapu and Karin 2009, Gerondakis and Siebenlist 2010, Liu et al. 2012). Activated IKKβ subsequently phosphorylates the inhibitory protein, IKBα, which is bound to NF-κB transcription factors to restrict them in the cytoplasm (Hacker and Karin 2006, Vallabhapurapu and Karin 2009, Gerondakis and Siebenlist 2010, Liu et al. 2012).

In this results chapter, we investigate whether the expression of constitutively active IKKβ can phenocopy the effects of gain-of-function CARD11 mutants in normal B cells and in the context where the B cells encounter self-antigen in vivo.

**Expression of gain-of-function IKKβ activate NF-κB signaling and results in B cell expansion in vitro**

To investigate the effects of oncogenically activated IKKβ on the survival and growth of activated B cells, we used a kinase-active mutant form of IKKβ in which the two regulatory serines at position 177 and 181 in the activation loop of the kinase catalytic site have been mutated to glutamic acid (Mercurio et al. 1997). The S177E and S181E mutations change these residues from uncharged to negatively charged, mimicking the physiological addition of negatively charged phosphates to the serine side-chains and constitutively activating the kinase activity of IKKβ (Mercurio et al. 1997). This activated form of IKKβ has been previously shown to activate NF-κB signalling, and increase the expression of pro-survival and growth-promoting factors such as Bcl2 and CyclinD in activated B cells (Sasaki et al. 2006). Moreover, transgenic mice engineered to express the S177E; S181E activated IKKβ mutant (IKKβ*) in B cells have increased marginal zone B cells in the spleens (Sasaki et al. 2006). The IKKβ* mutant B cells survive and develop without requiring BAFF, and proliferate extensively when stimulated in culture (Sasaki et
al. 2006). However, B cell lymphomas are only seen in mice where IKKβ* is expressed in activated B cells which lack BLIMP1/Prdm1, suggesting that the proliferation- or survival-inducing effects of IKKβ* are counter-regulated by BLIMP1-mediated differentiation of B cells (Calado et al. 2010).

In parallel we investigated a recently described, putative oncogenically activated IKKβ allele discovered in 3% of patients with splenic marginal zone lymphoma in humans (Rossi et al. 2011). The mutation results in the change from a positively charged lysine 171 residue to a negatively charged glutamic acid (Rossi et al. 2011). In the structure of constitutively active IKKβ, K171 lies in the β9 sheet next to the activation loop to form an integral part of the activation segment of the catalytic cleft, with the K171 side-chain very close to the phosphomimetic E177 side-chain (Xu et al. 2011, Polley et al. 2013). The K171E change has been hypothesised to give rise to a kinase active form of IKKβ, but this remains to be experimentally verified. Here, we compare the effects of the two mutated forms of IKKβ (IKKβS177E;S181E and IKKβK171E) with the gain-of-function CARD11Mut10 when introduced in activated B cells.

We retrovirally transduced activated B cells with bicistronic EGFP vectors encoding IKKβS177E;S181E, IKKβK171E, CARD11Mut10, or empty vector, and tracked the number of live EGFP+ B cells over a period of 3 days in culture (Fig 6.1a). The percentage of EGFP+ B cells expressing IKKβS177E;S181E, IKKβK171E, CARD11Mut10 increased from Day 0 to Day 3, while there was a reduction in the percentage of EGFP+ empty vector control B cells (Fig 6.1b). The relative numbers of EGFP+ cells also increased from Day 0 to Day 3, with a 6-fold increase in B cells transduced with IKKβS177E;S181E or with IKKβK171E, and a 7-fold increase in the B cells expressing CARD11Mut10 (Fig 6.1c). By contrast, the numbers of EGFP- B cells in all the experimental groups and the numbers of EGFP+ only expressing B cells gradually decreased over the time period (Fig 6.1c). In addition, the levels of phospho-p65 and phospho-JNK were also measured in total cell lysates of B cells expressing EGFP only (EV), wild-type CARD11 (WT), IKKβK171E and CARD11Mut10 (10) (Chapter 3, Fig 3.1d). The levels of phospho-p65 were increased comparably in B cells expressing IKKβK171E and CARD11Mut10, whereas more phospho-JNK was present CARD11Mut10 expressing B cells (Chapter 3, Fig 3.1d). Interestingly, the
levels of phospho-JNK were increased to comparable levels in B cells expressing CARD11\textsuperscript{WT} and IKKβ\textsuperscript{K171E} compared to the empty vector controls, indicating that enforced expression of a gain-of-function IKKβ protein may have effects on other signalling pathways (Chapter 3, Fig 3.1d). These results indicate that the naturally occurring lymphoma IKKβ\textsuperscript{K171E} mutant has the same effects in promoting growth of activated B cells as the artificially engineered IKKβ\textsuperscript{S177E,S181E} mutant.

**Elimination of B cells overexpressing constitutively active IKKβ in vivo**

Given that both mutated forms of IKKβ phenocopy the growth-promoting effects of CARD11\textsuperscript{Mut10} in activated cultured B cells, we asked whether the gain-of-function IKKβ mutants are able to prevent self-antigen mediated death in vivo. Activated Ig\textsuperscript{HEL}-transgenic B cells were transduced with empty vector, wild-type IKKβ, IKKβ\textsuperscript{S171E,S181E}, IKKβ\textsuperscript{K171E} and CARD11\textsuperscript{Mut10}, and the B cells were transferred into non-transgenic and HEL-transgenic recipients as described in Chapter 5 (Fig 5.1a). After 12 days, the spleens of the recipients were collected and the number of EGFP+ B cells were analysed. Interestingly, despite transferring equal numbers of EGFP+ B cells, the gain-of-function IKKβ transduced B cells did not expand in either the non-transgenic or the HEL-transgenic recipients (Fig 6.2a, b, c). By contrast, the B cells expressing CARD11\textsuperscript{Mut10} survived and expanded in the spleens of both types of recipients, and as we have previously observed more CARD11\textsuperscript{Mut10} expressing B cells accumulated in the HEL-transgenic recipients compared to the non-transgenic recipients (Fig 6.2a, b, c). Few EGFP+ IKKβ\textsuperscript{S177E,S181E} and IKKβ\textsuperscript{K171E} expressing B cells were detected in the spleens of the non-transgenic recipients, whereas the EGFP- non-transduced B cells were present in the spleens of the same recipients, indicating that the IKKβ-transduced B cells were being selected against (Fig 6.2a, b, c). These results were quite surprising given that the same B cells expanded by 6-fold from starting numbers when placed in culture (Fig 6.1). However, the counter-selection of IKKβ\textsuperscript{S177E,S181E} B cells in vivo potentially provides an explanation to the low levels of IgG and IgM antibodies detected in the serum of recipients, which was observed in Chapter 5 (Fig 6.5).
To explain these unexpected findings, we hypothesised that the expression of gain-of-function IKKβ^{S177E;S181E} or IKKβ^{K171E} enhances the expression of cell surface receptors such as FAS, which may engage with its ligands when transferred \textit{in vivo}, but not in culture (Strasser et al. 2009). Another possible explanation could be that IKKβ^{S177E;S181E} or IKKβ^{K171E} sensitises B cells to intrinsic death pathways regulated by p53 or intrinsic effectors of cell death (Vousden and Lane 2007, Vazquez et al. 2008, Hotchkiss et al. 2009). These possibilities are tested in the subsequent parts of this results chapter.

**Investigating the effects of FAS expression on IKKβ*-expressing B cells**

The cell surface receptor FAS (CD95) was originally discovered as the target of two monoclonal antibodies that trigger apoptotic cell death in certain human tumor-derived cell lines in culture. Cloning of the gene encoding FAS revealed that it is a member of the tumor necrosis factor receptor (TNFR) family (Itoh et al. 1991). Subsequently, the physiological ligand for FAS, called FAS ligand (FASL) was identified (Suda et al. 1993). CD95 plays a crucial role in regulating the immune system as mice carrying homozygous defects in \textit{Fas} or \textit{Fasl} develop lymphadenopathy and systemic lupus erythematosus-like autoimmune disease (Watanabe-Fukunaga et al. 1992, Takahashi et al. 1994). Moreover, patients with heterozygous inherited mutations in the \textit{FAS} gene develop an autoimmune lymphoproliferative syndrome (Fisher et al. 1995, Rieux-Laucat et al. 1995).

FASL expressed on activated T lymphocytes and natural killer cells contributes to their ability to kill target cells. Thus, ALPS patients and FAS- or FASL-deficient mice have an increased predisposition to lymphoma development (Straus et al. 2001, Strasser et al. 2009). Interestingly, increased FASL-mediated killing of healthy bystander cells expressing FAS has been implicated in certain immunopathological states, such as hepatitis induced by extensive T cell activation (Ando et al. 1997). Upon ligation of CD95, sequential association of the adaptor molecule FADD, pro-forms of caspases 8 and 10, and the caspase-8/10 regulator c-FLIP lead to the formation of a death-inducing signaling complex (DISC) (Peter and Krammer 2003). The oligomerization of procaspase-8 results in its activation,
autoproteolytic processing, and release of an active heterotetrameric enzyme into the cytosol (Peter and Krammer 2003). This apoptotic trigger kills cells by either active caspase-8 directly cleaving and activates caspase-3 or indirect cleavage of caspase-3 (Holler et al. 2000, Peter and Krammer 2003).

Interestingly, the levels of Fas mRNA was increased by approximately 2-fold in IKKβ* (IKKβS177E.S181E) expressing B cells compared to the empty vector controls and CARD11* (CARD11Mut10) (Fig 6.3a). These results were confirmed at the protein level as IKKβ*-transduced B cells expressed approximately 2-fold more FAS (CD95) on the surface of the B cells compared to the empty vector control (Fig 6.3b). To test whether the increased FAS expression on the surface of IKKβ*-B cells played a role in the elimination of the B cells in vivo, we transduced wild-type and Fas-deficient B cells with IKKβ*, CARD11* and an empty vector encoding for EGFP only. The lack of surface FAS expression on the transduced Fas-deficient B cells was confirmed by flow cytometry prior to transfer of the B cells in vivo (Fig 6.3c). There was no difference in the levels of CD25 on the surface of Fas-deficient and wild-type IKKβ*-expressing B cells, indicating that the absence of FAS does not affect the expression of this NF-κB-responsive protein in this context (Fig 6.3d).

Equal numbers of transduced Fas-deficient and wild-type IKKβ*-B cells were transferred into Rag1−/− recipients at Day 0 (Fig 6.4e). The percentage of Fas-deficient and wild-type EGFP+ IKKβ*- expressing B cells decreased from 22% at Day 0 to only 2% at Day 10 after transfer (Fig 6.3 e, f). By contrast, the percentage of EGFP+ Fas-deficient and wild-type empty vector control cells remained unchanged from Day 0 to Day 10, while the percentage of CARD11*-expressing B cells increased from 6% at the time of transfer to 50% at Day10 (Fig 6.3 e, f). Few Fas-deficient or wild-type IKKβ*-expressing B cells were detected in the spleens of the recipients at Day 10 after transfer, indicating that the absence of FAS expression fails to rescue the IKKβ*-expressing B cells (Fig 6.3f). The numbers of EGFP+ CARD11*-B cells of either genotype were approximately 150-fold higher than the EGFP only empty vector control B cells at Day 10, with 1.3 fold more CARD11* Fas-deficient B cells present in the recipients compared to the numbers of CARD11* wild-type B cells (Fig 6.3f). Thus, our findings suggest that the
increased expression of FAS receptor on the surface of IKKβ*-transduced B cells does not explain their disappearance in vivo.

**Investigating the effects of TNFSFR-mediated necroptosis**

Necrosis was originally described as a passive form of cell death in damaged tissues; however, programmed necrotic death in specific contexts has been recently found to be orchestrated in a cell-autonomous manner via receptor-interacting protein 1 [RIP1; (Holler et al. 2000)] and RIP3 (Cho et al. 2009, He et al. 2009, Zhang et al. 2009). The well characterized form of programmed necrosis, known as necroptosis, requires the assembly of a signalling complex of RIP1 and RIP3 (Cho et al. 2009, He et al. 2009, Zhang et al. 2009). RIP1- and RIP3-dependent necroptosis is activated when caspase 8 activity becomes compromised (Vandenabeele et al. 2010, Mocarski et al. 2012). This was recently revealed in mice with a germline disruption of the caspase 8 gene or the FAS-associated death domain protein gene (FADD), which both die during gestation at embryonic day 10-11 (Yeh et al. 1998, Zhang et al. 1998, Kang et al. 2008). This embryonic lethality was rescued in either Casp8−/−Rip3−/− or Fadd−/−Rip1−/− mice, indicating that Caspase 8 is involved in the suppression of necroptosis (Kaiser et al. 2011, Oberst et al. 2011, Zhang et al. 2011).

Necroptosis can be induced by the ligation of death receptors, including FAS (Vercammen et al. 1998), TNF receptor 1 (TNFR1) and TNFR2 (Laster et al. 1988, Vercammen et al. 1998, Chan et al. 2003). Interestingly, the study of cell lines and some primary cells, which express caspase inhibitors revealed a caspase-independent cell death pathway triggered by death receptors and resulting in necrotic cell death (Vandenabeele et al. 2010). Thus, we hypothesised that expression of IKKβ* could potentially directly, or indirectly inhibit Caspase 8 as a negative feedback mechanism which physiologically dampens TNFR1/2 signalling to activate NF-κB transcription factors (Wajant and Scheurich 2011, Workman and Habelhah 2013). The presence of such a feedback loop would render IKKβ*-transduced B cells more susceptible to necroptotic cell death in response to TNF-TNFR1/2 signalling in vivo.
To test this hypothesis, we transduced wild-type and \textit{Tnfsfr1/2}\textsuperscript{-}\textit{/-} activated B cells with empty vector encoding for EGFP and IKK\textbeta\textsuperscript{*}. Approximately 1.5 times more \textit{Tnfsfr1/2}\textsuperscript{-}\textit{/-} than wild-type EGFP only B cells and IKK\textbeta\textsuperscript{*}-expressing B cells were transferred into \textit{Rag1}\textsuperscript{-}\textit{/-} recipients at Day 0 (Fig 6.4a). At Day 10 after transfer, both wild-type and \textit{Tnfsfr1/2}\textsuperscript{-}\textit{/-} EGFP only-expressing B cells were detected in the spleens of the recipients, with about 1.5-fold more \textit{Tnfsfr1/2}\textsuperscript{-}\textit{/-} EGFP+ cells compared to wild-type EGFP+ present in the spleens of the recipients (Fig 6.4b). However, very low numbers of \textit{Tnfsfr1/2}\textsuperscript{-}\textit{/-} and wild-type IKK\textbeta\textsuperscript{*}-transduced B cells were found in the spleens of the recipients at Day 10, suggesting that the absence of TNFR1/2 did not affect the survival of the IKK\textbeta\textsuperscript{*}-expressing B cells \textit{in vivo} (Fig 6.4b). These results indicate that \textit{Tnfsrf1}-mediated necroptotic cell death does not play a role in suppressing the expansion of IKK\textbeta\textsuperscript{*}-transduced B cells \textit{in vivo}.

**Investigating the effects of NK cells in the elimination of IKK\textbeta\textsuperscript{*}-B cells in vivo**

Natural killer (NK) cells are one important component of the innate immune response to tumours and virus-infected cells (Vivier et al. 2008). NK cells were originally discovered because of their ability to kill certain tumour cells \textit{in vitro}. Depletion of NK cells \textit{in vivo} increases tumour formation in several mouse tumour models (Vivier et al. 2008). NK cells express several stimulatory and inhibitory NK cell receptors that enable NK cells to specifically target cells that increase or decrease expression of various ligands (Vivier et al. 2008). The ligands for NK cell stimulatory receptors are usually poorly expressed by healthy cells but are increased in expression on transformed, infected or stressed cells (Vivier et al. 2008, Raulet and Guerra 2009). The balance of stimulatory and inhibitory signaling controls NK cell activation when it engages the target cell (Vivier et al. 2008, Orr and Lanier 2010). Thus, even though normal cells can display stimulatory ligands, they are not killed by NK cells because their MHC class I molecules engage inhibitory receptors that counteract stimulatory signaling (Vivier et al. 2008, Raulet and Guerra 2009). Increased expression of stimulatory ligands by a target cell can overcome inhibitory signaling in the NK cell, resulting in target cell lysis. The well characterised NK cell activating receptor NKG2D can
be engaged by ligands such as Rae-1 (Guerra et al. 2008, Raulet and Guerra 2009). These interactions are strengthened by SLAM family proteins expressed on both NK cells and the target cells (Orr and Lanier 2010, Veillette 2010). Hence, we hypothesised that B cells expressing IKKβ* express increased levels of NK cell receptor activating ligands and some SLAM family members to enhance NK cell-target cell interactions.

At the time of transfer, IKKβ*-transduced B cells, on average, expressed approximately 10 times more SLAM on the surface of the cells compared to the empty vector control cells (Fig 6.5a). CARD11* expressing B cells also expressed more SLAM compared to the empty vector controls, but the levels were 2-fold lower than the IKKβ*-B cells (Fig 6.5a). The levels of Ly9 and Rae-1, which are both activating ligands for receptors on NK cells, were also increased on the surface of IKKβ*-expressing B cells compared to the empty vector and CARD11*-B cells (Fig 6.5b, c). However, the other SLAM family members: CD84 and Ly108 were expressed at lower or comparable levels to the empty vector and CARD11*-B cells (Fig 6.5d, e).

To investigate whether NK cell-mediated killing played a role in controlling the numbers of IKKβ*-B cells in vivo, we used two complementary approaches, which involved the transfer of transduced B cells into NK cell-deficient Rag1γC−/− recipients, and the depletion of NK cells in Rag1−/− recipients. Equal numbers of IKKβ*-expressing B cells were transferred into Rag1−/− and Rag1γC−/− recipients (Fig 6.5f). At Day 10 after transfer, lower numbers of IKKβ*-B cells compared to empty vector control B cells were detected in spleens of both types of recipients, indicating that the absence of NK cells did not affect the survival and expansion of IKKβ*-expressing B cells in vivo (Fig 6.5g). To confirm these findings, we repeated the transfer experiment using Rag1−/− recipients, which were either treated with an anti-NK1.1 depleting antibody or with an isotype control antibody. The recipients were treated with the NK cell depleting antibody or the isotype control 2 days prior to cell transfer and every 2 days after transfer up to Day 10. Equal numbers of IKKβ*-expressing B cells were transferred into the NK cell depleting antibody or the isotype control treated Rag1−/− recipients at Day 0 (Fig 6.5i). At Day 10, 4-fold less NKG2D+ NK cells were detected in the spleens of the Rag1−/− anti-NK1.1-
treated recipients compared to the isotype control treated recipients, indicating that partial depletion of NK cells occurred (Fig 6.5j). However, the numbers of IKKβ*-B cells remained lower than empty vector expressing B cells in spleens of both the NK cell-depleted or the control recipients, indicating that the reduction of NK cells in Rag1<sup>−/−</sup> did not influence the survival of IKKβ*-expressing B cells <em>in vivo</em> (Fig 6.5k). Together, these findings indicate that NK cell-mediated elimination does not account for the lack of expansion of IKKβ*-expressing B cells in Rag1<sup>−/−</sup> recipients.

**Investigating intrinsic mediators of cell death**

In addition to receptor-mediated pathways, cell death is also regulated by intrinsic pathways which are primarily controlled though the interaction between pro- and anti-apoptotic members of the BCL2 family (Youle and Strasser 2008). Apoptotic stimuli such as increased intracellular reactive oxygen species, DNA damage (p53-mediated), the unfolded protein response, and the deprivation of growth factors activate BCL2 family BH3-only proteins leading to the activation of pro-apoptotic molecules BAX and BAK and mitochondrial outer membrane permeabilisation (Tait and Green 2010). Anti-apoptotic BCL2 proteins prevent increased mitochondrial permeability by binding to BH3-only proteins and activated BAX or BAK (Tait and Green 2010). Following increase in mitochondrial membrane permeability, release of various proteins from the mitochondrial intermembrane space promotes caspase activation and apoptosis (Tait and Green 2010). Here, we investigate whether the p53-dependent (Vousden and Lane 2007, Vazquez et al. 2008) and the mitochondria-dependent cell death pathway (Tait and Green 2010) contribute to the <em>in vivo</em> elimination of B cells expressing IKKβ*.

We transduced B cells from wild-type mice and an ENU-induced loss-of-function p53 mutant strain, B-Blast. The B-Blast mouse strain bears an inactivating point mutation in the DNA binding domain of Trp53, which cripples the function of the protein (Yates 2003). Akin to p53-deficiency, mice carrying a homozygous p53 B-Blast mutation develop tumours such as B and T cell lymphoma and primitive haemopoietic leukaemia at 2-4 months of age (Yates 2003). Approximately equal
numbers of wild-type and homozygous B-Blast \((Trp53^{Bbl}/Bbl)\) IKKβ*-expressing B cells were transferred into \(Rag1^{-/-}\) recipients at Day 0 (Fig 6.6a). Wild-type and \(Trp53\) mutant B cells transduced with CARD11\(^{Mut10}\) (CARD11*) and empty vector were also transferred (Fig 6.6a). After 10 days, the number and percentage of EGFP+ B cells expressing CARD11* increased, while the numbers and percentage of EGFP+ B cells expressing IKKβ* decreased relative to the empty vector transduced controls (Fig 6.6b). However, approximately 5-fold more \(Trp53^{Bbl}/Bbl\) than wild-type IKKβ*-expressing B cells were present in the spleens of the recipients at Day 10, indicating that activation of the p53 pathway may play a role in restricting the expansion of IKKβ*-B cells \(in vivo\) (Fig 6.6c).

Next, we asked whether the enforced expression of the anti-apoptotic gene \(Bcl2\) from the \(Vav1\) promoter would be sufficient to prevent the elimination of IKKβ*-B cells \(in vivo\). The numbers of mature B cells in this \(Vav1-Bcl2\) transgenic strain is increased by approximately 4-fold (Ogilvy et al. 1999). The \(vav-Bcl2\) B cells survive for longer periods of time compared to wild-type cells when cultured in media alone (Ogilvy et al. 1999). Moreover, \(vav-Bcl2\) mice older than by 10 months develop follicular lymphoma (Egle et al. 2004). Thus, to test the hypothesis that increased expression of the anti-apoptotic molecule \(BCL2\) would prevent the elimination of IKKβ*-B cells \(in vivo\), we transferred equal numbers of wild-type and \(vav-Bcl2\) IKKβ*-expressing B cells into \(Rag1^{-/-}\) recipients at Day 0 (Fig 6.6d). Wild-type and \(vav-Bcl2\) B cells transduced with CARD11* and empty vector were transferred as controls (Fig 6.6d). At 10 days post-transfer, the number and percentage of EGFP+ B cells expressing CARD11* increased, while the numbers and percentage of EGFP+ B cells expressing IKKβ* decreased relative to the empty vector transduced controls as observed in the \(Trp53^{Bbl}/Bbl\) experiment (Fig 6.6c, e). Interestingly the number of \(vav-Bcl2\) IKKβ*-expressing B cells increased from Day 3 to Day 10, while the numbers of wild-type IKKβ*-expressing B cells decreased over the same time period (Fig 6.6f, g). There were approximately 10-fold more \(vav-Bcl2\) than wild-type IKKβ*-expressing B cells in the spleens of the recipients at Day 10 relative to Day 3 (Fig 6.6f, g). These findings indicate that overexpression of \(Bcl2\) could partially protect the IKKβ*-expressing B cells from counter-selection in \(Rag1^{-/-}\) recipients, although this was insufficient to restore growth of the B cells as
observed in vitro or phenocopy the expansion induced by expression of gain-of-function CARD11*.

**Discussion**

Here, we initially sought to investigate whether the expression of constitutively active IKKβ could phenocopy the effects of gain-of-function CARD11 mutants in normal B cells and in the context where the B cells encounter self-antigen in vivo. When introduced in activated B cells in culture, B cells expressing the gain-of-function IKKβS177E:S181E and IKKβK171E proliferated to approximately 6-fold the starting numbers over a period of 3 days (Fig 6.1). This result confirms that the IKKβK171E mutant allele found in 3% of splenic marginal zone lymphomas is indeed oncogenically activated comparably to the experimentally engineered IKKβS177E:S181E allele bearing two phosphomimetic mutations. However, paradoxically, when the same B cells were transferred in vivo almost no EGFP+ B cells expressing either of the activated IKKβ alleles could be detected in the spleens of the recipients, indicating that the IKKβ-transduced B cells were lost or unable to survive in vivo (Fig 6.2a, b, c). This result revealed a key difference between the effects of expression of gain-of-function IKKβ and CARD11 in activated B cells in vitro.

To explain these findings, we hypothesised that expression of gain-of-function IKKβ in activated B cells sensitises the cells to ‘die’ either through the upregulation of death-signalling receptors such as FAS and TNFR (Cho et al. 2009, He et al. 2009, Strasser et al. 2009, Zhang et al. 2009), or by a upregulation of ligands on the surface of the B cells which activate killer cells or phagocytes (Jadus et al. 1998, Vivier et al. 2008). These death-promoting factors or cells would only be present in vivo, and thus would not affect the expansion of the IKKβ-transduced B cells in vitro. We initially surveyed a number of different tissues at 24h and 72h after transfer, including the lymph nodes, bone marrow, peripheral blood and the lungs to ensure that the IKKβ*-expressing B cells were indeed being eliminated from the host, and not just being excluded from the spleen. We experimentally tested a number of different possibilities, and found that the increased expression of FAS
receptor on the surface of IKKβ*-transduced B cells and the absence of TNFR1/2 did not affect the survival of the IKKβ*-expressing B cells in vivo. We also found that NK cell-mediated elimination does not account for the lack of expansion of IKKβ*-expressing B cells in Rag1⁻/⁻ recipients. Moreover, we found that the p53 pathway may play a minor role in restricting the expansion of IKKβ*-B cells in vivo, as 5-fold more Trp53<sup>Bhi/Bbi</sup> than wild-type IKKβ*-expressing B cells were present in the spleens of the recipients at Day 10. Interestingly, overexpression of Bcl2 could partially protect the IKKβ*-expressing B cells from counter-selection in Rag1⁻/⁻ recipients, although this was insufficient to restore growth of the B cells as observed in vitro or phenocopy the expansion induced by expression of gain-of-function CARD11.

Given our results, the mechanisms by which the IKKβ*-expressing B cells are unable to proliferate and are eliminated in vivo remain unclear. The possibility that IKKβ*-B cells express a macrophage-stimulating ligand and are thus phagocytosed in vivo is yet to be tested. However, tumour-associated macrophages have been reported to have a dual role in neoplasms. Although macrophages may kill neoplastic cells following activation by stimulating cytokines such as IL-2, interferon and IL-12 (Brigati et al. 2002, Tsung et al. 2002), macrophages also produce a number of potent angiogenic and lymphangiogenic growth factors and proteases, which potentiate neoplastic progression (Schoppmann et al. 2002). Moreover, a tumour cell line expressing a membrane form of macrophage stimulating factor was shown to be efficiently phagocytosed by macrophages (Jadus et al. 1998). Thus, to specifically test whether IKKβ*-expressing B cells are phagocytosed by macrophages in vivo, cells could be transferred into control recipients or recipients treated with liposomes containing clodronate to deplete macrophages (Van Rooijen and Sanders 1994, Lundmark et al. 2013).

Moreover, to firmly rule out the possibility of death-receptor mediated elimination, the co-expression of cFLIP with IKKβ* in activated B cells should be tested. cFLIP has been characterised as a general blocker of receptor-mediated apoptosis as it has been shown to protect against apoptosis induced by several death receptors, including CD95, tumour-necrosis factor (TNF) receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptor 1, TRAILR2 and TNFR-related
apoptosis-mediating protein (TRAMP) (Han et al. 1997, Irmler et al. 1997, Srinivasula et al. 1997, Rasper et al. 1998). Moreover, given the possibility of a novel IKKβ*-induced death mechanism, an unbiased shRNA screen could be set up to identify potential pathways mediating the elimination of IKKβ*-B cells in vivo. For instance, shRNA libraries (obtained from Dr Marco Herold, WEHI, Melbourne; libraries provided by Cellecta) could be used to co-transduce IKKβ*-expressing B cells, which will transferred in vivo. The presence of any expanded B cell clone expressing IKKβ* (EGFP) would be monitored from the peripheral blood of the recipients. The identity of the shRNA and its target would then be determined by sequencing the FACS-sorted cell population using a set of specific primers. Interestingly, about 3% of cases with splenic marginal zone lymphoma express the IKKβ\textsuperscript{K171E} mutant, which we have shown to be a constitutively active mutant form of IKKβ that appears as active in B cells as the widely studied IKKβ\textsuperscript{S177E;S181E} allele (Rossi et al. 2011). It is notable that IKKβ\textsuperscript{K171E} has not been found in ABD-DLBCL, and this may be explained by the observation here that B cells expressing this allele are eliminated and do not proliferate in vivo, in contrast to B cells expressing activated alleles of CARD11 or MyD88 that are recurrently found in DLBCL (Jeelall et al. 2012, Wang et al. 2014). Insights to the mechanisms mediating the elimination of the IKKβ*-expressing B cells in vivo could potentially be obtained through the whole genome sequencing of human tumors driven by gain-of-function IKKB mutations. Moreover, transgenic mice expressing IKKβ\textsuperscript{S177E;S181E} in B cells only develop lymphomas when combined with absence of BLIMP1, after a long latency (Calado et al. 2010), indicating that additional mutations are required to transform the B cells into lymphoma. Similarly, an analysis of the whole genome of the lymphoma formed in this mouse model could be performed to determine the other pathways that cooperate with gain-of-function IKKβ*. 
Fig 6.1 Expression of gain-of-function IKKβ activates NF-κB signaling and results in B cell expansion in vitro. (a) Outline of the experimental strategy to examine the consequences of acquiring gain-of-function IKKβ mutations in antigen-activated B cells. (b) Flow cytometry plots analyzing the EGFP+ B cells transduced with the indicated retroviral vectors at Day 0 and Day 3. The numbers on the plots indicate the percentage of the EGFP+ population. (c) Relative number of live EGFP+ and EGFP- B cells in tissue culture without antigen or anti-CD40 for three days, expressed as percentage of the starting number on Day 0. Numbers are mean ± SEM of 3 independent cultures, and data are representative of 3 independent experiments.
(a) Diagram showing the vector with LTR, IKKβ, IRES, EGFP, and LTR. The controls are:
(i) Empty vector
(ii) CARD11^{Mut10}

(b) Flow cytometry graphs for different constructs:
- Empty vector
- IKKβ^{K171E}
- IKKβ^{S177E,S181E}
- CAR011^{Mut10}

Day 0:
- Empty vector: 47.5
- IKKβ^{K171E}: 25.7
- IKKβ^{S177E,S181E}: 25.3
- CAR011^{Mut10}: 3.16

Day 3:
- Empty vector: 35.6
- IKKβ^{K171E}: 52.4
- IKKβ^{S177E,S181E}: 49.2
- CAR011^{Mut10}: 71.2

(c) Graphs showing the number of EGFP+ cells over time for different constructs:
- Empty vector
- CARD11^{Mut10}
- IKKβ^{K171E}
- IKKβ^{S177E,S181E}
**Fig 6.2 Elimination of B cells overexpressing constitutively active IKKβ in vivo.** (a) Flow cytometry plots analyzing the EGFP+ B cells transduced with the indicated retroviral vectors at Day 10 post-transfer in non-transgenic and HEL-transgenic recipients on a Rag1−/− background. (b) Number of EGFP+ B cells expressing the indicated vectors at the time of transfer (input). Number of EGFP+ B cells expressing the indicated vectors detected in Rag1−/− recipients at the Day 10 post-transfer. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 5 independent experiments.
Fig 6.3 Increased FAS expression on IKKβ*-B cells does not mediate in vivo elimination. (a) Expression of in EGFP+ B cells transduced with the indicated vectors at Day 0 (n=3; data is representative of 2 independent microarray experiments). (b) Representative histogram overlay of FAS expression, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of FAS expressed on the surface of cultured B cells expressing the indicated vectors at Day 0 (n=3; experiment repeated 8 times). Representative histogram overlay of (c) FAS and (d) CD25 expression, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of FAS expressed on the surface of cultured wild-type and Fas-deficient B cells expressing the indicated vectors at Day 1 (n=3; experiment repeated 2 times). Representative flow cytometry plots of the EGFP+ wild-type and Fas-deficient B cell populations transduced with the indicated retroviral vectors at (e) the time of transfer Day 0 and (f) Day 10 post-transfer. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 2 independent experiments.
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**Fig 6.5 NK cell-mediated elimination is not involved in IKKB*-B cell elimination.** Representative histogram overlay of (a) SLAM, (b) Ly9, (c) Rae1, (d) CD84, (e) Ly108 expression, and corresponding column graph of the geometric mean fluorescence intensity (MFI) of the cell surface proteins on cultured B cells expressing the indicated vectors at Day 0 (n=3; experiment repeated 2 times). Representative flow cytometry plots of the EGFP+ wild-type B cell populations transduced with the indicated retroviral vectors at (f) the time of transfer Day 0 and (g) Day 10 post-transfer in *Rag1-/-* and *Rag1γC-/-* (NK cell deficient). The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 2 independent experiments. (h) Representative flow cytometry plots of the EGFP+ wild-type B cell populations transduced with the indicated retroviral vectors at the time of transfer Day 0. (i) Representative histogram and corresponding column graph with the number of NK cells present in the spleen of *Rag1-/-* recipients either treated with an anti-NK1.1 cell antibody (PK136) or with an isotype control antibody. (j) Representative flow cytometry plots of the EGFP+ wild-type B cell populations transduced with the indicated retroviral vectors in the spleen of *Rag1-/-* recipients either treated with an anti-NK1.1 cell antibody (PK136) or with an isotype control antibody at Day 10 post-transfer. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 2 independent experiments.
Fig 6.6 Elimination of IKKβ*-B cells cannot be prevented by loss-of-function p53 and overexpression of Bcl2 (intrinsic death pathways). Representative flow cytometry plots of the EGFP+ wild-type and p53^{BBlast/Bblast} (p53^{bbl/bbl}) B cell populations transduced with the indicated retroviral vectors at (a) the time of transfer Day 0 and (b) Day 10 post-transfer. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. (c) Number wild-type and p53^{bbl/bbl} of I IKKβ*-expressing B cells in the spleens of recipients at Day 10. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 2 independent experiments. (d) Number and percentage of EGFP+ wild-type and Vav-Bcl2 transgenic B cells at Day 0 (time of transfer). (e) Representative flow cytometry plots of the EGFP+ wild-type and Vav-Bcl2 transgenic B cell populations transduced with the indicated retroviral vectors at Day 10 post-transfer. The corresponding column graphs indicate the numbers and percentages of EGFP+ B cells. (f) Relative number of EGFP+ wild-type and Vav-Bcl2 transgenic B cells detected in Rag1^{-/-} recipients at the indicated time points (relative to input). (g) Relative number of EGFP+ wild-type and Vav-Bcl2 transgenic B cells detected in Rag1^{+/-} recipients at Day 10 relative to Day 3. Numbers are mean ± SEM of 3-5 recipients, and data is representative of 2 independent experiments.
Chapter 7: Characterisation of germline gain-of-function

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Introduction

The previous results chapters investigated gain-of-function *CARD11* alleles that had been found as somatic mutations in malignant B cell populations. Recently, gain-of-function *CARD11* mutations were discovered in the germ-line DNA of five patients with polyclonal B cell lymphocytosis, characterising a newly described disease referred to as BENTA (B cell Expansion with NF-κB and T cell Anergy)-like disease (Lenz et al. 2008, Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). Three of the five patients described were related (father and two children) and have the same E127G mutation (here referred to as Mut12) in the coiled-coil domain of *CARD11* (Snow et al. 2012). Note that the E127 residue refers to the numbering originally used by Snow et al and by the Staudt group in their earlier description of somatic *CARD11* mutations (Lenz et al. 2008, Snow et al. 2012). This numbering has been revised in the current Consensus Coding DNA Sequence (CCDS) annotation of CARD11, by addition of 7 amino acids to the N-terminus starting with the most 5’ methionine codon in the *CARD11* cDNA. For ease of reference to the published studies, the old numbering used by Snow et al and Lenz et al is used in this chapter to describe the mutations described in their publications (Lenz et al. 2008, Snow et al. 2012). To convert these to the current *CARD11* numbering, the old codon numbers need to be increased by 7.

In the patients with germline *CARD11* mutations, they all presented with splenomegaly and blood B cell lymphocytosis in infancy, with dramatically elevated levels of IgM+IgD+CD19+CD20+ circulating B cells, while T cell numbers were normal (Snow et al. 2012). In addition, CD10+ immature and transitional B cells were dramatically increased to comprise nearly half of the circulating B cells, while CD27+ memory and class-switched B cells were reduced in the blood of the patients. Interestingly, the two children failed to generate robust responses to polysaccharide-based vaccines and their serum IgM levels were below normal ranges (Snow et al. 2012). Histological examination of archival lymphoid organs from the father and tonsil samples from the children showed follicular hyperplasia with an excess of IgD+ naïve B cells (Snow et al. 2012).
Despite B cell lymphocytosis since infancy in 1971 the father only developed a B cell neoplasm in 2006, comprising a monoclonal expansion of CD5\textsuperscript{hi}, IgM/IgD\textsuperscript{low}, CD20\textsuperscript{low} B cells bearing a monoallelic deletion of tumor suppressor genes located on chromosome 13q14.3, consistent with B cell chronic lymphocytic leukemia (Snow et al. 2012). However, no evidence of autoimmune disease was found in these patients (Snow et al. 2012). The fourth unrelated patient had a germline G116S CARD11 mutation and presented with a similar B cell lymphocytosis-like disease as the other three patients, with greatly increased CD10+ circulating immature B cells and reduced CD27+ memory B cells (Snow et al. 2012). Staudt and colleagues also previously found the G116S CARD11 mutation (Mut3) in a ABC DLBCL sample, in this case occurring as a somatic mutation (Lenz et al. 2008). The effects of the Mut3 gain-of-function mutation in B cells were described in Chapters 3 and 5. However, unlike the other patients, the number of CD8 T cells and NK cells were elevated. Interestingly, this patient had anti-nuclear antibodies, anti-C3 antibodies and anti-neutrophil antibodies in the serum, indicative of systemic autoimmunity (Snow et al. 2012).

Both the E127G (Mut12) and G116S (Mut3) CARD11 mutants formed aggregates and increased NF-κB activation when introduced into a B cell line (Snow et al. 2012). E127G CARD11 formed spontaneous aggregates and more nuclear p65 were detected in naïve B cells isolated from the patients, indicating that NF-κB signalling was constitutively activated by gain-of-function CARD11. Moreover, the E127G CARD11 expressing B cells proliferated more in response to a range of B cell stimuli and expressed more NF-κB-responsive surface markers such as CD25, CD83 and CD86 compared to control B cells (Snow et al. 2012). In light of these findings, we aimed to further study this human disease by introducing the gain-of-function E127G CARD11 mutant in mouse bone marrow-derived hematopoietic stem cells and reconstitute irradiated recipients with the retrovirally-transduced cells. Given that we had already studied the G116S CARD11 mutant (Mut3), we focused on the E127G CARD11 (Mut12) in the experiments presented in this results chapter.
CARD11-transduced bone marrow-derived stem cells fail to reconstitute hematopoietic compartments

Since increased numbers of circulating immature and transitional B cells is the most striking abnormality in people with germ-line CARD11\textsuperscript{Mut12}, we sought to model this in mice by retrovirally expressing CARD11\textsuperscript{Mut12} in all stages of B cell development from hematopoietic stem cells (HSCs). To retrovirally transduce HSCs we adapted an optimised method used to produce TCR-retrogenic mice, by co-culturing bone marrow containing HSCs with a stable retrovirus particle producer cell line (Holst et al. 2006). GPE86+ producer cells were spin-infected with monocistronic retroviral particles containing pro-viral RNA encoding for a fusion protein comprising EGFP attached to the N-terminus of either CARD11\textsuperscript{WT}, CARD11\textsuperscript{Mut10}, or CARD11\textsuperscript{Mut12}, or encoding EGFP only (Fig 7.1a, b). The stably transduced EGFP+ GPE86+ cells were FACS sorted and either expanded in culture or stored at -80°C for future use. Bone marrow cells were collected from wild-type C57BL6 mice and cultured with IL-6, IL-3 and mSCF to stimulate the hematopoietic stem cells to proliferate (Fig 7.1c). The GPE86+ producer cells were irradiated at Day 0, and cultured to form a uniform monolayer, onto which activated hematopoietic stem cells were co-cultured 24 hours later. On Day 3 the mixture of transduced and non-transduced bone marrow cells was injected into the circulation of irradiated \textit{Rag1}^{-/-} recipients (Fig 7.1c, d).

The cells of the immune system are generated from self-renewing progenitors in the bone marrow (Morrison et al. 1995). Importantly, when transplanted into a lethally irradiated mouse, the donor HSCs colonize bone marrow niches to replace the irradiated recipient’s HSCs, undergo self-renewing division to establish lifelong marrow engraftment, and some of their progeny differentiate giving rise to cells of all hematopoietic lineages (Osawa et al. 1996). In addition, a phenotypically identical donor-derived population that maintains all of the functional characteristics of HSCs can be isolated from transplanted recipients (Osawa et al. 1996). On receiving differentiation signals, HSCs commit to either the lymphoid or the myeloid lineage (Morrison et al. 1995, Kondo et al. 2001). The cells of different lineages can be identified by the expression of unique cell surface proteins. For
instance, B cells, T cells and neutrophils can be differentiated as they uniquely express B220, TCRβ and Gr1 respectively (Morrison et al. 1995, Kondo et al. 2001).

We assessed efficiency of engraftment of the transduced hematopoietic stem cells and the fraction that carried the different vectors and produced EGFP by analyzing Gr1+ neutrophils in the blood 15 days post-reconstitution (Fig 7.2a). Gr1+ cells were used as an indicator of successful engraftment of transduced hematopoietic stem cells because neutrophils have a short lifespan of about 5 days and are rapidly replaced by newly differentiated neutrophils derived from bone marrow progenitors (Pillay et al. 2010, Amulic et al. 2012). At this early timepoint, EGFP+ Gr1+ cells were detected in the blood of recipients of bone marrow transduced with each of the vectors. By contrast, when the transplanted mice were bled and analysed again 8 weeks after marrow transplantation, EGFP-expressing Gr1+, B220+ and TCRβ+ cells were only detected in recipients that received the empty vector-transduced stem cells (Fig 7.2b). By contrast, no EGFP+ myeloid or lymphoid cells were detected in the recipients of CARD11-transduced bone marrow (Fig 7.2b). The presence of blood neutrophils EGFP-CARD11 fusion protein 15 days after marrow transplantation but not after 8 weeks could be explained by two possibilities. First, HSCs were transduced with the EGFP-only and EGFP-CARD11 vectors but those expressing the fusion EGFP-CARD11 fusion protein were unable to self-renew to maintain long-term neutrophil production. Alternatively, virus particles encoding the fusion protein were present in lower titre than those encoding EGFP alone, and the low titre was sufficient to transduce rapidly dividing myeloid progenitor cells but not the self-renewing HSCs. These results suggest that enforced expression of CARD11 in hematopoietic stem cells may be detrimental to stable engraftment. Therefore, we were unable to study the effects of gain-of-function CARD11 on the development and homeostasis of lymphoid cells. Similar problems with achieving stable marrow transplantation with CARD11-encoding vectors were observed in independently performed experiments by our collaborators (Dr A Snow and Dr M Lenardo, NIH, USA). The mechanism mediating this ‘toxicity’ remains unclear.
CARD11<sup>E127G</sup> increases CD25 expression, cell size and proliferation, but does not provide activated B cells with a survival advantage <i>in vitro</i>

To study the effects of the E127G (Mut12) CARD11 mutant in mature, already activated B cells, we transduced activated spleen B cells using the experimental system described in Chapter 3 (Fig 3.1). The percentage and number of CARD11<sup>Mut12</sup>-expressing B cells decreased from approximately 9% at Day 0 to 4% at Day 3, following a similar trend as the control B cells expressing EGFP only or CARD11<sup>WT</sup> (Fig 7.3a, b). These results indicated that the population of B cells with enforced CARD11<sup>Mut12</sup> does not increase in number from proliferation over a period of 3 days when cultured in media alone. However, CARD11<sup>Mut12</sup>-B cells expressed more of the NF-κB-responsive marker CD25 on the cell surface and had higher FSC-A compared to the EGFP only and CARD11<sup>WT</sup>-B cells at Day 0, indicating increased activation of NF-κB signalling (Fig 7.3c). In addition, B cells expressing CARD11<sup>Mut12</sup> went through one round of cell division as measured by dilution of cell trace violet (CTV), while the control B cells expressing EGFP only or CARD11<sup>WT</sup> did not divide in the three day period in culture (Fig 7.3d). The effects of CARD11<sup>Mut12</sup> on activation and growth inducing signals were milder compared to CARD11<sup>Mut10</sup>, as both the percentage and number of CARD11<sup>Mut10</sup>-B cells increased during the same time period (Fig 7.3a). Moreover, CARD11<sup>Mut10</sup>-B cells were larger in size and expressed more CD25 at Day 0, and went through two extra rounds of cell division compared to CARD11<sup>Mut12</sup>-B cells (Fig 7.3a, d, e, f). These results are consistent with the reported findings that CARD11<sup>Mut12</sup> acts as a gain-of-function version of CARD11. However the gain-of-function caused by Mut12 E127G is weaker than CARD11<sup>Mut3</sup>, which does stimulate some increase in the transduced population in culture (Fig 3.3 in Chapter 3). By the same assay, Mut3 G123S, which is present in the germline of patient 4 reported by Snow et al, is a weaker CARD11 gain-of-function than the most potently activating lymphoma somatic mutations Mut10 (L232LI) and Mut2 (L251P).
**CARD11\textsuperscript{E127G (Mut12)} prevents self-antigen induced B cell death *in vivo***

Since CARD11\textsuperscript{Mut12} appeared to be one of the most weakly activating of the coiled-coil mutations, we asked whether it would be able to protect activated B cells from self-antigen mediated deletion *in vivo*, which we found was a general property of gain-of-function lymphoma-derived CARD11 mutants (Chapter 5). B cells from Ig\textsuperscript{HEL} transgenic mice were transduced with EGFP-CARD11 fusion protein vectors encoding CARD11\textsuperscript{Mut12}, CARD11\textsuperscript{Mut10}, CARD11\textsuperscript{WT} or EGFP only, and the B cells were transferred into non-transgenic and HEL-transgenic recipients (Fig 5.4a, b). The EGFP+ B cells from the spleens of the recipients were analysed 10 days after transfer. In the non-transgenic recipients, which do not express HEL, B cells from all the transduction groups could be detected (Fig 7.4a). Consistent with the results from the *in vitro* growth assay, the EGFP+ subset of CARD11\textsuperscript{Mut12}-B cells and the control CARD11\textsuperscript{WT} and EGFP only B cells did not increase *in vivo*, measured as a percentage of the transferred B cells, whereas CARD11\textsuperscript{Mut10}-expressing B cells increased from 3.5% of the input B cells to reach 77.8% by day 10 (Fig 7.4a, b). Thus, CARD11\textsuperscript{Mut12} had little effect on the accumulation of B cells *in vivo* when the cells were not receiving antigen stimulation.

By contrast, the expression of CARD11\textsuperscript{Mut12} in B cells transferred into the HEL-transgenic protected the B cells from self-antigen induced B cells death, while control B cells expressing EGFP only or CARD11\textsuperscript{WT} were deleted in the presence of HEL self-antigen (Fig 7.4a, c). The frequency of EGFP+ cells expressing CARD11\textsuperscript{Mut12} increased from 4% of input B cells to reach 47.4% after 10 days in the HEL-transgenic recipients. Measured as total numbers, there were 3-fold more B cells expressing CARD11\textsuperscript{Mut12} and CARD11\textsuperscript{Mut10} in HEL-transgenic recipients compared to non-transgenic recipients, indicating that in the presence of self-antigen both mutant versions of CARD11 protected the B cells from death and induced proliferation of self-reactive B cells (Fig 7.4c).
Only B cells expressing high levels of CARD11^{E127G} expression differentiate into plasmablasts

In previous chapters, we observed that B cells expressing CARD11^{Mut10} adopted a FSC-A^{high} B220^{low} CD19^{low} phenotype brought about by BLIMP1-dependent differentiation into plasmablasts (Chapter 4). Thus, we examined whether CARD11^{Mut12}.B cells would also differentiate into plasmablasts when stimulated to increase in number in HEL-transgenic recipients. Interestingly, more than 80% of B cells expressing CARD11^{Mut12} retained expression of B220 at Day 10 in HEL-transgenic recipients, unlike CARD11^{Mut10}.B cells in HEL-transgenic recipients where less than 10% retained B220. There was a similar retention of CD19 by the majority of CARD11^{Mut12} expressing cells (Fig 7.5a). CARD11^{Mut12} nevertheless induced 20% of B cells to differentiate into B220^{low} plasmablasts, compared to control B cells expressing EGFP only in non-transgenic recipients where 95% still expressed B220 at Day 5 (Fig 7.5a). These results indicate that a much smaller fraction of CARD11^{Mut12}.B cells adopted a plasmablast phenotype than cells expressing any of the lymphoma-associated mutations, including those at the weaker end of the spectrum: Mut6 and Mut3.

To investigate why only a fraction of B cells expressing CARD11^{Mut12} differentiated into plasmablasts, the EGFP+ B cells were sub-divided into EGFP low and EGFP high fractions containing equal number of cells (Fig 7.5b). Since EGFP was fused to the N-terminus of the mutant CARD11 protein, the levels of EGFP in the B cells directly correlate with the relative amount of CARD11 expressed. Interestingly, CARD11^{Mut12}.B cells expressing high levels of EGFP adopted the FSC-A^{high}, B220^{low} and CD19^{low} phenotype, while the CARD11^{Mut12}.B cells in the EGFP^{low} fraction retained expression of B220 and CD19, and were smaller in size (Fig 7.5b). These results potentially highlight two signalling thresholds downstream of CARD11. The first threshold for maintaining the survival of self-reactive B cells which continuously engage with self-antigen potentially requires lower levels of CARD11 activity, as CARD11^{Mut12} protects B cells from self-antigen mediated death, irrespective of levels of expression. The second threshold drives the self-reactive B cells to differentiate into plasmablasts, requiring higher levels of CARD11 activity.
which in the case of the weak gain-of-function CARD11$^{\text{Mut12}}$ only occurs in B cells that express it at high levels.

**Characterization of a gain-of-function ENU-induced *Card11* (M365K) mouse strain**

The findings above provide information on the consequences of retroviral expression of CARD11 gain-of-function mutations that were found in the germline of people with unusually large numbers of circulating immature and naïve B cells, but did not provide an experimental model to investigate the developmental consequences for immature and naïve B cells. Since mRNA from the normal germline *Card11* gene increases dramatically during the differentiation of pre-B cells into immature B cells (Fig 7.6a), an ideal experimental model would introduce a gain-of-function coiled-coil mutation into the otherwise normal germline *Card11* gene.

As part of a large project to accumulate and bank ENU-induced mutations in mouse germline genes, identified by exome sequencing of first generation offspring of C57BL/6 mice exposed to ENU (N-ethyl-N-Nitrosourea), we have recently discovered a founder mouse with an A to T nucleotide substitution at position 140,889,709 on Chromosome 5. This A→T substitution results in a change from a methionine to lysine at amino acid position 365 (current CCDS numbering), which is part of the highly conserved region of the coiled-coil domain of CARD11 (Fig 7.6b). Interestingly, this CARD11 M365K substitution (referred to as CARD11$^{\text{Mut17}}$) has also been previously reported in several cases of ABC-DLBCL, suggesting that this amino acid change in the conserved coil-coiled domain of CARD11 may give rise to a gain-of-function protein, akin to a number of somatic mutations found in human lymphoma [(Shaffer et al. 2012); Fig 7.6c, d)]. CARD11Mut3 and CARD11Mut17 were found both in the germline of patients with BENTA disease and unrelated cases of ABC DLBCL, while CARD11Mut12 has only be described in three related patients with BENTA disease (Shaffer et al. 2012, Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014). Using the experimental system described in Chapter 3 (Fig 3.1), we found that CARD11Mut3 had more potent growth and
survival inducing effects than CARD11Mut17, which in turn provided more growth and survival signals than CARD11Mut12 in activated spleen-derived mouse B cells (Fig 7.6c). Moreover, CARD11Mut17, the germline ENU-induced Card11 mutation caused on average two rounds of cell division in the transduced B cells, while CARD11Mut3 and CARD11Mut12 induced a single round of division in transduced B cells over a period of 3 days in culture (Fig 7.6d). Thus these results indicate that CARD11$^{M365K}$ appeared to have stronger survival and growth-inducing effects than CARD11$^{E127G}$. In the subsequent part of this results chapter, we characterise the effects of germline CARD11$^{M365K}$ in mice bearing heterozygous and homozygous Card11 mutations, and compare this with the clinical phenotypes recently reported in patients with CARD11-driven polyclonal B cell lymphocytosis-like disease (Snow et al. 2012, Brohl et al. 2014, Turvey et al. 2014).

To investigate whether the germline Card11$^{M365K}$ mutation affected B cell output from hematopoietic stem cells, we compared the frequencies of B cells and their precursors in the bone marrow of Card11$^{M365K/wt}$, Card11$^{M365K/M365K}$ and littermate wildtype control mice (Fig 7.7). Mice homozygous and heterozygous for the Card11$^{M365K}$ mutation had comparable frequencies of total CD19+ cells to wildtype mice, with normal frequencies of immature B cell subsets. These results indicate that B cells development was unaffected by the M365K mutation (Fig 7.7a-i). Interestingly, there was a gene dose-dependent trend of reduced frequencies of recirculating IgM+IgD+ B cells in the bone marrow (Fig 7.7f). However, this trend needs to be confirmed in future experiments with more mice of each genotype (Fig 7.7b,f).

Once developing B cells in the bone marrow have acquired the ability to circulate in the blood, they migrate to B cell niches in secondary lymphoid organs such as the lymph nodes and the spleen where they further mature into follicular and marginal zone (MZ) subsets (Allman and Pillai 2008). To examine the effects of the expression of M365K CARD11 on the formation of the mature naïve B cell subsets, we analysed the spleens of Card11$^{M365K/M365K}$, Card11$^{M365K/Wt}$ and wild-type mice (Fig 7.8). The percentage and number of CD19+ cells in the spleen was comparable across the three genotypes (Fig 7.8a). There was a reduction in the percentage and number of CD93+ immature B cells in spleens of Card11$^{M365K/M365K}$, Card11$^{M365K/Wt}$,
with a reciprocal increase in the numbers of CD93- mature B cells (Fig 7.8b). The frequency and number of CD23+CD23+ follicular B cells were comparable in Card11M365K/M365K, Card11M365K/Wt and wildtype mice (Fig 7.8b). Interestingly, the number of B220+CD93-CD21highCD23low marginal zone B cells were slightly increased in Card11M365K/wt and Card11M365K/M365K spleens compared to wild-type controls, suggesting that MZ B cells may be sensitive to changes in CARD11 signalling (Fig 7.8c). However, unlike patients with BENTA disease, there was no accumulation of B cells, splenomegaly and lymphocytosis-like phenotype in mice with a germline gain-of-function Card11 mutation.

**Enhanced activation of Card11M365K/wt B cells following anti-IgM and LPS stimulation**

Given that CARD11 functions as a scaffold for the recruitment of signalling molecules following B cell receptor activation, we hypothesized that stimulation of Card11M365K/wt B cells with anti-IgM will result in increased expression of NF-κB-regulated markers such as CD25. Thus, we investigated whether the expression of CARD11M365K would affect the activation of splenic B cells by anti-IgM and LPS in vitro. Total splenocytes from wild-type, Card11M365K/wt and Card11ko/ko mice (mice bearing a naturally occurring mutation in the intronic region regulating expression of Card11 resulting in complete loss of CARD11 protein expression; Keisuke Horikawa; Unpublished data. Mice were obtained from Dr Daniel Grey, WEHI, Melbourne) were cultured with increasing concentrations of anti-IgM or LPS, or media alone and B220+7AAD- cells were analysed 24 hours after stimulation. The wild-type B cells expressed higher levels of CD25 with increase in concentration of IgM, whereas there was a more steep increase in the levels of CD25 expressed by B cells from Card11M365K/wt mice (Fig 7.9a). By contrast, there was no increase in CD25 expression on the surface of Card11ko/ko B cells compared to the unstimulated controls (Fig 7.9a). The levels of CD25 on unstimulated B cells of all three genotypes were comparable, indicating that increase in CD25 expression on B cells only occurs after B cell stimulation (Fig 7.9a). The levels of CD25 expressed on LPS-stimulated Card11M365K/wt B cells were increased compared to wild-type and Card11ko/ko B cells (Fig 7.9b), indicating that LPS stimulation may act...
cooperatively with gain-of-function CARD11 to increase signalling to NF-κB transcription factors. Moreover, unlike the B cells stimulated with anti-IgM, the expression of CD25 on B cells activated with LPS did not follow a dose-response relationship as the levels of CD25 did not increase further as the concentration of LPS was increased (Fig 7.9b). Interestingly, the average FSC-A MFI of the LPS stimulated B cells were comparable at 1μg/ml LPS, with only a slight increase in FSC-A of Card11M365K/wt B cells with higher doses of LPS (Fig 7.9c, d). The average FSC-A MFI of the anti-IgM stimulated wild-type and Card11M365K/wt B cells increased with higher concentrations of anti-IgM, with the Card11M365K/wt B cells having higher FSC-A MFI irrespective of the concentration of anti-IgM (Fig 7.9c).

To extend these findings, we also measured CD25 expression and FSC-A MFI on wild-type activated B cells retrovirally transduced with CARD11M365K (also referred to as CARD11Mut17), CARD11WT and EGFP alone (Empty vector). As observed with the Card11M365K/wt B cells, the CARD11Mut17 B cells expressed higher levels of CD25 and had increased FSC-A MFI compared to the EGFP alone and CARD11WT control B cells (Fig 7.9e). In addition, we also compared the effects of enforced expression of CARD11Mut17 to one of the most activating CARD11 mutants, CARD11Mut10 (Chapter 3). CARD11Mut10 B cells expressed 2.5-fold more CD25 and had significantly increased FSC-A MFI compared to B cells expressing CARD11Mut17, indicating that CARD11M365K is a less active gain-of-function than CARD11Mut10. These findings establish that CARD11M365K mutant causes a moderate gain-of-function.

**CARD11M365K increases survival and proliferation of activated B cells in vitro**

Next, we asked whether enforced expression of gain-of-function CARD11M365K (Mut17) would affect the survival and proliferation of activated B cells. We used a similar survival assay as described in Chapter 3, and tracked the numbers of EGFP+ cells cultured in media only for a period of three days. The percentage of CARD11Mut17 B cells was maintained constant over the 3-day period, whereas the percentage of EGFP+ B cells in both control groups gradually decreased over time (Fig 7.10a). The numbers of CARD11Mut17-expressing B cells decreased to 40% of
the starting numbers by Day 3, while the numbers of the CARD11\textsuperscript{WT} and empty vector-transduced B cells reduced to 10% and 20% respectively at the same time-point (Fig 7.10b). These results indicated that the enforced expression of CARD11\textsuperscript{Mut17} in activated B cells provide the cells with some survival advantage. However, CARD11\textsuperscript{Mut17} induce lower growth-inducing signals in activated B cells compared to the lymphoma-derived CARD11\textsuperscript{Mut10}, which causes the increase in the both the percentage and numbers of B cells over a period of three days in culture with media alone (Fig 7.10c, d). The lack of an increase in B cells with CARD11\textsuperscript{Mut17} parallels the findings with other weak gain-of-function alleles like the human germ-line Mut12 described earlier in this chapter (Fig 7.3).

By labeling the cultured B cells with the proliferation-tracking dye, Cell Trace Violet, we measured the average number of cell divisions over the period of 3 days in culture without exogenous growth stimuli. Interestingly, based on a model described in Chapter 3, the CARD11\textsuperscript{Mut17}-expressing B cells made on average two rounds of cell division, while CARD11\textsuperscript{Mut10} B cells made three divisions (Fig 7.10e). B cells transduced with the control EGFP-only or CARD11\textsuperscript{WT} vectors did not undergo any rounds of cell division (Fig 7.10e). These findings indicate that gain-of-function CARD11\textsuperscript{Mut17} induce proliferation signals, but the dividing B cells survive relatively less well compared to those expressing CARD11\textsuperscript{Mut10} so that there is no net increase in EGFP+ frequency and an overall decline in number.

To investigate the effects of germ-line CARD11\textsuperscript{Mut17} on B cell survival and proliferation, we cultured primary B cells from mice with either wild-type, Card11\textsuperscript{M365K/wt} or Card11\textsuperscript{ko/ko} germ-line genes in media only or media supplemented with anti-IgM or LPS. The percentage of B220+7AAD- B cells cultured in media only decreased over the period of 4 days irrespective of genotype, indicating that the germline Card11\textsuperscript{M365K/wt} mutation does not enhance survival nor induce spontaneous proliferation in culture (Fig 7.11a). However, the Card11\textsuperscript{ko/ko} B cells cultured in media only decreased more rapidly compared to Card11\textsuperscript{M365K/wt} and wild-type B cells (Fig 7.11a). This result suggest that the expression of CARD11 provides pro-survival signals to B cells, such that in the absence of extrinsic survival factors such as BAFF, B cells die at a faster rate (Mackay et al. 2003). The provision of a sub-mitogenic dose of 1ug/ml anti-IgM did
not affect the rate of reduction of the Card11\textsuperscript{ko/ko} B cells, suggesting that other pathways downstream of the B cell receptor cannot compensate for the lack of CARD11-induced survival signals (Fig 7.11b). The Card11\textsuperscript{M365K/wt} B cells, however, seemed to survive better than wild-type B cells stimulated with 1\,\mu g/ml anti-IgM, indicating that CARD11\textsuperscript{M365K} may depend on signals from the B cell receptor for its full activation as observed in experiments with lymphoma-derived CARD11 mutants ([Lenz et al. 2008]; Chapter 5- Fig 5.3, 5.4).

The splenocytes were also labeled with Cell Trace Violet (CTV) and the number of cell divisions was analysed after 3 days in culture in the presence of 10\,\mu g/ml anti-IgM or LPS. The wild-type and Card11\textsuperscript{M365K/wt} B cells divided up to 5 times over a period of 3 days of stimulation with 10\,\mu g/ml anti-IgM, while 95\% the Card11\textsuperscript{ko/ko} B cells failed to divide (Fig 7.11c). A larger fraction of Card11\textsuperscript{M365K/wt} B cells than wild-type cells had divided three times or more in response to anti-IgM stimulation, indicating that CARD11\textsuperscript{M365K} amplified the proliferation signals provided by anti-IgM (Fig 7.11c). By contrast, there was no difference in the proliferation response of wild-type and Card11\textsuperscript{M365K/wt} B cells stimulated with 10\,\mu g/ml LPS (Fig 5.8h). Interestingly, the absence of CARD11 in Card11\textsuperscript{ko/ko} B cells affected the proliferation response to 10\,\mu g/ml LPS, with lower fractions of B cells dividing five times or more compared to the wild-type controls (Fig 7.11d). These results indicate that CARD11 could either play a direct or indirect role in LPS-induced proliferation of B cells. The findings further support the gain-of-function phenotype of CARD11\textsuperscript{M365K} as its expression enhances the survival and proliferation of B cells \textit{in vitro} provided they are stimulated via their BCR.

\textbf{Antigen enhances proliferation of CARD11\textsuperscript{M365K} B cells \textit{in vitro}}

In Chapter 5, we have seen that expression of lymphoma-derived CARD11 mutants in self-reactive B cells reversed the effects of HEL, made as a self-antigen, so that instead of inducing B cell death it induced the B cells to proliferate. Here, we asked whether CARD11\textsuperscript{Mut17} (CARD11\textsuperscript{M365K}), which we have established to be a \textit{bona fide} gain-of-function CARD11 mutant, could also prevent antigen induced B cell death. To address this question, we developed an \textit{in vitro} assay to assess the effects of
self-antigen-B cell receptor engagement. Immunoglobulin HEL-transgenic B cells were activated and transduced with CARD11\textsuperscript{Mut17}, CARD11\textsuperscript{Mut10} or CARD11\textsuperscript{WT} as previously described (Chapter 3). At Day 0, the mixture of transduced (EGFP+) and non-transduced (EGFP-) B cells were labeled with Cell Trace Violet (CTV) and cultured in either media only or in media containing 100ng/ml HEL. In cultures with HEL the B cells were constantly stimulated by the antigen but, since they receive no TLR signal or T cell help, "signal 1" from antigen alone induces apoptosis. The survival and proliferation of the B cells were assessed after four days in culture.

The population of total 7AAD-viable B cells in the CARD11\textsuperscript{WT} transduction group decreased six-fold in frequency in cultures with HEL compared to those without (Fig 7.12a). This decrease in viable B cells was fully inhibited in cultures transduced with CARD11\textsuperscript{Mut10} but was only partly inhibited in the CARD11\textsuperscript{Mut17} transduced cells, where the frequency of 7AAD- B cells decreased 3-fold. When the frequency of EGFP+ cells was measured among the remaining viable B cells, EGFP+ cells expressing CARD11\textsuperscript{Mut17} preferentially survived in cultures with HEL, so that their frequency increased from 8% in the absence of HEL to 47% in its presence (Fig 7.12b). These results indicate that CARD11\textsuperscript{Mut17} and, as previously observed in an in vivo transfer assay with CARD11\textsuperscript{Mut10}, the gain-of-function CARD11 mutants are able to prevent antigen induced death.

To further examine the effects of the presence of self-antigen on B cells, we first compared the average FSC-A of B cells expressing CARD11\textsuperscript{WT}, CARD11\textsuperscript{Mut10} and CARD11\textsuperscript{Mut17} at Day 4 in culture. In the presence of HEL, B cells expressing both CARD11\textsuperscript{Mut17} and CARD11\textsuperscript{Mut10} had increased FSC-A, with a fraction of B cells expressing CARD11\textsuperscript{Mut17} having higher FSC-A than CARD11\textsuperscript{Mut10} B cells (Fig 7.12c). By contrast, in the absence of HEL, B cells expressing CARD11\textsuperscript{Mut10} had the highest FSC-A MFI, and cells with CARD11\textsuperscript{WT} or CARD11\textsuperscript{Mut17} had comparable FSC distributions (Fig 7.12c). In addition, as measured by the dilution of CTV, the majority of CARD11\textsuperscript{Mut17} B cells had undergone more rounds of cell division than B cells expressing CARD11\textsuperscript{Mut10} in the presence of HEL (Fig 7.12d). However the reverse was true in the absence of HEL, where B cells expressing CARD11\textsuperscript{Mut10} diluted CTV more than CARD11\textsuperscript{Mut17} B cells (Fig 7.12d). Interestingly, the presence
of HEL seems to restrict the dilution of CTV by \( \text{CARD11}^{\text{Mut10}} \)-B cells, while \( \text{CARD11}^{\text{Mut17}} \)-B cells further dilute CTV (Fig 7.12d). Moreover, the \( \text{CARD11}^{\text{Mut17}} \) B cells which proliferated extensively in the presence of HEL seem to express more EGFP-\( \text{CARD11}^{\text{Mut17}} \) fusion protein compared to the same cells in the absence of HEL, indicating that B cells with a certain amount of \( \text{CARD11}^{\text{Mut17}} \) have preferentially expanded (Fig 7.12e). Collectively these results indicate that continued stimulation by antigen alone, in the absence of “signal 2” from TLRs or T cell help, markedly enhances the growth and proliferation signals induced by \( \text{CARD11}^{\text{Mut17}} \). In addition, the level of \( \text{CARD11}^{\text{Mut17}} \) protein expressed in activated B cells may influence the expansion of B cells in response to self-antigen.

**Discussion**

The findings presented in this results chapter demonstrate that B cells expressing the germline E127G\( \text{CARD11} \) mutant (Mut12), discovered in patients with polyclonal B cell lymphocytosis-like disease, presented with features consistent with increased NF-\( \kappa \)B signalling. \( \text{CARD11}^{\text{Mut12}} \)-B cells expressed more of the NF-\( \kappa \)B-responsive marker CD25 on the cell surface, had higher FSC-A and, akin to lymphoma-derived\( \text{CARD11} \) mutants, E127G \( \text{CARD11} \) protected the B cells from death and induced accumulation of self-reactive B cells. Interestingly, only \( \text{CARD11}^{\text{Mut12}} \)-B cells expressing high levels of EGFP adopted the FSC-A\textsuperscript{high}, B220\textsuperscript{low} and CD19\textsuperscript{low} phenotype, suggesting that levels of gain-of-function \( \text{CARD11} \) expression could affect differentiation of B cells. However, we were not able to model the human disease and study the effects of \( \text{CARD11}^{\text{Mut12}} \) on the development and homeostasis of lymphoid cells, as enforced expression of \( \text{CARD11} \) in hematopoietic stem cells resulted in a failure of reconstitution of both myeloid and lymphoid hematopoietic compartments.

In the clinics, hematopoietic stem cells are important targets for somatic gene therapy, given their availability and adaptability for *in vitro* manipulation and transplant. Gene therapy involving manipulation of HSCs has clearly shown clinical benefits in a number of diseases such as X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al. 2002), and more recently,
Metachromatic leukodystrophy disease (Biffi et al. 2013) and Wiskott-Aldrich syndrome (Aiuti et al. 2013). However, the broader application of gene therapy has been hampered by the oncogenic potential of the random integration of genes and promoters in the genome, and the issue of ‘transgene toxicity’ (Baum et al. 2003, Woods et al. 2006, Aiuti et al. 2012, Cavazzana-Calvo et al. 2012). One example of ‘transgene toxicity’, which potentially parallels what we have observed with CARD11-transduced HSCs, is the expression of the galactosylceramidase (GALC) gene, which is mutated in Globoid cell leukodystrophy (a demyelinating lysosomal storage disease) (Gentner et al. 2010). GALC overexpression was found to be toxic to HSCs, whereas expression in mature haematopoietic lineages, such as lymphocytes and microglia, was well tolerated (Gentner et al. 2010). In this particular case, the technical issue was elegantly overcome by regulating the expression of exogenous GALC through the addition of miR-126 binding sites to the sequence encoding for GALC (Gentner et al. 2010). MiR-126 is a microRNA expressed and functionally active in mouse HSCs but less active in mature haematopoietic cells (Gentner et al. 2010). An alternative option would be to use CARD11 vectors containing a floxed-STOP cassette to regulate the expression of the gene in cells expressing the Cre recombinase enzyme (Lakso et al. 1992).

Recently, Snow and colleagues described the first cases of CARD11-driven polyclonal B cell lymphocytosis and BENTA disease in humans (Snow et al. 2012, Turvey et al. 2014). The disease in these patients was characterised by onset in infancy of splenomegaly and polyclonal expansion of B cells, with defective antibody responses (Snow et al. 2012). The drastic increase in numbers of B cells appeared to result from increased B cell output from the bone marrow, rather than from increased survival or proliferation in the periphery (Snow et al. 2012). However, the analysis of the B cell compartment in mice expressing a heterozygous ENU-induced gain-of-function Card11 mutation (M365K) revealed no B cell lymphocytosis. It is unlikely that the absence of B cell lymphocytosis is due to the germ-line M365K causing less gain-of-function than the germ-line E127G mutation in patients 1-3, because when the different mutations were retrovirally expressed in B cells (Fig7.6c) CARD11M365K appeared to have stronger survival and growth-inducing effects than CARD11E127G. Alternatively, the lack of B cell lymphocytosis in mice with a germ-line gain-of-function Card11 gene could be
that CARD11 is required at different stages of B cell development in humans and mice. In support of this possibility, there are striking differences between humans and mice in the requirement for the cytokine IL-7 and the BCR signaling enzyme BTK during B cell development, as discussed further below (Peschon et al. 1994, Puel et al. 1998). However, the fact that different CARD11 residues were affected could simply account for the differences in the phenotypes in this particular comparison, even though three different CARD11 mutations have now all been found to cause massive increases in circulating naïve and immature B cells (Snow et al. 2012, Brohl et al. 2014).

The development of mature B cells is dependent on coordinated survival and differentiation signals at specific stages of development (Busslinger 2004, Nutt and Kee 2007). In both humans and mice, the interleukin 7 receptor α (IL-7Rα) chain is expressed by early B cell progenitors, and signalling via IL-7Rα paired with the common gamma chain activates signal transducer and activator of transcription 5 (STAT5) (Johnson et al. 2005). In mice, B cell development requires the IL-7Rα chain, and IL-7 signalling, whereas in humans several mutations in the IL7Ra gene have been described that preserve normal numbers of peripheral CD19+ B cells but greatly diminish the numbers of peripheral T cells and natural killer cells (Peschon et al. 1994, von Freeden-Jeffry et al. 1995, Puel et al. 1998, Giliani et al. 2005). Likewise, common gamma chain deficiency in mice causes profound B and T cell deficiency but only T cell deficiency in humans (Noguchi et al. 1993, Cao et al. 1995, Pepper et al. 1995, Tassara et al. 1995). By contrast, mature B cells continue to develop – albeit accumulating in lower numbers – in mice with a null mutation in Btk (Rawlings et al. 1993, Thomas et al. 1993) whereas BTK null mutations in human cause complete failure of B cells to mature (Tsukada et al. 1993, Vetrie et al. 1993).

The fact that mouse B cell development is more dependent on IL-7R signaling whereas human B cell development depends more on BTK signaling may explain the differences in B cell development in humans and mice with a germ-line gain-of-function CARD11 allele. One possible explanation is that tonic BTK signaling is stronger in immature human B cells than in their mouse counterparts, resulting in higher basal phosphorylation of CARD11 by PKCβ. The experiments in this chapter
and in earlier chapters show that the effects of subtle gain-of-function CARD11 mutations are greatly enhanced in B cells that receive constant antigen stimulation. Alternatively, the effects of gain-of-function CARD11 in developing mouse B cells may be neutralised by counter-regulatory mechanisms that are less effective in developing human B cells. Alternatively, CARD11 mRNA and protein may be expressed at higher levels in developing human B cells. In the experiments here, the effects of weaker gain-of-function CARD11 mutations were largest in retrovirally transduced B cells with higher amounts of EGFP-CARD11 fusion protein. Thus, an analysis of the similarities and differences in the transcription factor binding sites and regulatory elements controlling CARD11 expression in human and mouse B cells may provide some insight into the latter hypothesis.

The availability of an ENU-induced CARD11M365K mutant mouse strain presents unique opportunities to further study how gain-of-function CARD11 disrupts the antibody repertoire and function of B cells. Comparative studies could allow us to gain insight into whether regulation of CARD11 expression and signalling are different between mice and humans. Future experiments could also examine the contributions of gain-of-function CARD11 in the development of lymphoma and autoimmunity. This mouse strain could provide interesting parallels to the work done by Rajewsky and colleagues using a gain-of-function IKKβS177E;S181E mouse model. These studies showed that B cells expressing IKKβS177E;S181E could develop and mature without the pro-survival factor BAFF, and these B cells could develop into ABC DLBCL-like disease when combined with loss of Prdm1 (Sasaki et al. 2006, Calado et al. 2010). The consequences of the cooperation of additional genetic lesions such as increased expression of Bcl2, loss-of-function Tnfaip3 or Btg2 with CARD11M365K could also be studied.
Fig 7.1 Experimental strategy for enforced expression of CARD11 in all hematopoietic cells. (a) Outline of the experimental strategy to produce stable GPE86+ retrovirus producer cell lines producing retrovirus particles containing genes encoding for EGFP only (empty vector), wild-type CARD11, CARD11 Mut10, and CARD11 Mut12 (E127G) representing a human germline mutation found in a father and two daughters with polyclonal B cell lymphocytosis. (b) Representative flow cytometry plots of GPE86+ cells lines stably transduced with the indicated vectors. (c) Outline of the experimental strategy to transduce bone marrow-derived hematopoietic stem cells with retrovirus particles. (d) Representative flow cytometry plots of bone marrow cells transduced with the indicated vectors at Day 3. The numbers on the plots indicate the percentage of the EGFP+ population.
(a) **LTR**  **EGFP**  **CARD11**  **LTR**

**Controls:**
(i) Empty vector  
(ii) CARD11\(^{WT}\)

Mut10: L232L1
Mut12: E127G

GPE86+ cell line  (Day 0)

Retroviral transduction  (Day 1)

FACS sort EGFP+ GPE86+ cells  (Day 3)

Expand cells in culture  
Store cells at -80°C

(b) 

![Flow cytometry images](a)

(c) 

Harvest bone marrow cells  (Day 0)

Culture with 50ng/ml IL6, 20ng/ml IL3 and 50ng/ml mSCF

Irradiated GPE86+ cells  
Co-culture GPE86+ cells with cultured bone marrow cells  (Day 1)

Transfer transfused bone marrow cells into irradiated \(\text{Rag}^1\) recipients  (Day 3)

(d) 

![Flow cytometry images](b)
Fig 7.2 CARD11-transduced bone marrow-derived stem cells fail to reconstitute hematopoietic compartments. (a) Representative flow cytometry plots of Gr1+ cells circulating in the blood of irradiated Rag1−/− recipients 15 days after reconstitution with bone marrow-derived stem cells transduced with the indicated vectors. Numbers show the percentage of GR1+ cells that were EGFP+. (b) Representative flow cytometry plots of Gr1+, B220+ and TCRβ+ cells circulating in the blood of irradiated Rag1−/− recipients 50 days after reconstitution with bone marrow-derived stem cells transduced with the indicated vectors. Data are representative of 5 recipients per transduction group. Experiment was performed 2 times.
Fig 7.3 CARD11^{E127G} (Mut12) increases CD25 expression, cell size and proliferation, but does not provide activated B cells with a survival advantage \textit{in vitro}. (a) Representative flow cytometry plots showing the percentage of viable B cells that were EGFP+ expressing the indicated retroviral vectors on Day 0 and Day 3 of culture without anti-CD40. (b) Relative number of live EGFP+ B cells expressing the indicated vectors in tissue culture without antigen or anti-CD40 for three days, expressed as percentage of the starting number on Day 0. Numbers are mean \pm SEM of 3 independent cultures, and data are representative of 3 independent experiments. (c) Geometric mean fluorescence intensity (MFI) of CD25 expression and FSC-A of cultured B cells expressing the indicated vectors at Day 1 (n=3; experiment repeated 3 times). (d) Representative histogram overlay of Cell Trace Violet (CTV) fluorescence in EGFP+ cells with the indicated vectors, and corresponding graph of a model constructed based on the theoretical CTV MFI per cell division, with the average CTV MFI of cultured B cells expressing the indicated vectors at Day 3 (n=3; experiment repeated 2 times). (e) Geometric mean fluorescence intensity (MFI) of CD25 expression and FSC-A of cultured B cells expressing the indicated vectors at Day 1 (n=3; experiment repeated 3 times). (f) Relative number of live EGFP+ B cells expressing the indicated vectors in tissue culture without antigen or anti-CD40 for three days, expressed as percentage of the starting number on Day 0. Numbers are mean \pm SEM of 3 independent cultures, and data are representative of 3 independent experiments.
Fig 7.4 CARD11<sup>E127G</sup> prevents self-antigen induced B cell death <i>in vivo</i>. (a)
Representative flow cytometry plots analyzing B cells transduced with the indicated retroviral vectors in the spleen on Day 10 post-transfer into non-transgenic and HEL-transgenic recipients on a <i>Rag1</i><sup>-/-</sup> background. The upper set of eight panels are gated on FSC and show the percentage of lymphocytes expressing the donor-specific surface IgM<sup>a</sup>. The lower set of panels are gated on IgM<sup>a</sup> positive spleen cells and show the percentage that are EGFP+. (b) Number of EGFP+ B cells expressing the indicated vectors injected into each mouse on Day 0. (c) Number of EGFP+ B cells expressing the indicated vectors in the spleens of non-transgenic and HEL-transgenic recipients at Day 10 post-transfer. Numbers are mean ± SEM of 3-5 recipients, and data are representative of 2 independent experiments.
Fig 7.5 B cells expressing high levels of CARD11$^{E127G}$ differentiate into plasmablasts. (a) Representative flow cytometry analysis of CD19 and EGFP expression by B cells transduced with the indicated vectors, analysed in the spleen on Day 10 post-transfer to non-transgenic or HEL-transgenic recipients. Corresponding representative histogram gated on EGFP+ cells, showing the distribution of B220 expression and percentage remaining B220+. Column graphs show mean and s.e.m percent of CD19low cells among EGFP+ and the %B220+ among EGFP+ respectively. (b) Representative histogram overlays of CD19, B220 and FSC-A comparing expression levels on EGFP high (blue) and EGFP low (red)-CARD11$^{\text{Mut}12}$-transduced B cells present in the spleen of HEL-transgenic recipients at Day 10 post-transfer.
Fig 7.6 ENU-induced M365K substitution in the coil-coiled domain of CARD11.
(a) Expression of Card11 in B cell subsets [data obtained and graph modified from Immgen; BM- bone marrow, Sp- spleen, MLN- mesenteric lymph node, LN- lymph node, PC- peritoneal cavity]. (b) Schematic illustrating the position of M365K mutation in the coil-coiled domain of CARD11 protein. Methionine 365 of CARD11 protein is conserved between human, mouse, chicken and zebrafish. (c). Relative number of live EGFP+ B cells transduced with the indicated retroviral vectors in tissue culture without antigen or anti-CD40 for five days, expressed as percentage of the starting number on Day 0. (d) Graph of a model constructed based on the theoretical CTV MFI per cell division, with the average CTV MFI of cultured B cells expressing the indicated vectors at Day 3 (n=3; experiment repeated 2 times).
Data obtained from http://www.immgen.org/databrowser/index.html
**Fig 7.7 Normal B cell output from the bone marrow of Card11$^{M365K}$ mice.**

Representative flow cytometry plots of bone marrow cells in 8-12 weeks old mice with wild-type Card11, heterozygous and homozygous mutant Card11 M365K (n=2-5 mice of each genotype), showing the percentage of (a) CD19+ B cells, and (b) gated on CD19+ cells to show surface IgM and IgD and the percentage of B cells expressing different amounts of IgD or IgM. (c) Representative flow cytometry plots analyzing CD19+IgM-IgD- populations of each genotype. Scatter plots of the percentage of (d) CD19+ bone marrow cells and (e-i) the indicated B cell subsets present among the CD19+ cells (n= 2-5 mice of each genotype).
**Fig 7.8 Card11\textsuperscript{M365K} mice have decreased immature B cells and increased marginal zone B cells.** Representative flow cytometry plots of spleen cells in 8-12 weeks old mice with wild-type Card11, heterozygous and homozygous mutant Card11 M365K (n= 2-5 mice of each genotype), showing (a) the population of CD19+ B cells, (b) the populations of CD19+CD93+ immature B cells and CD19+CD93- mature B cells, (c) the CD21+CD23- marginal zone and CD21+CD23+ follicular mature B cell populations. The corresponding scatter plots show the percentage and numbers of the B cell populations gated on the flow cytometry plots.
Fig 7.9 Enhanced activation of B cells bearing $Card11^{M365K}$ (Mut 17) following anti-IgM and LPS stimulation. (a-d) Analysis of cultured B cells from mice with germ-line gain- or loss-of-function mutations in the endogenous $Card11$ gene. Representative histogram overlays of CD25 expression (a, b) or FSC-A (c, d) on the surface of B cells stimulated with: (a, c) 10ug/ml anti-IgM; or (b, d) 10ug/ml LPS for 24hours in culture. Corresponding dose-response graph of CD25 mean geometric fluorescence intensity with increasing amounts of anti-IgM and LPS. (e, f) Analysis of normal B cells transduced with retroviral vectors encoding $Card11^{WT}$, $Card11^{M365K}$ (Mut17), or $Card11^{L225LI}$ (Mut10). Expression of CD25 and FSC-A MFI of EGFP+ B cells expressing the indicated vectors at Day 0 in culture. Numbers are average MFI from 3 replicate cultures; experiment performed twice.
Fig 7.10 Analysis of the effects of Card11$^{M365K}$ (Mut17) constitutively expressed from an EGFP retroviral vector in activated B cells in vitro

(a, c) Percentage and (b, d) relative number of live EGFP+ B cells expressing the indicated vectors in tissue culture without antigen or anti-CD40 for three days, expressed as percentage of the starting number on Day 0. Numbers are mean ± SEM of 3 independent cultures, and data are representative of 3 independent experiments. (e) Representative histogram overlay of fluorescence from the Cell Trace Violet (CTV), and corresponding graph of a model constructed based on the theoretical CTV MFI per cell division, with the average CTV MFI of cultured B cells expressing the indicated vectors at Day 3 (n=3; experiment repeated 2 times).
**Fig 7.11** CARD11<sup>m365k</sup> increases survival and proliferation of splenic B cells in culture. (a, b) Percentage B220+ live cells with the indicated germline Card11 genotype, after culture for the indicated number of days either: (a) in media only; or (b) in media containing 1ug/ml anti-IgM. (c, d) Representative histogram overlays of Cell Trace Violet (CTV) dilution profiles of B cells of the indicated germline Card11 genotype after 3 days in culture with either: (c) 10ug/ml anti-IgM; or (d) 10ug/ml LPS (n=3; experiment repeated 2 times).
Fig 7.12 Antigen enhances proliferation of B cells with retrovirally expressed CARD11$^{M365K}$ in vitro. Representative flow cytometry profiles of anti-CD40 activated Ig-transgenic spleen cells, transduced with the indicated vectors, and then cultured for 4 days without anti-CD40 in the presence or absence of 100ng HEL. (a) Percentage of viable cells among cultured B cells. (b) Percentage of viable cells expressing EGFP. (c-e) Representative histogram overlays of gated viable EGFP+ cells, showing: (c) FSC-A; (d) CTV dilution; and (e) EGFP expression.
(a) Day 4

No HEL

+ 100ng HEL

CARD11^WT

CARD11^Mut10

CARD11^Mut17

FSC-A

7AAD

(b) No HEL

+ 100ng HEL

FSC-A

EGFP

(c) No HEL

+ 100ng HEL

WT

Mut10

Mut17

FSC-A

(d) No HEL

+ 100ng HEL

WT

Mut10

Mut17

CTV

(e) No HEL

+ 100ng HEL

WT

Mut10

Mut17

EGFP
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