Role of Transcription Factor NFATc1 in Development, Survival and Function of B Lymphocytes

Die Bedeutung des Transkriptionsfaktors NFATc1 für die Entwicklung, das Überleben und die Funktion von B-Lymphozyten

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Summary

The Nuclear Factors of Activated T cells (NFATs) are critical transcription factors playing major roles in the control of the cell cycle, apoptosis and, probably, also cancerogenesis. Of all the four genuine NFATc family members, NFATc1 has the unique induction property which appears to be essential for T and B cell development, along with its considerable role in cytokine gene expression and function in non-lymphoid tissues and during organ development (such as in the development of muscle and heart cells). A number of studies have proved the potential role of NFATc1 protein in development of lymphomas and leukemias and provided evidence of differential expression of the same gene in different tumours (Suppression in classical Hodgkin lymphomas but overexpression in T-ALLs). Although the most commonly accepted pathway is the dephosphorylation of NFAT by calcineurin upon a rise in intracellular Ca^{++} leading to nuclear translocation followed by transcription of *Il2* gene and related cytokines, it is quite possible that signaling mechanisms other than (or in addition to) calcineurin activation lead to NFATc1 induction as well.

One of the major isoforms of NFATc1, NFATc1/αA, is the short inducible factor, produced upon full T and B cell activation. Here we used two different conditional knock-out mice as our study model. Inactivation of the murine *Nfatc1* gene in bone marrow (of *Cd79a/mb-1-cre x Nfatc1*$^{flx/flx}$ mice) and spleen (of *Cd23-cre x Nfatc1*$^{flx/flx}$ mice*)* resulted in complete ablation of NFATc1 expression in splenic B cells. Although no severe developmental defects were found for the generation of 'conventional' B2 cells, NFATc1 inactivation in bone marrow B-cells led to a strong decrease in the peritoneal B1a cell population.

In-vitro studies showed a clear-cut decrease in proliferation and an increase in Activation Induced Cell Death (AICD) of NFATc1$^{-/-}$ splenic B cells upon BCR stimulation. While NFATc1 appears to control directly the AICD of peripheral B cells, further studies revealed an effect of NFATc1 on proliferation by a sustained differentiation program controlling Ca^{++} flux and calcineurin activity which are needed to maintain transcription

and proliferation of primary B cells. Re-expression of NFATc1 at a low dose could protect cells against AICD, whereas at a higher dose it initiated AICD. These data suggest an important dual role of NFATc1 in controlling proliferation and apoptosis of peripheral B lymphocytes.

NFATc1 ablation also impaired the Ig class switch to IgG3 by T cell-independent (TI) type II antigens and impaired $IgG3^+$ plasmablast formation when studied *in-vivo* by NP-Ficoll immunization or *in-vitro* using an *in-vitro* class-switch model. Contrary to the immunizations with TI-type II antigen, no significant differences were documented in Ig class switch upon immunization with NP-KLH, a T-cell dependent (TD) antigen. Taken together, the data indicate NFATc1/αA as a crucial player in the activation and function of splenic B cells upon BCR stimulation. Missing or incomplete NFATc1/αA induction appears to be one reason for the generation of B cell unresponsiveness, whereas uncontrolled NFATc1/αA expression could lead to unbalanced immune reactions and autoimmune diseases.

Zusammenfassung

Die Transkriptionsfaktoren der NFAT-Proteinfamilie (Nuclear Factor of Activated T cells, NFATc1-4) sind an entscheidender Stelle in die Regulation des Zellzyklus, des programmierten Zelltodes und der Kanzerogenese involviert. NFATc1 nimmt innerhalb dieser Familie eine Sonderrolle ein, da dessen Aktivität auch durch eine stark induzierbare Expression gesteigert werden kann. Dies ist insbesondere für die Differenzierung und Funktion von T- und B-Lymphozyten von Bedeutung. Weiterhin ist NFATc1 für die Muskel- oder Herzentwicklung notwendig. Eine Reihe von Arbeiten belegen darüber hinaus eine Beteiligung dieses Transkriptionsfaktors an der Entstehung von Leukämien und Lymphomen. Während klassische Hodgkin-Lymphome allerdings durch eine abgeschaltete NFATc1-Expression gekennzeichnet sind, wird für T-ALL (Akute Lymphatische Leukämie der T-Zelle) eine Überexpression beschrieben. Die Kernlokalisation dieses Transkriptionsfaktors erfolgt nach Dephosphorylierung des zytoplasmatischen Proteins durch die Phosphatase Calcineurin. Deren Phosphataseaktivität wird durch einen Anstieg des intrazellulären Ca^{++}-Spiegels aktiviert. Inwiefern die Calcineurin-abhängige Kerntranslokalisation den einzigen Aktivierungsmechanismus für NFAT-Faktoren darstellt, ist noch nicht eindeutig geklärt.

Nach optimaler Aktivierung von T- bzw. B-Zellen ist die kurze, induzierbare Isoform NFATc1/αA das Hauptprodukt des *Nfatc1*-Gens. In dieser Arbeit wurden für die gezielte Deletion des *Nfatc1*-Gens in der Maus zwei verschiedene konditionelle Systeme verwandt. Hierzu wurden Tiere, die ein mit „flox“-Sequenzen versehenes drittes Exon des *Nfatc1*-Gens in der Keimbahn tragen, mit verschiedenen Cre-Rekombinase expremierenden Linien verkreuzt. Der Verlust funktionellen NFATc1-Proteins erfolgt dann früh in der B-Zell-Differenzierung im Knochenmark (*Cd79a/mb-1-cre x Nfatc1*$^{flx/fl}$) bzw. in reifen B-Zellen (*Cd23-cre x Nfatc1*$^{flx/flx}$). Während in keiner dieser Linien signifikante Defekte in der Differenzierung “konventioneller B2” B-Lymphozyten beobachtet wurden, hatte die frühe Inaktivierung des *Nfatc1*-Gens im Knochenmark den Verlust der B1a-Zell-Population im Peritoneum zur Folge.

In vitro zeigten *Nfatc1*$^{-/-}$-B-Zellen aus der Milz nach Aktivierung über den B-Zell-Rezeptor deutliche Defekte in der Zellteilung bei einer gleichzeitigen Zunahme des aktivierungsinduzierten Zelltodes (AICD, activation induced cell death). Die vergleichende Transkriptomanalyse identifizierte wichtige Gene des Ca^{++}/Calcineurin-Signalweges als NFATc1-Zielgene und mitverantwortlich für die Proliferationsdefekte.
In NFATc1-defizienten B-Zellen konnte Re-Expression von NFATc1 in geringer Konzentration den aktivierten Zelltod inhibieren, wohingegen hohe Konzentrationen diesen noch weiter förderten. Zusammengenommen lässt sich daher schließen, dass NFATc1 entscheidend an der Kontrolle von Proliferation und Zelltod peripherer B-Lymphozyten beteiligt ist.

Eine weitere wichtige Funktion kommt NFATc1 beim Klassenwechsel im Immunglobulin-Lokus zu. In den untersuchten Mäusen war die IgG3-Produktion nach Immunisierung mit NP-Ficoll (einem T-Zell-unabhängigen Antigen des Typs II) deutlich reduziert, wenn das *Nfatc1*-Gen in den B-Lymphozyten funktionslos war. Auch die Bildung von $IgG3^{+}$-Plasmablasten war gehemmt. Zu ähnlichen Ergebnissen führten Untersuchungen an isolierten B-Lymphozyten in einem *in vitro* Klassenwechsel-Modell. Demgegenüber zeigten Immunisierungen der Tiere mit NP-KLH (einem T-Zell-abhängigen Antigen) keine signifikanten Abweichungen im Klassenwechsel.

Zusammengefasst zeigen diese Daten die große Bedeutung des Transkriptionsfaktors NFATc1 für das Überleben und die Funktion peripherer B-Lymphozyten.

1. Introduction

"It stands to the everlasting credit of science that by acting on the human mind it has overcome man's insecurity before himself and before nature."

Albert Einstein

The concept of humoral immunity dates back to the 1900s when the German scientist Paul Ehrlich postulated the "side chain theory". He argued that cells have specific receptors with a unique structure that work like locks for the cell, and only substances identical to this structure could penetrate the cell (Schwartz 2004). As the research progressed, humoral immunity got its recognition as a major source of the body's defense mechanism where a specialized type of white blood cell called the B cell, or B lymphocyte, could produce *humoral* substances, i.e. antibodies or the immunoglobulins upon stimulation.

The primary purpose of B cell development is to introduce a diverse population of peripheral B cell pool in the immune system that is both self tolerant and reactive to invaders by the means of antibody production. The bone marrow is the primary site of B lymphopoiesis. This process is generally divided into four chronological phases:

1) Early development in the bone marrow
2) Maturation of transitional B cells during their transit to the periphery
3) Entry into the mature B-cell compartments
4) Antigen-dependent differentiation into plasma cells and/or memory B cells (Cain, Kondo et al. 2009)

1.1 B-cell developmental stages

B cells are originated from multipotent, self-renewing hematopoietic stem cells (HSC) in the fetal liver and bone marrow and transit through a series of maturation steps and developmental checkpoints before leaving the bone marrow for the spleen or other

peripheral lymphoid tissues where they complete their development (Cain, Kondo et al. 2009)

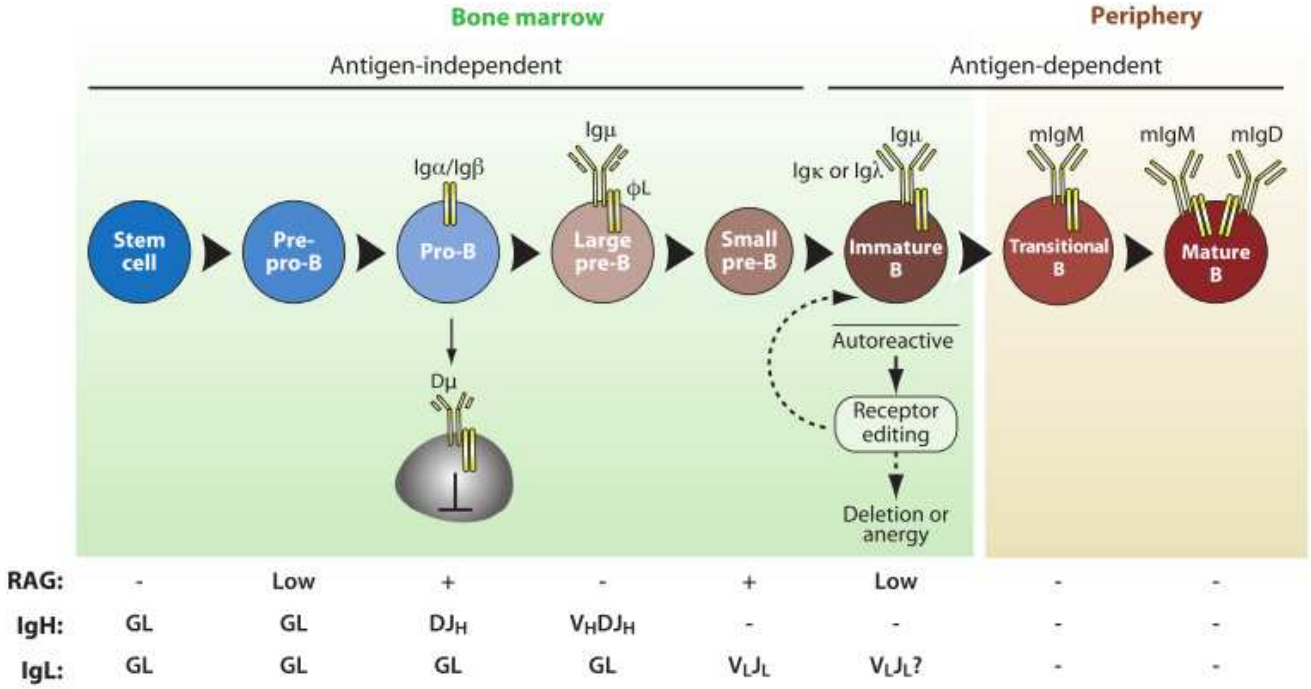


Fig.1 B cell differentiation scheme (Kurosaki, Shinohara et al. 2010)

Pre-pro-B cells, as the name indicates, are the precursor cells for Pro-B cells with unarranged germline configuration of *IgH* and *IgL* genes. The recombination activating genes (*Rag1* and *Rag2*) are also found in low levels at this stage (Kurosaki, Shinohara et al. 2010).

Pro-B cells are the first to initiate the D_H-to-J_H rearrangements on one *Igh* allele (Cain, Kondo et al. 2009) followed by $V_H \rightarrow D_H J_H$ recombination with the help of cytokine IL-7, the transcription factors Pax5 and YY1 (a zinc-finger protein), and high levels of Rag1/Rag2 (Kurosaki, Shinohara et al. 2010). If the initial $V_H D_H J_H$ rearrangements fail, a second $V_H D_H J_H$ rearrangement attempt takes place on the alternative allele to compensate the failure (Cain, Kondo et al. 2009). Increases in surface expression of

CD19 and HSA along with Igα/Igβ heterodimers in association with calnexin (Pro-BCR) mark the pro-B cell stage (Kurosaki, Shinohara et al. 2010). Both activation of B-lineage specific genes (such as BLNK, CD19 and Igα) and suppression of non-lineage genes (Notch1) by PAX5 are crucial for the lineage commitment at this stage (Wang and Clark 2003).

Pre-B cells express the surface pre-BCR which is composed of the transmembrane form of μHC (mIg μ) as a result of a successful $V_HD_HJ_H$ rearrangement, the invariant surrogate light chain (comprising the VpreB and λ5 polypeptides) and Igα/Igβ heterodimers (Kawano, Yoshikawa et al. 2006) .

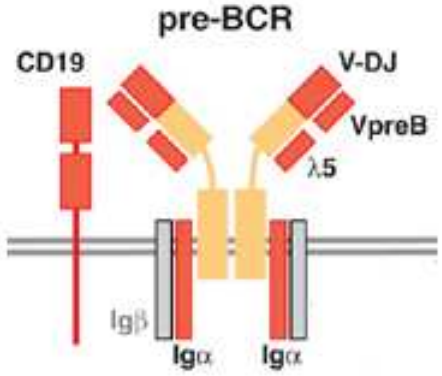


Fig.2 Schematic diagram of the pre-BCR (Holmes, Pridans et al. 2008)

The primary role of pre-BCR includes allelic exclusion at the heavy-chain locus and clonal expansion of the pre-B cells (Kurosaki, Shinohara et al. 2010) by providing constitutive survival and proliferation signals. As *large pre-B cells,* they show a coincident reduction in RAG1/2 expression before they become *small pre-B cells* followed by G1 cell cycle arrest (Cain, Kondo et al. 2009). Small pre-B cells express an elevated level of RAG1/2 to drive Vκ-to-Jκ light (L)-chain rearrangements in an IL-7 independent manner, resulting in the cell surface expression of IgM BCR by the replacement of SLC with an Igκ or Igλ polypeptide (Wang and Clark 2003).

Immature B cells are typically IgM^{+} and $IgD^{lo/-}$ cells, capable of recognizing exogenous antigens, and reside in the immature compartment for 3.5 days on average before migrating into splenic red pulp via the bloodstream, where they are referred to as transitional B cells (Kurosaki, Shinohara et al. 2010). At this stage, immature B cells are subjected to receptor-mediated negative selection to ensure complete removal of the auroreactive B cells from the immune repertoire. The three mechanisms of immunological tolerance usually include (1) apoptotic deletion, in response to high-avidity ligands (or without any ligand: "death by neglect"), (2) anergy, in response to low-avidity ligands, whereas (3) receptor-editing stimulates secondary immunoglobulin gene arrangement to substitute the autoreactive receptors (Wang and Clark 2003).

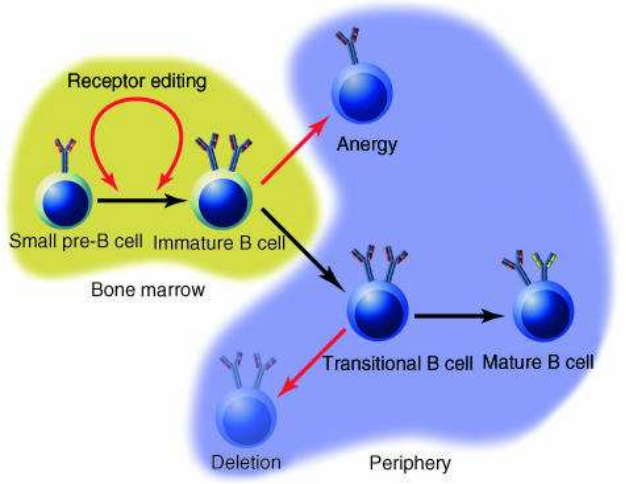


Fig.3 Mechanisms of negative selection during B-cell development (Wang and Clark, 2003)

The final stages of B-cell maturation take place in the spleen, where *transitional B cells*, an intermediate form between immature and mature phenotypes, undergo selection in peripheral B-cell compartments (Cain, Kondo et al. 2009). Based on their surface-marker expressions, they can be sub-divided into populations, referred to as T1 ($AA4^{+}CD23^{-}sIgM^{high}$), T2 ($AA4^{+}CD23^{+}sIgM^{high}$), and T3 ($AA4^{+}CD23^{+}sIgM^{low}$) (Allman, Lindsley et al. 2001). T1 B cells are unable to carry out receptor editing on exposure to self-antigen ligands but endure apoptosis following BCR ligation. T2 B cells, on the other hand, proliferate and differentiate upon BCR-mediated signalling (Cain, Kondo et al. 2009). Studies have revealed the significance of factors regulating the development of T1 into T2 B cells and finally, to the mature B cell pool. These are tonic BCR signals, along with

BAFF-mediated survival signals (Kurosaki, Shinohara et al. 2010) and the microenvironment of the adult spleen (Loder, Mutschler et al. 1999).

Mature B cells down-regulate AA4.1 and co-express IgM and IgD on their surface. Out of the 2 x 10^7 IgM^+ B cells that develop in the mouse bone marrow at a regular basis, 10% arrive at the spleen and only 1–3% enter the long-lived mature B cell compartment (Loder, Mutschler et al. 1999). Mature B cells exist in three major forms: B-1, follicular (FO), and marginal zone (MZ) B cells (Carey, Moffatt-Blue et al. 2008).

Depending on the differential BCR signaling strength (signal strength hypothesis), either FO or MZ B cells develop. It is hypothesized that FO cells are a product of strong BCR signaling, whereas a weak signal results in MZ cells (Giles, Bender et al. 2009). BAFF-receptor (BAFF-R) mediated signals are also crucial for the maturity of both FO and MZ B cells, along with the transcription factors c-Myb for FO B cells, Notch-2 and Aiolos for MZ B cells and Aiolos and c-Myb for B-1 cells (Yu, Quinn et al. 2008).

1.2 B-cell subsets

B cell subsets are composed of two sub-populations, B-1 and B-2 cells, based on their anatomical location, surface marker expression, and function (Duber, Hafner et al. 2009). They are competent to undergo class switch recombination (CSR) to generate IgG, IgE and IgA antibody subclasses (Babbe, McMenamin et al. 2009). In principle, follicular (FO) and marginal zone (MZ) B cells are collectively referred to as B-2 B cells, whereas B-1 cells are again divided into B-1a ($CD5^+$) and B-1b ($CD5^-$) cells (Babbe, McMenamin et al. 2009).

Unlike B2 cells, which develop in BM after birth, B1 cells are thought to be generated predominantly in the fetal liver (Carey, Moffatt-Blue et al. 2008) and maintained via self-renewal (Hardy 2006). The origin of B-1 cells has been interpreted by two different models: the lineage model and the selection model. According to the lineage model, B-1 and B-2 B cells are derived from distinct progenitors, while the selection model suggests

the existence of one common progenitor with differences in antigen recognizing pattern (Montecino-Rodriguez and Dorshkind 2006).

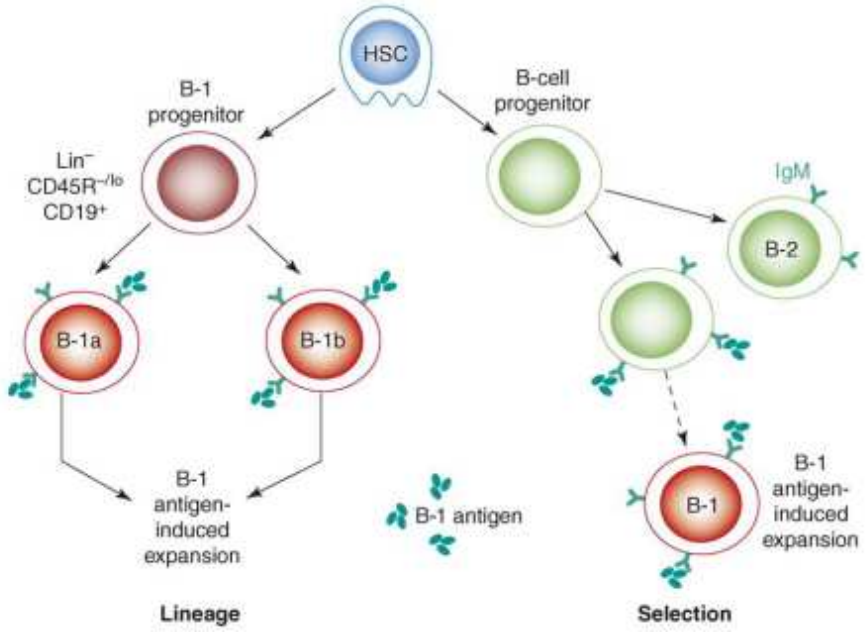


Fig.4 Models of B-1 B-cell development (Montecino-Rodriguez and Dorshkind 2006)

FO B cells ($IgM^{lo}IgD^{hi}CD21^{+}CD23^{+}CD19^{+}$) reside in the follicles of spleen and lymph nodes and circulate through the body including the bone marrow (Babbe, McMenamin et al. 2009). MZ B cells ($IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo/-}CD19^{+}$), on the other hand, are restricted to the marginal zone (Carey, Moffatt-Blue et al. 2008). Although B-1a cells ($IgM^{hi}IgD^{lo}CD43^{+}$) are predominantly found in the peritoneal cavity and pleural body serosa (Babbe, McMenamin et al. 2009), a small population also exists in the spleen and in various parts of the intestine (Montecino-Rodriguez and Dorshkind 2006).

B-1a cells are well-known for their capacity to produce “natural IgM antibodies” without antigen challenge, whereas B-1b cells can only manifest antibody production in response to antigenic stimulation (Duber, Hafner et al. 2009). Both B-1a and MZ B cells provide a rapid “first line of defense” against bacterial pathogens (Babbe, McMenamin et al. 2009). MZ B cells produce short-lived plasma cells in response to T cell-independent antigens

(TI), while FO B cells recognize T cell-dependent antigens (TD), participate in the germinal centre reaction (discussed later), and give rise to long-lived plasma cells and memory B cells (Yu, Quinn et al. 2008). TI antigens are again of two types, TI type-1 (TI-1) and TI type-2 (TI-2). TI-1 antigens are polyclonal B-cell activators and essentially mitogenic whereas TI-2 antigens are non-mitogenic and include pathogens like *Haemophilus influenzae* b, *Streptococcus pneumoniae* and *Nisseria meningitides* (Fairfax, Kallies et al. 2008). Activated B1 cells migrate to the spleen or gut, loose CD5 expression and become plasma cells upon up-regulation of Blimp1 expression (Shapiro-Shelef and Calame 2005).

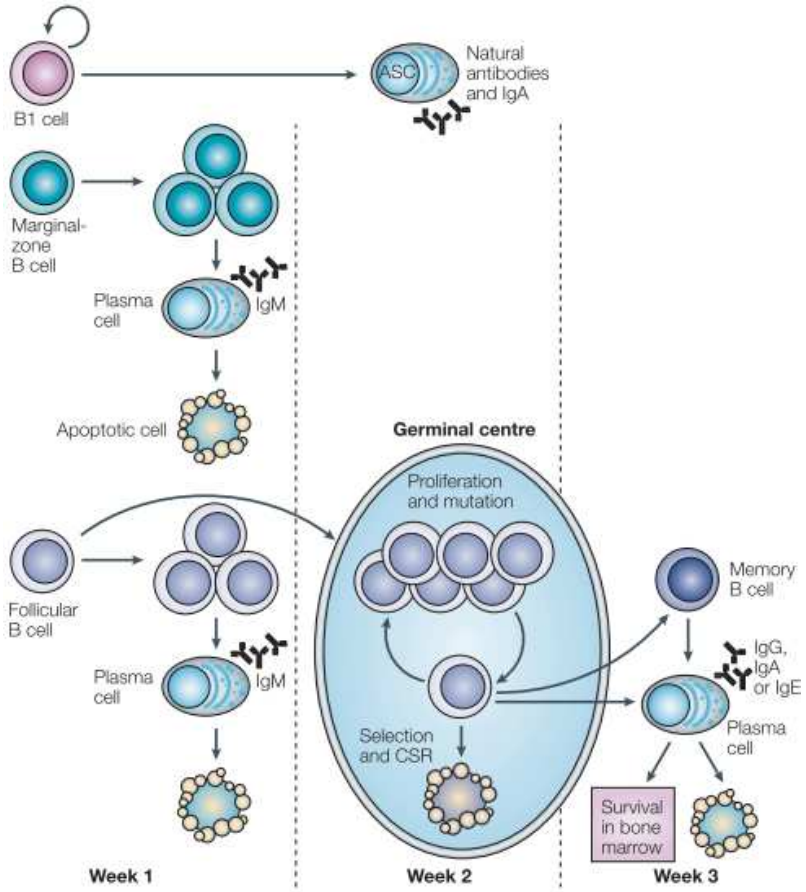


Fig.5 Participation of B cell subsets in immune response (Shapiro-Shelef and Calame 2005).

1.3 B-cell receptor signaling

The B-cell antigen receptor (BCR) was first identified in 1970 (Wang and Clark 2003). It is a multimeric complex composed of membrane immunoglobin (mIg) heavy and light chains and is non-covalently coupled with Igα/Igβ (CD79a/CD79b) heterodimers (Harwood and Batista 2008). The signal-transducing unit is located inside the cytoplasmic domains of the heterodimers which transduces signals followed by antigen binding to the mIg subunits (Patterson, Kraus et al. 2006).

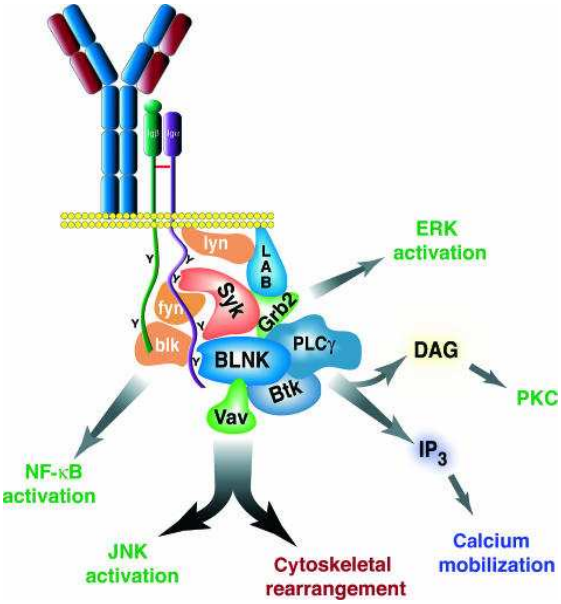


Fig.6 B-cell receptor-mediated signaling pathways (Wang and Clark 2003)

Only the cytoplasmic tails of membrane IgG contain signaling capacity. Antigen recognition leading to receptor aggregation stimulates phosphorylation of the two ITAM tyrosines (Wang and Clark 2003) by Src-family kinases, such as Lyn, Fyn, Blk, or Lck (Dal Porto, Gauld et al. 2004). Upon BCR engagement, a supramolecular complex, also known as B cell signalosome, is formed by the coordinated assembly of a definite proximal BCR signaling molecules, such as Vav, Bruton's tyrosine kinase (Btk), phosphoinositide 3-kinase (PI3K) and phospholipase C-γ2 (PLCγ2), along with the tyrosine-phosphorylated form of adaptor proteins, such as BLNK, SLP-65, or BASH.

The signalosome initiates a multitude of cellular responses including regulation of gene expression, re-organization of the cytoskeleton, and BCR mediated antigen internalization (Patterson, Kraus et al. 2006).

1.4 BCR engagement and B-cell activation

After binding of exogenous antigen to the BCR, the antigen-dependent activation of naïve mature B cells takes place. At this stage, each B cell gets a chance to "decide" and choose between survival and death, finally leading to the formation of germinal centre (GC) B-cells, plasma cells or memory B-cells (Goodnow, Vinuesa et al. 2010).

1.4.1 Development of GC and role of Bcl-6

Within 1-6 hours after antigen binding, B cells move to the T-cell zone of the peripheral lymphoid tissues in spleen, lymph nodes or Peyer's patches (Goodnow, Vinuesa et al. 2010) and get fully activated once CD40(on B-cells)-CD40L(on helper T-cells)-mediated interactions take place (Klein and Dalla-Favera 2008). Activated B cells also interact via CD86-CD28 and initiate T cell-mediated synthesis of pro-survival cytokines, which not only protect B cell from FasL-induced apoptosis but also support its proliferation by a weak proliferative signal, delivered by FasL (Goodnow, Vinuesa et al. 2010). These cells can either directly develop into antibody-secreting cells or mature into GC-precursor B cells which undergo vigorous proliferation for a few days to form GC (Klein and Dalla-Favera 2008). Early GC B cells are IgD^{+} and as they enter the GC, a rapid down-regulation of IgD, increase in Fas expression and in receptor specificity to PNA are observed (Carter and Myers 2008). Bcl-6, the master transcriptional regulator of GC B cells, plays a major role by (1) supporting the high proliferation rate of these B cells while inhibiting (2) pre-mature B cell activation as well as (3) differentiation into plasma cells and memory B cells (Klein and Dalla-Favera 2008).

Within 3 days after antigen encounter, fully developed GCs are formed with anatomically distinct dark and light zones (Natkunam 2007). The dark zone contains densely packed proliferating B cells, known as centroblasts, which undergo somatic hypermutation (SHM), the basis for affinity maturation (Klein and Dalla-Favera 2008). SHM can also

occur outside GCs. This has been shown by a number of studies where GC formation was impaired; for example mice deficient for lymphotoxin-alpha (Matsumoto, Lo et al. 1996) or the TNF-receptor1 (TNFR1) fail to form GCs. However, they produce high affinity antibodies (Kim, Kim et al. 2006). The light zone, on the other hand, harbors differentiated B cells known as non-dividing centrocytes that undergo clonal selection based on antigen affinity. A subset of centrocytes goes through immunoglobulin class-switch recombination (CSR) in the light zone followed by differentiation into plasma cells or memory B cells (Klein and Dalla-Favera 2008).

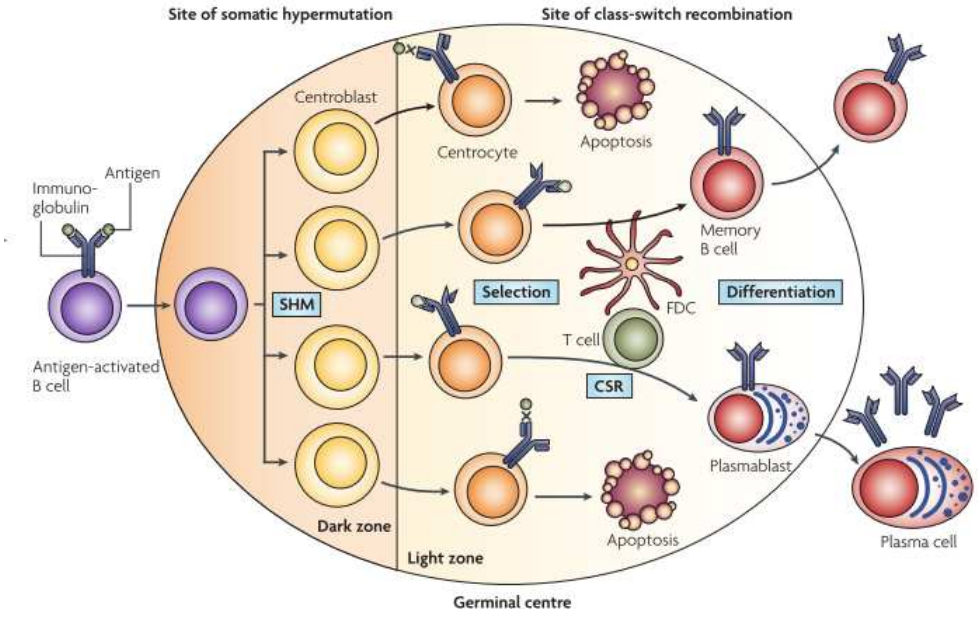


Fig.7 Development of GC (Klein and Dalla-Favera 2008)

In mice, the classical "dark-zone light-zone pattern" is less well-defined (Klein and Dalla-Favera 2008) and B cells are found to transit both intra-zonally and bi-directionally between the zones with well-observed capacity to proliferate (Natkunam 2007). It takes approximately two weeks for the GC to attain its maximal size before undergoing slow involution and disappearance over several weeks (Klein and Dalla-Favera 2008).

1.4.2 Post-GC events: Generation of memory B cells and plasma cells

Generation of plasmablasts, long-lived plasma cells and long-lived memory B cells are crucial for the maintenance of serum concentration of specific antibodies. GC-derived memory B cells that are produced late in the immune response are supposed to be more efficient, exhibiting an increased affinity for antigen and contributing to the critical memory B-cell population (Tarlinton 2006). Co-existence of both short-lived and long-lived memory B cells have also been documented (Dorner and Radbruch 2005). Plasma cells exit GC by down-regulating the chemokine receptors CXCR5 and CCR7 and migrate to different sites according to the expression of different chemokine receptors (Fairfax, Kallies et al. 2008). In order to receive survival signals, including CXCL12, IL-6, BAFF and/or APRIL, and TNF it is important for plasmablasts to migrate to survival niches in the bone marrow within a week to become long-lived plasma cells. Both memory B cells and long-lived plasma cells express class switched, affinity matured antibodies. However, memory B cells do not secrete antibody but can respond quickly to a subsequent antigen challenge by differentiating into plasmablasts (Radbruch, Muehlinghaus et al. 2006).

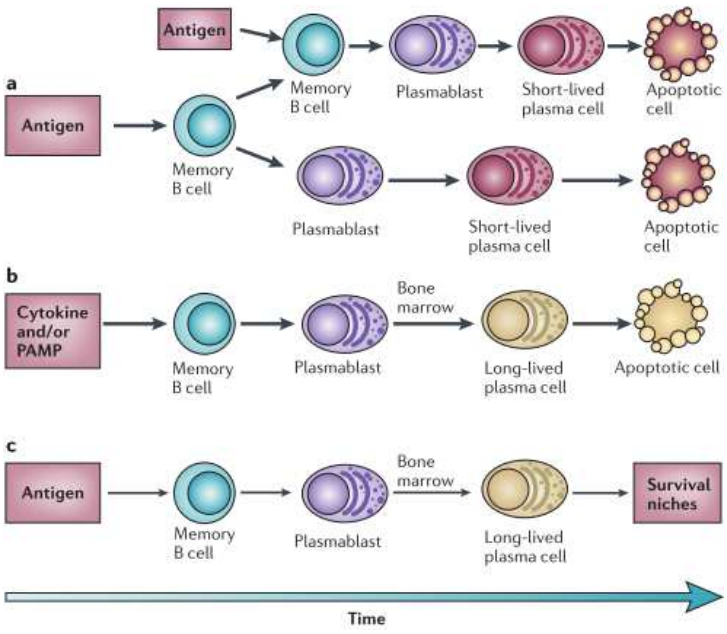


Fig.8 Generation of plasmablasts, and short-lived and long-lived plasma cells from memory B cells upon antigen stimulation and/or cytokine signaling (Radbruch, Muehlinghaus et al. 2006)

Plasma cell development depends on several factors, which includes IL-21 and the activation of *PRDM1* encoding the transcription factor BLIMP1 (Radbruch, Muehlinghaus et al. 2006). Blimp1 represses *PAX5* and *Bcl6,* allowing the expression of XBP1 and *IgJ*. In order to escape from apoptosis, plasma cells are also found to up-regulate anti-apoptotic genes encoding Bcl2, Mcl and Bclw (Fairfax, Kallies et al. 2008).

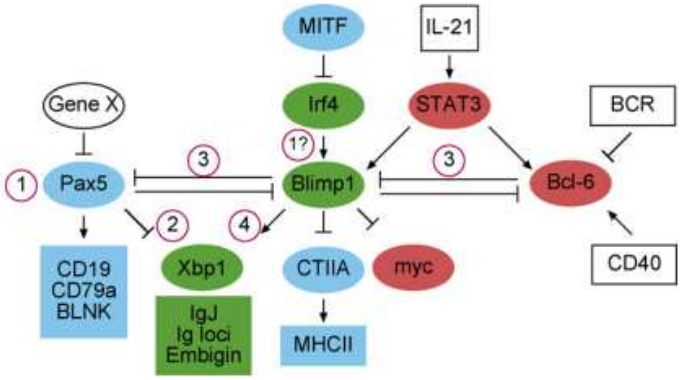


Fig.9 Genetic regulation of plasma cell differentiation (Fairfax, Kallies et al. 2008)

On the basis of Blimp1 expression levels on their surface and at a given time, plasma cells can be subdivided into two sub-populations. Both populations, Blimp1-high (Blimp1^{hi}), also known as the long-lived plasma cells, and Blimp1-intermediate (Blimp1^{int}), the short-lived plasma cells, co-exist in the spleen of naïve adult mice In general, bone marrow plasma cells, which form the long-lived population, are Blimp1^{hi} missing B cell markers, while plasma cells which are found in blood, are Blimp1^{int} and retain the surface expression of B220, CD19, CD22 and MHCII (Fairfax, Kallies et al. 2008).

1.5 Nuclear Factor of Activated T-cells (*NFAT*)

NFATs are critical transcription factors having major roles in the control of the cell cycle, apoptosis and, probably, carcinogenesis. NFAT was first identified as a nuclear protein in Jurkat T leukemia cells which binds to the human IL-2 promoter upon activation (Shaw, Utz et al. 1988). Not only immune cells, such as T cells, B cells, mast cells, basophils, macrophages and NK cells express NFATs (Rao, Luo et al. 1997), non-immune cells,

like skeletal muscle, osteoclasts, cardiac muscle cells and many others are also known to express NFATs (Hogan, Chen et al. 2003).

1.5.1 NFAT family members

The NFAT family of transcription factors comprises NFATc1 (also designated as NFAT2/NFATc), NFATc2(NFAT1/NFATp), NFATc3(NFAT4/NFATx), NFATc4(NFAT3) and NFAT5 (Rao, Luo et al. 1997; Serfling, Klein-Hessling et al. 2006). These proteins share with the Rel/NF-kB factors the so-called Rel-like DNA binding domain which spans approximately 300 amino acids (aa) (Rao, Luo et al. 1997) . Except NFAT5 which is already found in Drosophila (Hogan, Chen et al. 2003) and regulated by osmotic stress (Ranjbar, Tsytsykova et al. 2006), all the other NFATc family members are regulated by the Ca^{++}/calmodulin-dependent phosphatase, calcineurin (CN) (Serfling, Klein-Hessling et al. 2006).

The *Nfatc1* gene has two promoters, P1 and P2, which direct transcripts encoding either the N-terminal α peptide of 42 aa, starting at exon 1, or the β peptide of 29 aa, starting at exon 2, respectively. Due to alternative splicing and poly A site usage, C-terminal peptides of varying lengths are generated, which in turn get polyadenylated at one of the two polyadenylation sites (pA1 and pA2) to form three isoforms: Isoform A, isoform B or isoform C. Among the six isoforms (NFATc1/αA, αB, αC, and βA, βB and βC), NFATc1/αA, the short isoform, is strongly induced upon activation (Serfling, Chuvpilo et al. 2006).

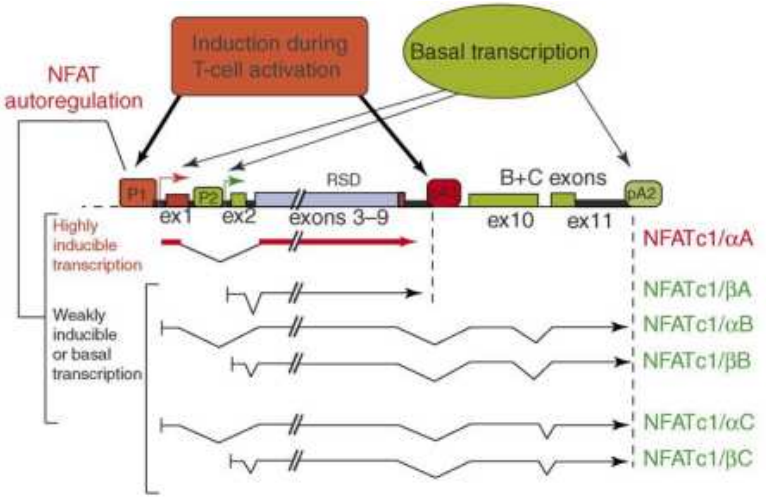


Fig. 10 A model of *Nfatc1* gene expression (Serfling, Chuvpilo et al. 2006)

1.5.2 The calcium (Ca^{2+})/calcineurin/NFAT signaling pathway

Ligation of cell surface receptors, such as immunoreceptors, activate tyrosine protein kinases and PLCγ, while G-protein-coupled receptors (GPCR) activate PLCβ. Activated PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP3) that activate RasGRP, protein kinase C (PKC), Ras, MAP kinases (MAPK), PI-3 kinase (PI3K) etc. InsP3 binds to the InsP3R, located on the endoplasmic reticulum (ER), to release calcium from internal stores (Hogan, Chen et al. 2003). Store depletion leads to the opening of store-operated calcium-release-activated calcium channels (CRAC, e.g. the ORAI/STIM complex) in the plasma membrane (Gachet and Ghysdael 2009) allowing constant "store-operated" Ca^{++} entry (Hogan, Chen et al. 2003). Finally, calcium binds to calmodulin and activates calcineurin (Macian 2005), which in turn dephosphorylates multiple phosphoserines on NFAT (Hogan, Chen et al. 2003) and exposes their nuclear-localization signal (NLS) (Gachet and Ghysdael 2009). The two most common immunosuppressants used in clinics, namely CsA and FK506, can inhibit NFAT translocation by inhibiting calcineurin (CN) activity (Serfling, Klein-Hessling et al. 2006). In the nucleus, NFAT factors participate in the regulation of numerous genes which are important for cell survival, proliferation and apoptosis.

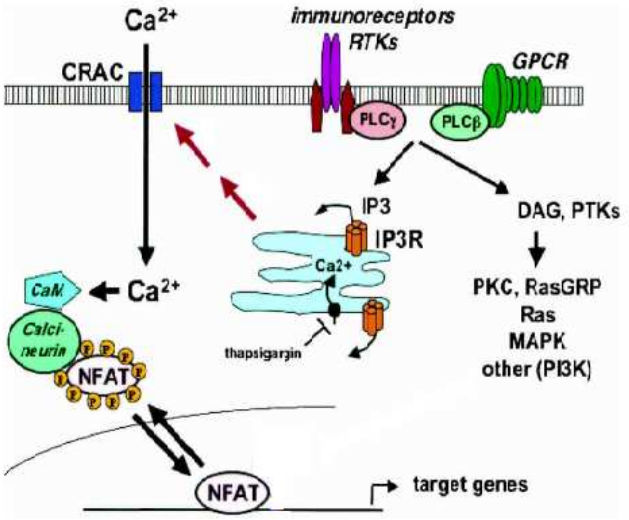


Fig. 11 Schematic view of the regulation of NFAT activation (adapted from (Hogan, Chen et al. 2003)

The shuffling of NFATs between cytoplasm and nucleus is known to be regulated by several protein kinases, such as casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3) and dual-specificity tyrosine-phosphorylation regulated kinase (DYRK) (Serfling, Klein-Hessling et al. 2006).

1.5.3 Role of NFATs

It is well documented that the inactivation of the *Nfatc1* gene can lead to early embryonic death. This shows the importance of NFATc1s as an endocardial transcription factor (Zhou, Wu et al. 2005). Severe defects in heart and thymocyte development are also observed in these mice, in contrast to NFATc2$^{-/-}$ mice, in which normal heart and thymus development is not impaired (Hodge, Ranger et al. 1996; de la Pompa, Timmerman et al. 1998; Ranger, Grusby et al. 1998). The short inducible isoform NFATc1/αA also plays an important role in osteoclast formation, while NFATc2 is necessary for their further differentiation (Asagiri, Sato et al. 2005). They are also known to play a significant role in TCR-mediated FasL induction (Latinis, Norian et al. 1997) and mice deficient for NFATc1 fail to resist rapid AICD, leading to cell death and impaired proliferation (Bhattacharyya, Deb et al. 2011). A number of cytokine genes (IL-2, IL-3, IL-4, IL-5, IL-13, INF-γ, TNF-α, GM-CSF) as well as membrane receptors (CD40L and CTLA-4) are known to be NFAT targets (Kuklina and Shirshev 2001).

Aim of the project

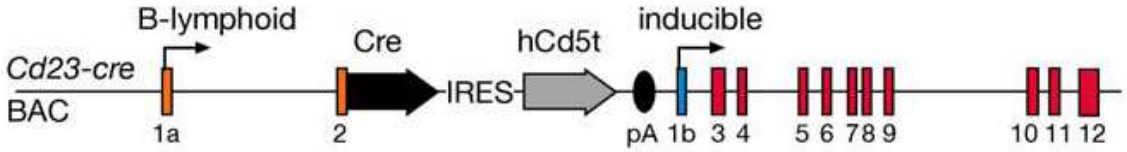
NFAT factors are well known for their major role in controlling the immune response, NFATc1 and NFATc2 being the most prominent players in lymphocyte activation. Induction of NFATc1 upon TCR stimulation in $CD4^+$ T cells has been known for years. In this study we focused mainly on B-cell fate following BCR engagement and tried to address the following points using B cell stage-specific NFATc1 knockout mice:

1) Role of NFATc1 in B-cell development
2) Role of NFATc1 in B cell survival and proliferation
3) Role of NFATc1 in B cell function, such as in antibody production.

2. Materials and Methods

2.1 Generation and maintenance of mice for the conditional inactivation of NFATc1 in B cells

A *Cre-IRES-hCd5t* expression cassette was inserted into exon 2 of a *Cd23* BAC to make *Cd23-cre* transgenic mice (Kwon, Hutter et al. 2008).



mb1-cre transgenic mice were created by inserting hCre into the *mb-1* WT locus between exon 2 and 3 (Hobeika, Thiemann et al. 2006)

Nfatc1^{flx} mice were generated by K. Mark Ansel, M. Mueller and A. Rao (Harvard Medical School) by flanking *Nfatc1* exon 3 with loxP sites. The cre lines mentioned above were crossed with *Nfatc1*^{flx} mice and continued to breed in our mice facility until the generation of *mb1-cre x Nfatc1*$^{flx/flx}$ and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice, respectively. Other knock-out mice (*Pdcd1*$^{-/-}$, *Tnfsf14*$^{-/-}$, *Slp65*$^{-/-}$, *Cd22*$^{-/-}$, OT-II) used in this study were kindly provided by our collaborative groups: Dr. M. Busslinger (Vienna), G. and P. Matthias (Basel), M. Reth (Freiburg), S. Scheu (Düsseldorf), L. Nitschke (Erlangen), H. Wiendl (Würzburg) and M.Lutz (Würzburg).
All the mice are of C57BL/6 background.

2.2 Genotyping of mice bearing *Nfatc1*$^{-/-}$ *B cells*

Genotyping was done on 4 weeks old mice. The male and female pups were separated from their parents and kept in separate cages. The tip of the tail was cut using a scalpel and put in an eppendrof tube. 30 µl of proteinase K (Fermentas; #EO0491) was added per

1 ml genomic lysis buffer and 20 µl of the cocktail was added to each tail sample following overnight incubation at 56°C. The next day 480µl of water was added to each sample and incubated for another 10 min at 95°C. Following centrifugation, 3 µl of DNA sample was used to run PCR using the following primer pairs:

Detection of Cre gene
for 5'-ACCTCTGATGAAGTCAGGAAGAAC-3'
rev 5'-GGAGATGTCCTTCACTCTGATTCT-3'

Detection of NFATc1 *flx/flx* allele
for 5'-CCTATTTAAACACCTGGCTCCCTGCG-3'
rev 5'-CCATCTCTCTGACCAACAGAAGCCAG-3'

Detection of *mb1* WT locus
for 5'-CTGCGGGTAGAAGGGGGT-3'
rev 5'-CCTTGCGAGGTCAGGGAGCC-3'

Genomic lysis buffer:
300mM NaCl
25mM EDTA
50mM Tris (pH 8.0)
0.2% SDS

2.3 Lymphocyte isolation, surface marker staining and cell sorting

Age (6-8 weeks old) and sex matched animals were sacrificed and dissected to obtain peritoneal cells using PBS and glass pipettes followed by the removal of spleen and bone marrow (BM). Using a cell strainer (Hartenstein), single splenic cell populations were obtained and erythrocytes were lysed with TAC buffer (Roth). Naïve splenic B cell isolation was done using Miltenyì's B cell isolation kit (#130-090-862) according to manufacturer's instructions with a yield of 95-98% pure B cell population. BM cells were flushed out using a syringe and PBS.

Cells were blocked with mouse Fc receptor specific mAb (BD, #553142) and stained with the following antibodies for flowcytometric analysis: CD22-APC (BD, #533145), IgG1-Bio (BD, #553952), IgG3-Bio (BD, #553401), CD138-PE (BD, #553714), CD19-Bio/FITC/PE (BD, #553784/557398/557399), B220-Bio/PE (BD,

#553085/553090), CD43-FITC (BD, #553270), BP1-PE (BD, #553735), CD24-Bio (BD, #555296), IgD-PE (Southern Biotech, #112009), IgM-FITC (BD, #553437), CD21/CD35-PE (BD, #552957), CD23-FITC (BD, #553138), CD5-PE (eBioscience, #12-0051-82), AA4.1-Bio (eBioscience, #13-5892-81), GL7-FITC (BD, #553666), PNA-Bio (Vector laboratories, #B-1075), CD38-FITC (BD, #553714), Fas-PE (BD, #554258), Strptavidin APC (BD, #554067).

In order to sort GC B cells and plasma cells, SRBC-immunized mice (200 µl i.p; Dunn Labortechnik GmbH, #IC100-0210-15) were sacrificed on day 10 and subjected to B cell purification. Purified B-cells were stained with B220-PE and GL7-FITC, marker for GC B cells, while to sort plasma cells, CD19 FITC-CD138 PE staining was done. The following populations: $B220^{+}GL7^{+}$, $B220^{+}GL7^{-}$ and $CD19^{+}CD138^{+}$, $CD19^{+}$ $CD138^{-}$, were sorted using FACSVantage (BD Biosciences) in lysis buffer containing dithiothreitol, BSA, 10% Triton, RNAsin, oligo(dT) primers, and distilled H_2O(Roche).

2.4 Cell culture, protein extraction and western blotting

$5x10^6$ cells were cultured in 1 ml X-vivo 15 medium (BE04-418Q; Lonza) and stimulated by LPS (10 µg/ml; Sigma, #L5293), α-IgM (10 µg/ml; Jackson Lab., #115-006-020), α -CD40 (2 µg/ml; R&D, #MAB440) or TPA (Sigma, #79346) and ionomycin (Invitrogen, #I24222) for different time periods. The cells were harvested and washed twice with cold PBS.

PBS
8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4
Dissolved in 800 ml ddH_2O; adjust pH to 7.4 with HCl, add ddH_2O to 1 Liter.

Whole protein extract was prepared by incubation of cells on ice with a cocktail of RIPA and PMSF (Sigma) followed by centrifugation.

RIPA buffer

150 mM	NaCl
1.0%	NP-40 (Sigma)
0.5%	sodium deoxycholate
0.1%	SDS
50 mM	Tris, pH 8.0

PMSF
0.1 M in 100 % Ethanol

Bradford Assay (BIORAD) was done to estimate the protein concentration. A minimum of 25-30 µg protein was loaded per sample per lane for the separation on SDS-PAGE gels. Nitrocellulose membranes (WHATMAN, #BA 85) were used for transfer of protein. Membranes were blocked using 4% non-fat dry milk for 45 min-1 hr. The membranes were incubated with primary antibodies over night at 4^{o}C on a shaker. The following primary antibodies were used in this study: NFATc1 (7A6 mAb; BD, #556602), NFATc1/αA (IG-457 pAb; ImmunoGlobe), PLCγ2 (Sc-407 pAb; Santa Cruz). Secondary antibodies (α-mouse or α-rabbit) were used based on the source of primary antibodies. Signals were developed in dark room using Super Signal^{TM} kit (Pierce Chemical Company, CA). To confirm equal loading, the filters were re-blotted with β-actin (AC-15 mAb; Sigma, #A1978) or ERK (mAb; Santa Cruz, #Sc-154).

10% Resolving gel (10 ml)

H_2O	4.0 ml
30% Polyacrylamide (Applichem)	3.3 ml
1.5 M Tris (pH 8.8)	2.5 ml
10% Ammonium persulfate (APS) (Sigma)	0.1 ml
10% Sodiumdodecyl sulfate (SDS) (Roth)	0.1 ml
TEMED (Sigma)	0.004 ml

1.5 M Tris (Roth)
90.825g Tris
300ml ddH_2O
pH adjusted to 6.8 and 8.8 with conc. Hcl
ddH_2O added to 1 Liter

Stacking gel (3 ml)

H_2O	2.1ml
30% Polyacrylamide (Applichem)	0.5 ml
1.5 M Tris (pH 6.8)	0.25 ml
10% Ammonium persulfate (APS) (Sigma)	0.3 ml
10% Sodiumdodecyl sulfate (SDS) (Roth)	0.3 ml
TEMED (Sigma)	0.003 ml

10% SDS
100g SDS
900ml ddH_2O
Heat to 68°C to assist dissolution
pH adjusted to 7.2 with conc. Hcl
ddH_2O added to 1 Liter

Running buffer (1X TG Buffer)

10x TG buffer
30.2g Tris
188g Glycine (Roth)
100ml 10% SDS
ddH_2O added to 1 Liter

1x TG buffer
100ml 10X TG Buffer
ddH_2O added to 1 Liter

1 x transfer buffer (store at 4° C)
72.5g Glycine
14.5g Tris
1 Liter methanol
ddH_2O added to 5 Liter and chill to 4° C

Wash buffer (TBS Tween)
2.42g Tris
8g NaCl
ddH_2O added to 1 Liter and add 450µl of Tween.

2.5 Proliferation and apoptosis assay

Purified splenic B cells were incubated with CFSE (Invitrogen, #C34554) at 37^oC for 3 min. The cells were then washed with RPMI 1640 (Invitrogen), re-suspended in X-vivo 15 medium (BioWhittaker) and cultured in a 96-well plate with various stimuli including LPS (10 µg/ml), α-IgM (10 µg/ml) or α-CD40 (2 µg/ml) for 72hr at 37^oC. To measure the effect of $Ca2^+$ and Cn inhibitors, cells were co-cultured with α-IgM (10 µg/ml) and $CaCl_2$ (10 mM/ml), and/or CsA (100 ng/ml, Sigma; #C3662) and NCI3 (1 µM and 10 µM/ml). The NFAT inhibitor 11R-Vivit (Calbiochem, #480401), dissolved in DMSO (Sigma), was also added to the cell culture along with α-IgM (10 µg/ml). T cells from WT and OT-II mice were isolated using a $CD4^+$ T cell isolation kit (Miltenyi; #130-090-860) and co-cultured (B: T= 50,000:50,000) with WT or KO B cells with or without Ova protein (75 µg/ml; Sigma).

For apoptosis assay, the cells were cultured under the same conditions for 48 hr and stained with Annexin V (BD, #550475) for 15 min at RT followed by staining with

propidium iodide (PI, 125µg/ml, Sigma). FACS Calibur (BD) was used to measure CFSE dilution and Annexin V positive cells.

10x Binding buffer

0.1 M	HEPES
1.4 M	NaCl
25mM	CaCl2

0.5 M HEPES

11.915g HEPES
100ml ddH2O
pH adjusted to 7.4

5 M NaCl

292.2 g NaCl
800 ml ddH2O
ddH_2O added to 1 Liter

0.5 M CaCl2

7.351g CaCl2
100ml ddH2O

2.6 Calcium flux: Measurement of intracellular Ca^{2+}

Freshly prepared splenic B cells ($1x10^7$) were re-suspended in 1400µl RPMI 1640 containing 5% FCS. 14µl Indo-Mix was mixed with the cell suspension and incubated for 25 min on a shaker at 30°C. Then, 1400µl RPMI medium containing 10% FCS was added to the solution and re-incubated for another 10 min at 37°C. The cells were washed twice with 2 ml Krebs-Ringer (Molecular Probes) solution and re-suspended in 800µl Krebs-Ringer solution. Prior to measurement, the tubes were pre-warmed using a 37°C water-bath and a baseline measurement of 50 second was recorded per sample. Further 2 min recordings were done after the addition of α-IgM (10 µg/ml) followed by Ca^{++} (1 mM). LSR II (BD Biosciences) was used for data acquisition and 5 µl ionomycin was used to check proper loading.

Indo-Mix

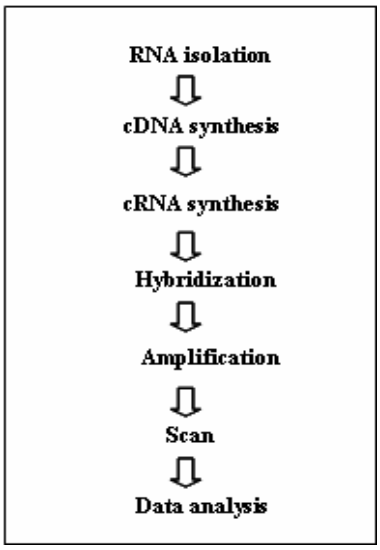
1 Stubb	Indo-1-AM (Molecular Probes; #I1223)
458µl	DMSO
37µl	20% Pluronic F-127 (Molecular Probes, #P3000MP)

Krebs Ringer

10mM	HEPES (pH 7.0)
140mM	NaCl
4mM	KCL
1mM	MgCl2
1mM	CaCl2
10mM	Glucose

2.7 Gene expression profiling

The GeneChip® Mouse Genome 430 2.0 Array from Affymetrix (Affymetrix, Santa Clara, CA, USA), which supports identification of over 39,000 transcripts on a single array, was used to study the gene expression profiling of splenic B cells from wild type and NFATc1-deficient mice. Splenic B cells were incubated with or without α-IgM (10 µg/ml) for 3, 8 or 16 h and subjected to the following steps:



The experiments were performed in triplicates and data analysis was done by the Gene Chip Operating Software (GCOS). Genes with at least two-fold difference in expression profiling, as determined by overlap analysis using Access, were further analyzed with Gene set enrichment analysis (GSEA) which is a computational method for interpreting gene expression data.-These experimentes were performed in collaboration with Dr. Ellen Leich in Prof. Dr. A. Rosenwald's Laboratory at the Institute of Pathology.

2.8 Nucleofection of primary B cells

Either empty vector or caNFATc1/αA of varying concentrations was used to transfect isolated KO B cells using Mouse B Cell Nucleofector Kit (Lonza, #VPA-1010) as per instruction. Briefly, isolated murine splenic B cells were stimulated for 24 hours in pre-Nucleofection medium supplemented with 10 µg/ml α-IgM. 1 ml of plating medium was added per well in a 12-well plate and allowed to get pre-incubated. $3x10^6$ cells/100 µl Nucleofector Solution was then combined with 2 µg DNA, transferred into certified

cuvette and subjected to Nucleofector program Z-001 (NuFe II). Approximately 500 µl of the pre-equilibrated plating medium was added to the cuvette and immediately transferred into the 12-well plate using the pipettes supplied. The cells were then stimulated with α-IgM and LPS and incubated at 37°C /5% CO_2 for additional 24 hrs.

2.9 RT-PCR and qRT-PCR

Stimulated or un-stimulated B cells were harvested after specified time periods, and RNA isolation was done using Trizol reagent (Invitrogen). In brief, cells were suspended into 1 ml Trizol and stored at -70°C. Prior to RNA extraction, the tubes were allowed to thaw at RT and 200 µl of chloroform (Roth) was added to the samples. The tubes were vortex for 10 s following 5 min incubation at RT and then subjected to centrifugation in the cold room at the maximum speed for 10 min. The aqueous upper phase was then carefully transferred into a new autoclaved (RNase free) eppendrof tube and 500 µl of isopropanol (Roth) was added. After a short vortex, the cells were incubated at RT for 10 min and centrifuged at the maximum speed for 30 min. The supernatant was removed and the pellet was washed with 1 ml of 75% Ethanol (Roth) and air-dried before dissolving in 10-12 µl of DEPC (Roth) water. The newly extracted RNA was mixed with 1 µl of 6x loading dye and run on 1% agarose gel to check for any degradation.

1% agarose gel

1g	Agarose (Applichem)
100 ml	TAE Buffer (1x)
10 µl	Ethidium bromide (Roth)

50x TAE buffer

242 g	Tris base
57.1 ml	Acetic Acid
100ml	0.5 M EDTA

ddH_2O added to 1 Liter

6x DNA loading dye

3ml glycerol (30%)
25mg bromophenol blue (Roth)
ddH_2O added to 10 ml

1 µg of total RNA was used to synthesize cDNA using the iScript™ cDNA Synthesis Kit (BIO-RAD). cDNA preparation from sorted cells was done using Titan One Tube RT-PCR System (Roche Diagnostics, Mannheim,Germany).

PCR Master Mix (2x; #K0171) and Gene Ruler™ 100bp ladder were purchased from MBI Fermentas. FastStart Universal Probe master (ROX, Roche; #04913949001) was used for qRT-PCR studies.

RT-PCR Primers

Cd22: for 5'-TCCCAGACTTTCCCCTCCCTA-3'
rev 5' -CAACCACTTTTACCCACTGA-3'
Nfatc2: for 5'-GGGTTCGGTGAGTGACAGTT-3'
rev 5'-CTCCTTGGCTGTTTGGGATA-3'
Nfatc3: for 5'- CACGCCGATGACTACTGCAAACTG-3'
rev 5'-CCTTGGAGCTGAAATGATGGTGAC-3'
Mcl-1: for 5'-TCCGGAAACTGGACATTAAA-3'
rev 5'-AGTCCCCTATTGACATCACA-3'
β-actin: for 5′-CCCAACTTGATGTATGAAGG-3′
rev 5′-TTGTGTAAGGTAAGGTGTGC-3′

qRT-PCR TaqMan Assay (Applied Biosystems)

Universal Probe Library, Probe #15 (Roche)
Fasl: Mm00438864_m1
Pdcd1: Mm01285676_m1
Rcan1: Mm00627762_m1
Spp1: Mm00436767_m1
Tnfsf14: Mm00444567_m1
GAPDH: VIC-MGB

2.10 Immunization and enzyme-linked immunosorbent assay (ELISA)

Age and sex-matched WT and KO mice (4 in each group) were immunized with NP-Ficoll (10 μg/mouse in PBS; Bioresearch Technologies, #F-1300-10-27), NP-KLH (100 μg/mouse, Alum precipitated; Bioresearch Technologies, #N-5060-5) or SRBC by intraperitoneal injection (i.p.). The immunized mice were re-challenged either after 21 days or 4 months, except for SRBC. All mice were bled prior to immunization, or as specified, and serum samples were collected. They were sacrificed on different time points and flowcytometric analysis was performed to study the generation of B-cell populations before or after immunization.

For ELISA based studies, maxisorb plates (Nunc), coated with NP (16)-BSA or NP (4)-BSA (10 μg/ml; Bioresearch Technologies), were used to detect the NP-specific antibody

level, whereas polysorb plates (Nunc) coated with isotype specific Abs were used to detect total Ig titers from non-immunized mice. Samples were serially diluted and a pool of sera from day 7 and day 21 was used as a standard for NP-specific antibody detection. Monoclonal isotype Abs (Southern Biotech; IgM, #1021-01; IgG1, #1035-01; IgG2a, #1080-04; IgG2b, #109-04; IgG3, #1041-03) were used for total Ig measurement in naïve mice.

The plates were coated with coating solution and kept at 4°C overnight. Followed by 2hr incubation at 37°C with blocking solution and 3 times washing with wash buffer, samples were added to the plates. A serial dilution of sera was done using dilution buffer and incubated for another 2hr at 37°C. Secondary antibodies were then added to the plates and left overnight at 4°C. Next day, substrate was added to each plate and allowed to develop colour. The plates were read using a plate reader (Molecular Devices) and quantifications were done against the standard curve.

Coating solution	NP-BSA in PBS; working conc. 10µg/ml
Blocking solution	1% BSA (Sigma) in PBS + 0.05% sodium azide (Sigma)
Dilution buffer	0.1% BSA in PBS + 0.05% sodium azide
Wash buffer	PBS+0.05% Tween 20
Substrate	p-Nitrophenylphosphate (Sigma)/1 ml diethanolamine buffer

Diethanolamine buffer (pH 9.8)

0.1g	MgCl2
0.2g	NaAzide
97ml	Diethanolamine

ddH_2O added to 1 Liter

2.11 *In-vitro* generation of GC B cells and plasma cells followed by class-switching assays

Induction of GC B cells were achieved by stimulating splenocytes with anti-CD3 (BD) pre-coated plates for 2 days. FACS analysis was done after staining with CD19-PE, GL7-FITC and PNA-Bio (Vector Laboratories).

LPS (10 µg/ml) stimulated splenocytes were harvested after 3 days and stained with CD19, CD138 and IgG1 and/or IgG3 for the detection of in-vitro plasma cell formation and class switched antibodies using flowcytometry. The same study was also done by ELISAs in which 2.5 x 10^5 splenic cells/ ml in X-vivo medium were stimulated with or without LPS (10 µg/ml) for 6 days and supernatants were used to perform ELISA using unlabelled goat anti-mouse IgM (Southern Biotech, # 1220-01) and IgG3 (Southern Biotech, #1100-01) coated plates. Mouse IgM (0.1 mg/ml, Southern Biotech; #0101-01) and mouse IgG3 (0.1 mg/ml, Southern Biotech; #0105-01) were used as standards along with alkaline phosphatase coupled isotype specific Abs for detection.

In order to identify circular transcripts, murine splenocytes were cultured at a concentration of 1 x 10^6 cells/ ml in X-vivo medium and incubated with or without LPS (10 µg/ml) and LPS (10 µg/ml) + IL-4 (10ng/ml; PeproTech; #315-05) for 2 days at 37°C. Followed by RNA extraction and c-DNA synthesis, PCR reactions were carried out with the following primers as described by Kinoshita et al (Kinoshita, Harigai et al. 2001).

Ig3F: 5'-TGGGCAAGTGGATCTGAACA-3'
Ig1F: 5'-GGCCCTTCCAGATCTTTGAG-3'
CuR: 5'-AATGGTGCTGGGCAGGAAGT-3'

2.12 Intracellular IL-10 staining

Splenocytes were stimulated in the absence or presence of LPS for 4 days. Then, they were treated with T+I and Golgiplug (BD) for the final 6 h. The cells were harvested, washed and blocked with mouse Fc receptor specific mAb (BD) and stained with CD19 and CD138 antibodies (BD). Cells were fixed and permeabilized (eBioscience fixing and permeablizing kit) and washed followed by staining with anti-mouse IL-10 APC Ab (eBioscience). Prior to FACS measurement, they were re-washed and re-suspended in FACS buffer.

2.13 Statistical analysis

Error bars in figures represent ±SEM (standard error of mean). Unpaired t-test was performed to evaluate the statistical significance of the data set. A value of $p < 0.001$(***) is considered highly significant, while $p<0.05$(*) as well as $p<0.005$(**) are known to be statistically significant. The statistical analysis was done using GraphPad Prism.

Instruments/accessories

Balance machine	Chyo
Centrifuge	Eppendorf
Cold centrifuge	Heraeus
Culture plates	Greiner
Cuvettes	Brand
Gel documentation system	Herolab
Heating blocks	Hartenstein
Light microscope	Olympus, Leica
Microcentrifuge	Eppendorf
Multichannel pipette	Eppendorf
Nucleofector II	Amaxa
Pipette tips	Eppendorf
PCR machine	Primus 96
pH meter	WTW
Real-Time RCR machine	ABI Prism 7000
SDS-PAGE apparatus	Hoefer
Spectrophotometer	Pharmacia
Vortexer	Eppendorf
Waterbath	Heidolph
Western blot apparatus	Hoefer
Whatman filter paper	Hartenstein
X-ray film	Kodak

3. Results

3.1 NFATc1/αA isoform is highly inducible upon BCR stimulation

It is established that expression of NFATc1 is highly inducible in T cells upon stimulation with TPA/ionomycin (T/I) or anti-CD3+CD28. In order to characterize the induction of NFATc1 in B cells, splenic B cells from 6-8 weeks old WT mice were stimulated by LPS or α-IgM (Fig. 3.1A) and α-CD40 or T/I (Fig. 3.1B) for 8, 24 and 48h. *In-vitro* stimulation with both α-IgM and T/I led to a stronger NFATc1 induction after 24h, whereas LPS and α-CD40 appeared to be weak stimuli.

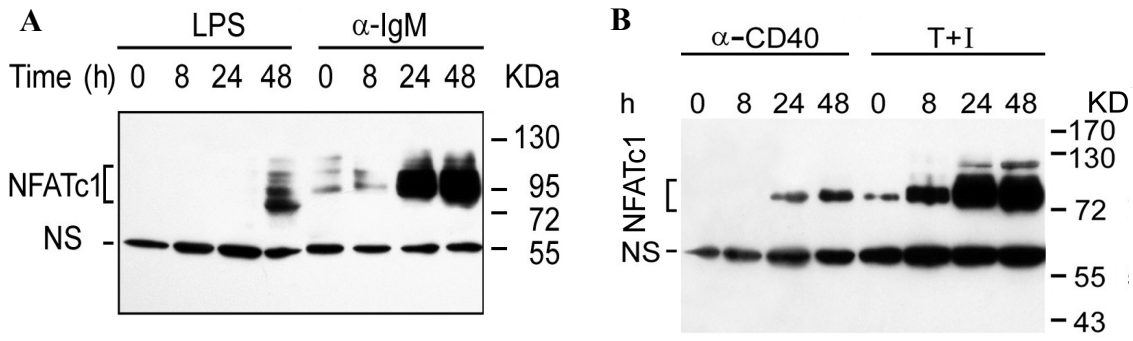


Figure 3.1 Induction of NFATc1. Western blots of whole cell protein extracts showing induction of NFATc1 expression upon stimulation with LPS or α-IgM (A) and α-CD40 or T/I (B). For each (A) and (B) one typical blot out of three is represented.

α-IgM mediated induction of short NFATc1 protein reacted upon stimulation with an antibody raised against the NFATc1-α peptide, a component of NFATc1/αA, which, however, failed to react with LPS-induced NFATc1 protein (Fig. 3.2).

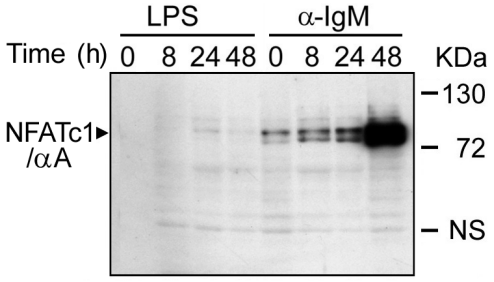


Figure 3.2 Induction of NFATc1/αA upon BCR stimulation. Western blot of whole cell protein extract showing the induction of NFATc1/αA upon α-IgM stimulation using an antibody specific for the N-terminal α peptide of NFATc1/αA proteins. Figure shows one blot out of more than three experiments.

Antigen cross linking of BCR leads to activation of a number of adaptor proteins including SLP-65. Mice lacking SLP-65 in B cells, a key component of BCR transducer complex, fail to induce NFATc1 upon LPS or α-IgM stimulation, confirming the role of BCR signals in NFATc1 induction (Fig. 3.3).

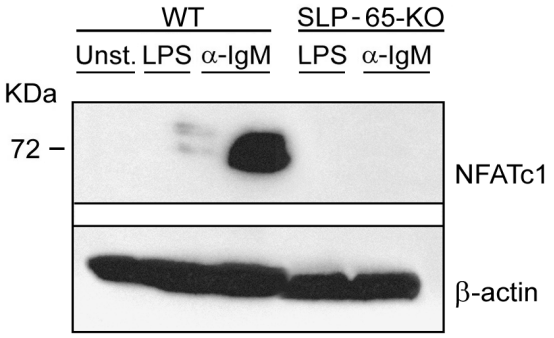


Figure 3.3 NFATc1 induction is mediated via BCR cross linking. Western blot showing abolished expression of NFATc1 in SLP65$^{-/-}$ B cells compared to WT. Figure represents one typical blot out of two experiments.

3.2 Complete loss of NFATc1 in splenic B cells from *mb1-Cre x Nfatc1$^{flx/flx}$* and *Cd23-Cre x Nfatc1$^{flx/flx}$* mice

To study the role of NFATc1 in B cell physiology, we created B cell stage-specific KO mice as described in "Material and Methods". BM specific KO (*mb1-Cre x Nfatc1$^{flx/flx}$*) (Fig. 3.4A) and splenic B cell specific KO (*Cd23-Cre x Nfatc1$^{flx/flx}$*) mice (Fig. 3.4B) had a complete loss of NFATc1 in splenic B cells, as detected by western blot analysis after stimulating splenic B cells with α-IgM.

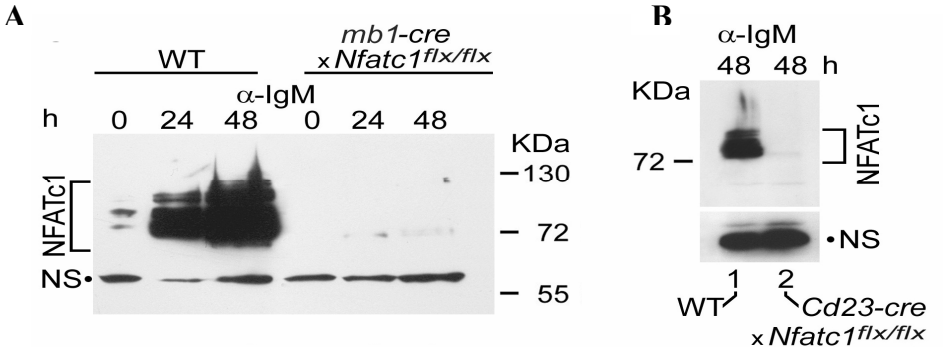


Figure 3.4 Complete ablation of NFATc1 upon inactivation of the *Nfatc1* gene in bone marrow or spleen. Western blots showing complete loss of NFATc1 expression in B cells from *mb1-Cre x Nfatc1$^{flx/flx}$* (A) and *Cd23-Cre x Nfatc1$^{flx/flx}$* mice (B). For (A) and (B), figure represents one typical blot out of more than three experiments.

3.3 NFATc1 is essential for peritoneal B1a cell generation when deleted in BM B cells

To investigate the role of NFATc1 in B cell development, next we studied the B cell compartments from WT and *mb1-cre* x *Nfatc1*$^{flx/flx}$ by flow cytometry. Cells, isolated from BM, spleen and lymph nodes (LN), were stained with specific surface markers followed by flow cytometric analysis. No significant differences were found between the two mice with respect to their BM precursor B cells (B220^{+} CD43^{hi}) and BM immature and mature B cells (B220^{+} CD43^{lo}) (Fig. 3.5A), percentage of peripheral B cells in LN (IgD^{+} IgM^{+})(Fig. 3.5B) splenic MZ (CD21^{+} CD23^{-}), FO (CD21^{lo} CD23^{+}) (Fig. 3.5C) or splenic immature (B220^{+} AA4.1^{+}) and mature (B220^{+} AA4.1^{-}) B cell population (Fig. 3.5D). As described previously (Berland and Wortis 2002), we also observed a 5-10 fold decrease in peritoneal B1a (CD5 ^{lo} IgM^{+}) cells in *mb1-cre* x *Nfatc1*$^{flx/fl}$ mice compared to WT mice (Fig. 3.5E).

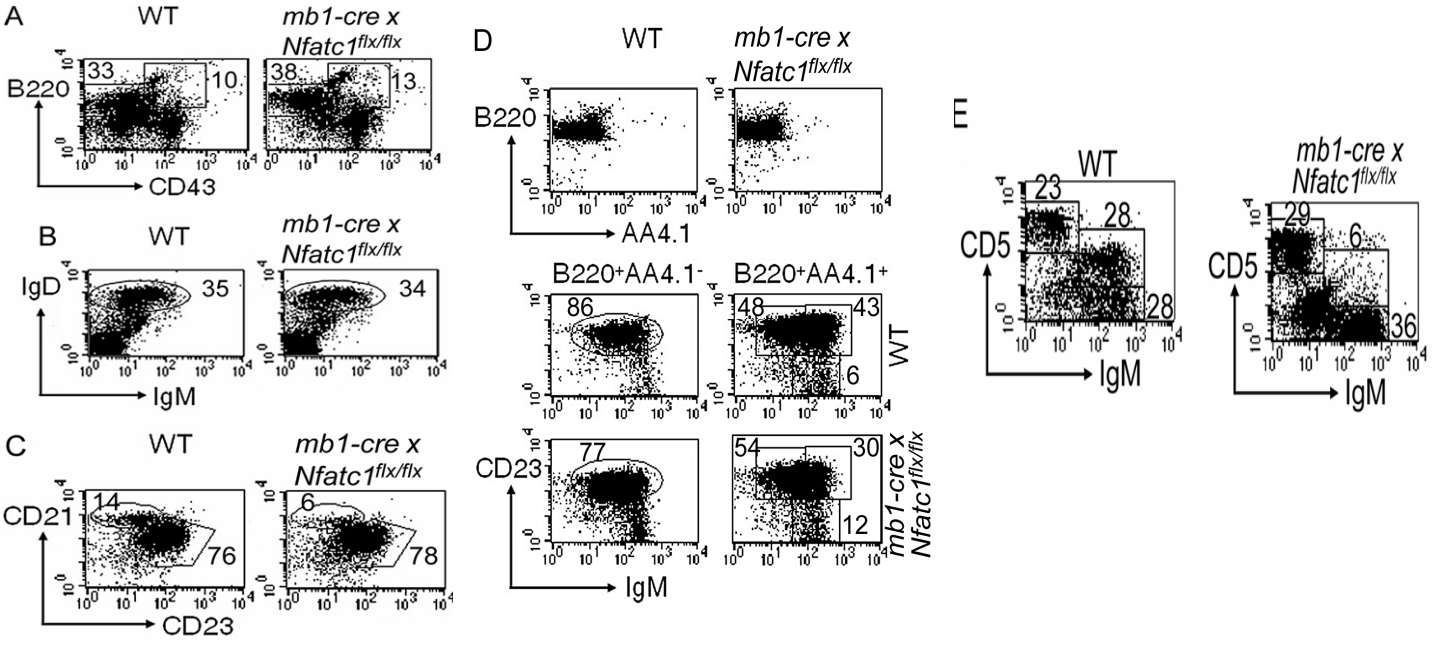


Figure 3.5 Effect of NFATc1 ablation on B cell development. Flowcytometric analysis represents percentage of BM precursor, immature and mature B cells (A), peripheral B cells from LNs (B), splenic MZ and FO B cells (C), or splenic immature and mature B cells (D) and peritoneal B1a cells (E) from WT and *mb1-cre x Nfatc1*$^{flx/flx}$ mice. Each experiment was performed with more than three animals.

3.4 NFATc1 deficiency leads to impaired BCR-mediated proliferation and increased AICD of splenic B cells

In CFSE dilution assays we observed an impaired B cell proliferation for NFATc1$^{-/-}$ compared to WT B cells, when stimulated with α-IgM for 72h. In contrast, stimulation with LPS and α-CD40 had no significant effect on proliferation suggesting a role of NFATc1 in BCR-mediated cell proliferation (Fig. 3.6).

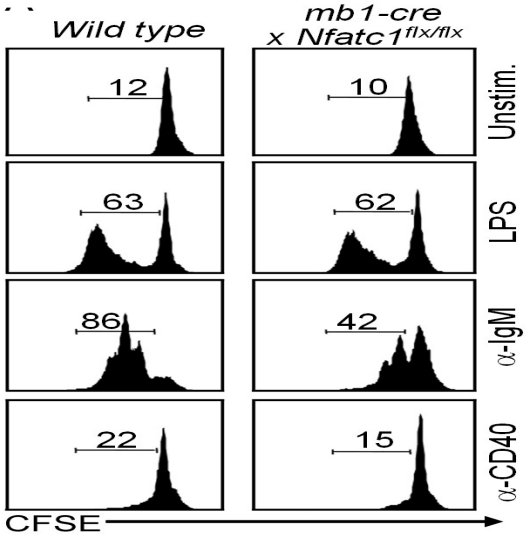


Figure 3.6 NFATc1 plays an important role in BCR-mediated proliferation. Isolated B cells were stained with CFSE and stimulated with or without α-IgM, LPS and α-CD40 for 3 days. Histograms represent flowcytometric detection of CFSE dilution for proliferating WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells. Figure shows one typical example out of three.

In parallel to defective proliferation of α-IgM-induced B cells we detected an increase in cell death as evidenced by the appearance of higher number of Annexin V positive apoptotic cells in *mb1-cre x Nfatc1*$^{flx/flx}$ B cell cultures when stimulated with α-IgM rather than LPS or α-CD40. These observations confirm a role of NFATc1 in AICD (Fig. 3.7).

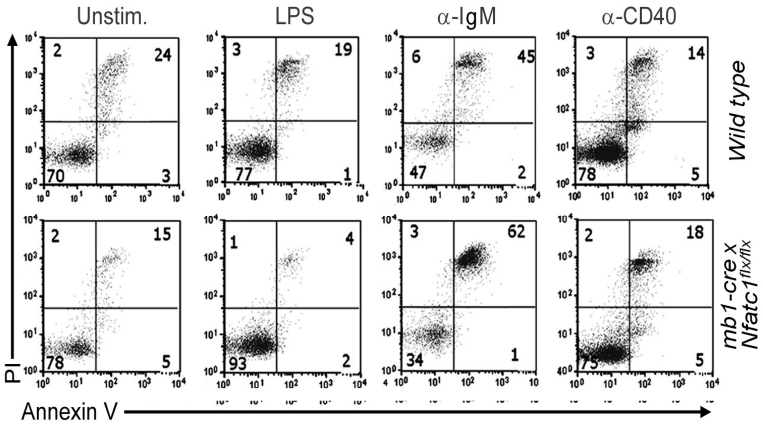


Figure 3.7 NFATc1 deficiency supports AICD. Isolated WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells were cultured with or without α-IgM, LPS or α-CD40 for 48 hr. Cells were stained with Annexin V and PI, dot plot represents percentage of apoptotic cells detected by flow cytometry. Figure represents one typical experiment out of three.

In order to address the issue of impaired proliferation of B cells from *mb1-cre x Nfatc1*$^{flx/flx}$ mice, we measured the calcium influx in WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells. BCR engagement activates signaling pathways that initiate Ca^{2+} flux required for proliferation and survival of B cells. To determine the status of store operated Ca^{2+} release in WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells, we measured Ca^{2+} flux using cells suspended in Krebs-Ringer solution supplemented with EGTA (in order to chelate external Ca^{2+}). On the other hand, extracellular Ca^{2+} influx was measured by addition of 1 mM Ca^{2+} in medium devoid of EGTA. A significant decrease was observed in intracellular (store operated) (Fig. 3.8A) as well as extracellular (Fig. 3.8B) Ca^{++} influx upon α-IgM stimulation in *mb1-cre x Nfatc1*$^{flx/flx}$ B cells.

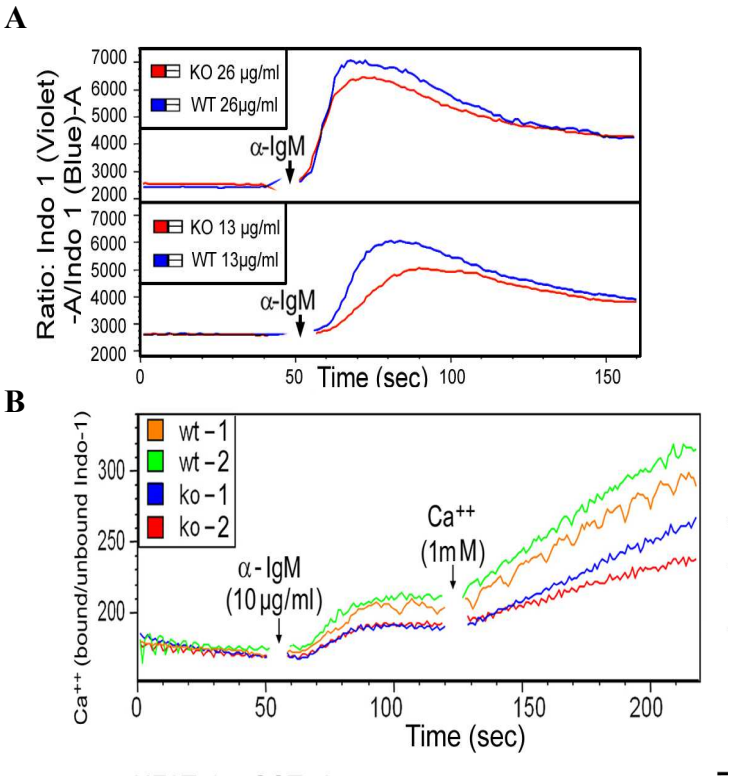


Figure 3.8 Impaired calcium flux is observed in NFATc1 deficient B cells. Isolated WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells were stained with Indo-1 and stimulated with an indicated concentration of α-IgM. Intracellular (A) and extracellular (B) Ca^{2+} levels were measured by flow cytometry. Each experiment was conducted with minimum of three animals in each group.

The positive effect of calcium in B cells was further evaluated by culturing splenic B cells in X-vivo medium, in a 1:1 mixture of X-vivo and RPMI media (the latter is a Ca^{++}-poor medium) or by supplementing the culture medium with $CaCl_2$. The selective use of RPMI which contains about one third of Ca^{2+} of X-vivo resulted in impaired proliferation in WT and *mb1-cre x Nfatc1*$^{flx/flx}$ B cells, the later being more severely affected. Addition of Ca^{2+} in the form of $CaCl_2$ could restore their proliferative capacity, while addition of CsA, a well known calcineurin inhibitor, had a strong suppressive effect on B cell proliferation when added at the start of the experiment or one day later (Fig. 3.9).

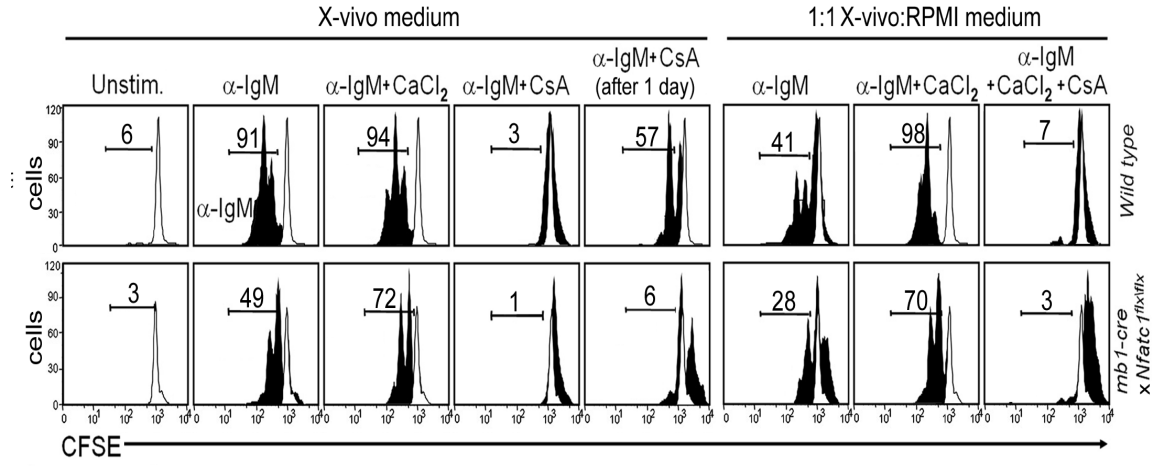


Figure 3.9 Effect of calcium and calcineurin on B cell proliferation. Splenic B cells from WT or *mb1-cre x Nfatc1*$^{flx/flx}$ mice were stained with CFSE and treated with 10 μg/ml α-IgM in the absence or presence of Ca^{2+} or CsA in X-vivo medium or 1:1 X-vivo+RPMI medium which contains less Ca^{2+}. Numbers indicate the percentage of cycling cells. Experiments were repeated at least three times

We used also NCI3 to inhibit calcineurin and to study its effect on B cells. NCI3 is an inhibitor that binds to calcineurin but does not interfere with its phosphatase activity. It inhibits NFAT dephosphorylation and blocks NFAT signaling (Sieber, Karanik et al. 2007). When B cells were incubated with higher doses of NCI3, strong suppression of proliferation was observed in a dose dependent manner (Fig. 3.10A). The VIVIT peptide, Vivit-11R, is a selective inhibitor of NFAT-calcineurin interaction. Like NCI3, it also does not inhibit calcineurin phosphatase activity as CsA while inhibiting NFAT activation. Therefore, Vivit allows the progression of other calcineurin-independent pathways while inhibiting the activity of NFATs (Aramburu, Yaffe et al. 1999). B cells incubated with different doses of VIVIT peptide resulted in decreased proliferation (Fig.3.10B) and increased B cell death (Fig.3.10C).

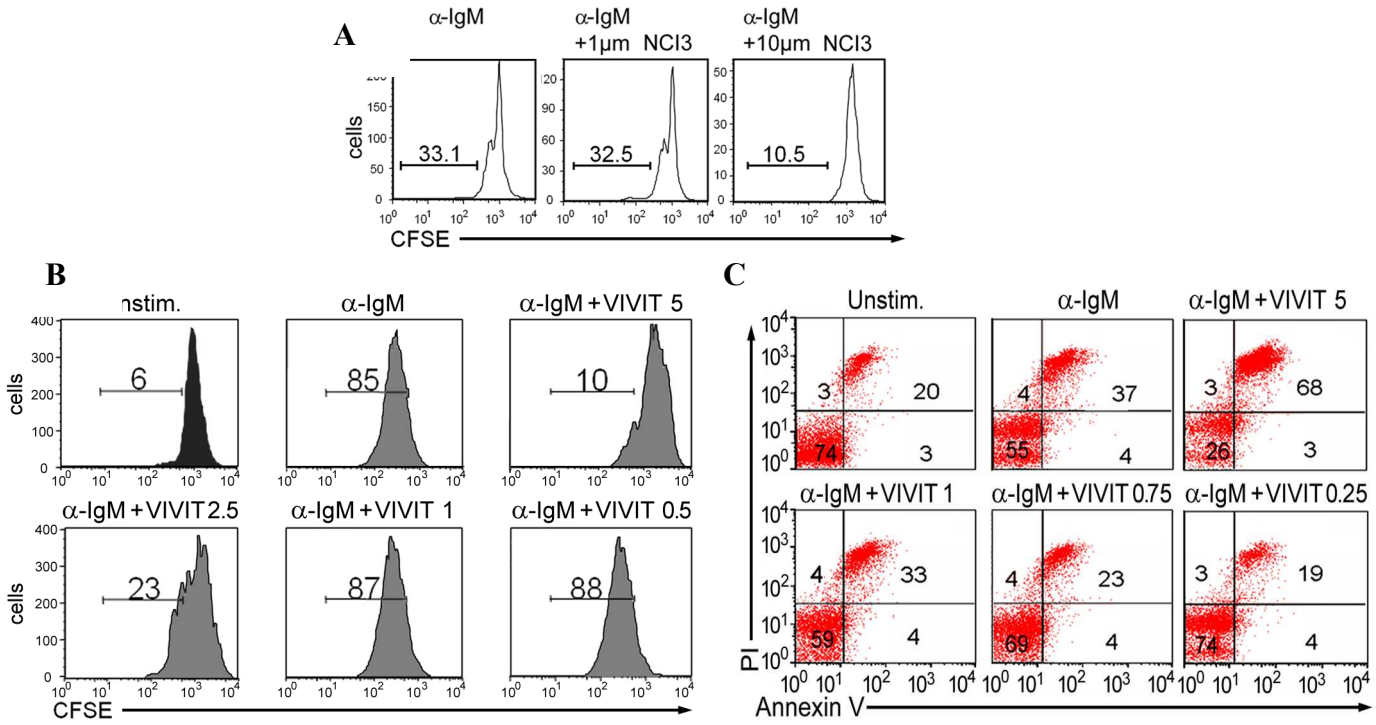


Figure 3.10 Inhibition of calcineurin and of NFATs results in decreased proliferation and increased cell death of splenic B cells. (A) Histogram represents flow cytometric detection of CFSE dilution in isolated splenic B cells treated with different concentrations of NCI3, a novel Cn inhibitor, and 10 μg/ml of α-IgM for 3 days. Numbers represent percentage of proliferating cells. Experiments were repeated twice. (B) Isolated splenic B cells were left un-stimulated or stimulated by α-IgM in presence of 0.5-5 μM Vivit-11R for 3 d. Proliferation of B cells was detected using the CFSE dilution assay. (C) Isolated splenic B cells were incubated with 10 μg/ml of α-IgM in absence or presence of Vivit-11R as indicated, for 48 hr. AICD was determined by flowcytometry of annexin-V/PI staining. Figure (B) and (C) represents one typical example out of three such experiments.

3.5 Most prominent NFATc1 target genes in splenic B cells

DNA microarray studies were done to identify NFATc1 target genes having role in proliferation and/or apoptosis of splenic B cells. The following set of genes, known to encode apoptosis regulators and having a role in cell signalling, were detected amongst the enhanced "top ten" genes in B cells from wild type mice versus mice bearing NFATc1-deficient B cells: *Spp1* / osteopontin, *Rcan1* / calcipressin, *Pdcd1* / PD-1, *Tnfsf14* / LIGHT and *Fasl* (Fig. 3.11A) . They were undetected in naïve B cells and induced upon α-IgM stimulation. A rapid induction of *Spp1* and *Rcan1* expression was noticed in samples treated for 3h with α-IgM, following a decrease in *Spp1* RNA level with no notable change in *Rcan1* expression in NFATc1$^{-/-}$ B cells in respect to WT. On

the other hand, expression of *Pdcd1, Tnfsf14* and *Fasl* genes increased strongly after 16h of α-IgM stimulation in WT with little to no induction in NFATc1$^{-/-}$ B cell (Fig. 3.11B).

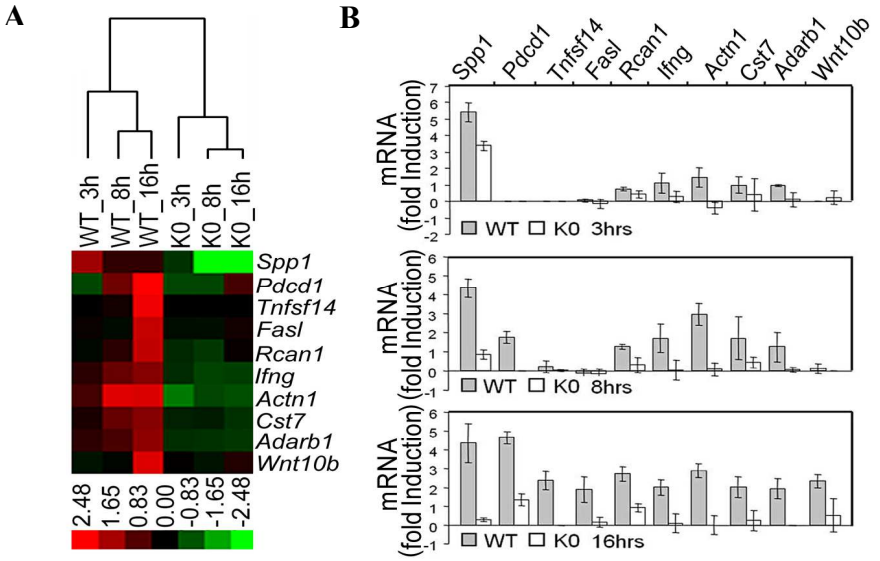


Figure 3.11 NFATc1 target genes in murine splenic B cells. (A) WT and *mb1-cre x Nfatc1$^{flx/flx}$* splenic B cells were stimulated with α-IgM as indicated. Gene expression was analyzed by DNA microarray assays. Key at the bottom indicates the fold changes in gene expression (log2 values). (B) Induction of 10 NFATc1 target genes by α-IgM stimulation in WT and NFATc1$^{-/-}$ splenic B cells. Experiments were repeated three times.

We then performed a qRT-PCR analysis with WT B cells to support the DNA microarray studies. The findings also indicate a time dependent induction pattern of the genes as indicated in data of DNA microarray assays (Fig. 3.12).

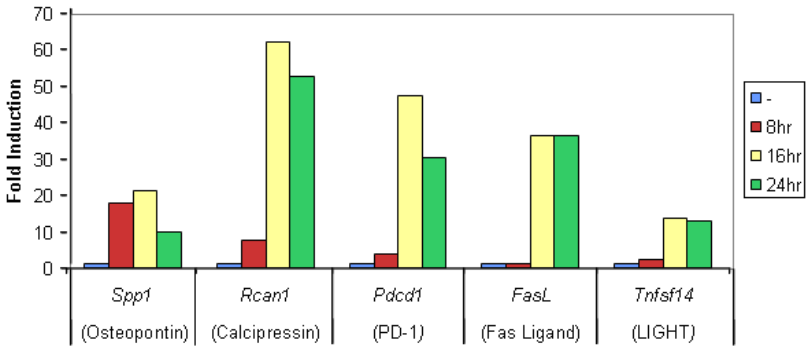


Figure 3.12 qRT-PCR supports DNA microarray data. Splenic B cells were left unstimulated or treated with α-IgM (10µg/ml) for indicated time period and subjected to qRT-PCR analysis. Graph showing differential regulation of five genes among the enhanced "top-ten" gene set which was determined in DNA microarray studies. Figure shows one typical RT-PCR result out of two.

In order to determine the functional aspects of PD1 and LIGHT, two of the most prominent NFATc1 targets - as confirmed by DNA microarray and qRT-PCR analyses - we used *Pdcd1*$^{-/-}$ and *Tnfsf14*$^{-/-}$ animals. Mice lacking PD-1 (*Pdcd1*$^{-/-}$ mice) and LIGHT (*Tnfsf14*$^{-/-}$ mice) showed a strong reduction in AICD (Fig. 3.13A) with no defect in proliferation (Fig 3.13B) when treated with α-IgM, indicating their pro-apoptotic function in B cells.

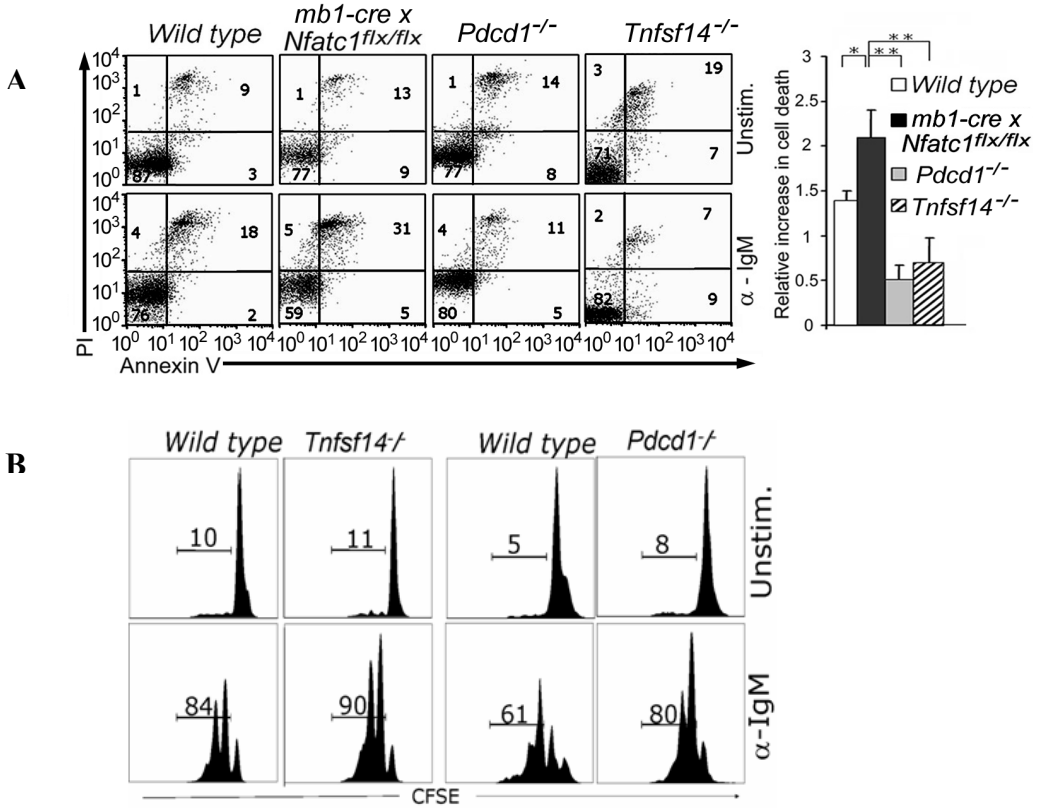


Figure 3.13 PD-1 and LIGHT are pro-apoptotic proteins. Splenic B cells from WT, *mb1-cre x Nfatc1* $^{flx/flx}$, *Pdcd1*$^{-/-}$ and *Tnfsf14*$^{-/-}$ mice were left unstimulated or treated with α-IgM (10 μg/ml) for 48 hr and subjected to Annexin V/PI staining for flowcytometric detection of AICD (A) or CFSE dilution assay to assess proliferation (B). Each experiment was repeated three times.

It is noteworthy that the most prominent target genes that are positively regulated by NFATc1 (*Fasl*, *Pdcd1* and *Tnfsf14*) are pro-apoptotic in nature, which seems to be in stark contrast to the phenotype of NFATc1 *mb1-cre x Nfatc1*$^{flx/flx}$ B cells that are highly susceptible to AICD. When we analyzed our DNA microarray data to look for the suppressed genes by NFATc1, we identified *Cd22* among the most strongly suppressed

genes (Fig. 3.14). *Cd22* is a negative regulator of BCR signaling and known to inhibit BCR-mediated Ca^{2+} signaling (Nitschke 2009).

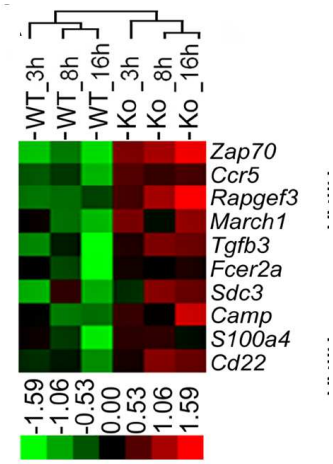


Figure 3.14 NFATc1 negatively regulates CD22. (A) DNA microarray assay showing 10 genes whose expression was enhanced in B cells from *mb1-cre x Nfatc1*$^{flx/flx}$ mice, compared to WT B cells. Expression analysis was done with B cells from three WT and three KO animals.

Our finding that NFATc1 suppresses CD22 expression is supported by RT-PCR and flowcytometric analysis. CD22 expression was much higher in NFATc1$^{-/-}$ B cells compared to the WT in either at RNA level as observed by RT-PCR (Fig. 3.15 A) or protein level as measured by flowcytometry (Fig. 3.15B). It is notable that CD22 expression declined upon BCR engagement in WT B cells but not in NFATc1 *Cd23-cre x Nfatc1*$^{flx/flx}$ B cells.

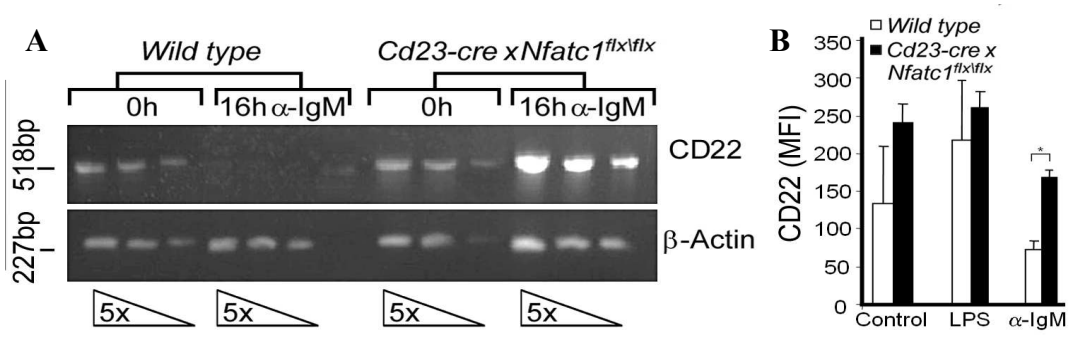


Figure 3.15 NFATc1 suppresses CD22 expression. Semi-quantitative RT-PCR assay showing the generation of CD22 RNA in WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ splenic B cells (A) and graph representing the flowcytometric detection of CD22 expression on WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ splenic B cells left unstimulated or stimulated with LPS or α-IgM for 24 h (B). Experiments were repeated twice.

Western blot analysis of splenic B cells from CD22$^{-/-}$ mice revealed strong NFATc1 expression in basal level compared to the WT B cells. The induction of the short isoform NFATc1/αA was also found in LPS treated CD22$^{-/-}$ B Cells in contrast to the WT B cells which upregulated NFATc1/αA expression only after α-IgM treatment (Fig. 3.16).

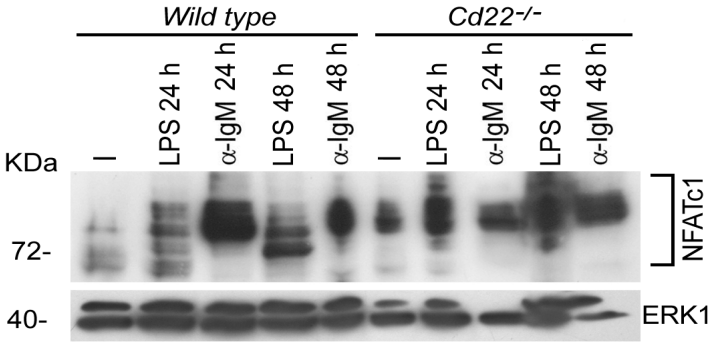


Figure 3.16 NFATc1 expression is high in CD22$^{-/-}$ mice. Immunoblots of whole cell extract, showing expression of NFATc1/αA in un-stimulated, IgM and LPS-treated splenic B cells from two WT and CD22$^{-/-}$ mice.

As we know sustained level of calcium entry is important for cells and also required for NFAT activation, we wanted to find out if impaired Ca^{2+} flux in NFATc1$^{-/-}$ B cells had any considerable effect on the generation of other NFATs. RT-PCR studies showed no difference in NFATc2 RNA level between WT and NFATc1$^{-/-}$ B cells. However, a slight decrease in NFATc3 RNA level was observed in *Cd23-cre x Nfatc1$^{flx/flx}$* splenic B cells (Fig. 3.17). In view of the fact that no significant increase in NFATc3 RNA level was identified in stimulated WT B cells, NFATc1 emerges as an indirect regulator of *Nfatc3* expression.

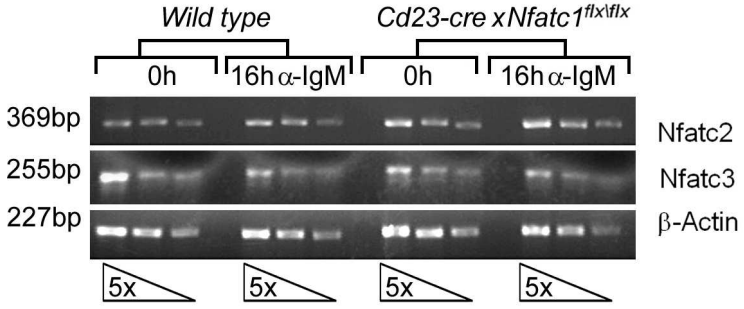


Figure 3.17 NFATc1 affects *Nfatc3* expression. Semi-quantitative RT-PCR assay showing *Nfatc2* and *Nfatc3* expression level in WT and *Cd23-cre x Nfatc1$^{flx/flx}$* splenic B cells. Figure represents one typical result out of three.

The apparent discrepancy between the DNA microarray data and phenotype of NFATc1-deficient B cells prompted us to closely scrutinize the microarray data. In addition to the *Ccnb1/*Cyclin B1 gene amongst the list of top 53 genes whose expression was twofold or more increased upon BCR stimulation in WT compared to NFATc1$^{-/-}$ B cells we did not identify genes which directly control cell cycle and proliferation. However, when we subjected the DNA microarray data to gene set enrichment analysis (GSEA), eleven most prominent signatures among numerous other gene signatures that regulates cell cycle and proliferation, were found to be significantly enriched in WT B cells compared to NFATc1$^{-/-}$ B cells (Table 1). Therefore, our findings imply that even though NFATc1 augments the expression of genes associated with proliferation only to moderate levels, the cumulative effect of these genes on B cell survival and/or proliferation is much more prominent.

Pathways/ Signatures	References	Enriched in WT	Enriched in *Nfatc1*$^{-/-}$ B cells	Nominal p-value	FDRq value
Cell Cycle	(Brentani, Caballero et al. 2003) (Cho, Huang et al. 2001) (Whitfield, Sherlock et al. 2002) (Liu, Umbach et al. 2004)	YES	NO	<0.0001	<0.07
Proliferation	(Shaffer, Wright et al. 2006) (Rosenwald, Wright et al. 2002) (Su, Wiltshire et al. 2004)	YES	NO	<0.0001	<0.09
Myc targets	(Zeller, Jegga et al. 2003) (Yu, Cozma et al. 2005)	YES	NO	<0.01	<0.08
Anti-Apoptosis	(Wu, Kirschmeier et al. 2002)	YES	NO	≤0.01	<0.08
Wnt signaling	(Kenny, Enver et al. 2005)	YES	NO	<0.031	0.24

Table 1. NFATc1 enhances expression of numerous genes linked to proliferation only to moderate levels. Gene set enrichment analysis (GSEA) of microarray data of splenic B cells from WT and *mb1-cre x Nfatc1*$^{flx/flx}$ mice stimulated for 16 h by α-IgM using 81 lymphoma associated signatures from the signature data base of the Staudt laboratory (http://lymphochip.nih.gov/signaturedb) and 1.687 curated gene sets (c2) from the Molecular Signatures Database (MSigDB)

3.6 Ectopic expression of NFATc1 can protect B cells from AICD

In order to determine the reason for the apparent contrast between the anti apoptotic property of NFATc1 as evidenced by increased AICD in NFATc1$^{-/-}$ B cells on the one hand and identification of pro-apoptotic genes as most prominent NFATc1 target in DNA microarray assays on the other, we introduced increasing amounts of a constitutively active (ca) version of NFATc1/αA and an extra *Egfp* marker gene into splenic B cells via nucleofection. The cells were cultured in α-IgM (and low dose of LPS for survival)–containing medium for 24 h and analysed for apoptotic cells. GFP$^+$ cells but not GFP$^-$ cells had pronounced changes in AICD induction depending on the concentrations of *caNfatc1/αA* plasmid. Cells transfected with low plasmid concentrations (0.1-0.75 μg/ml) showed protection against AICD whereas at high concentrations (2μg/ml) an increase in AICD was observed among the transfected cells (Fig. 3.18). These findings imply that (i) in the absence of any NFAT activity B cells are prone to apoptosis and (ii) at moderate to high levels NFATc1 supports B cell proliferation and protects against AICD, whereas (iii) at very high concentrations, NFATc1 can also eliminate B cells by supporting AICD.

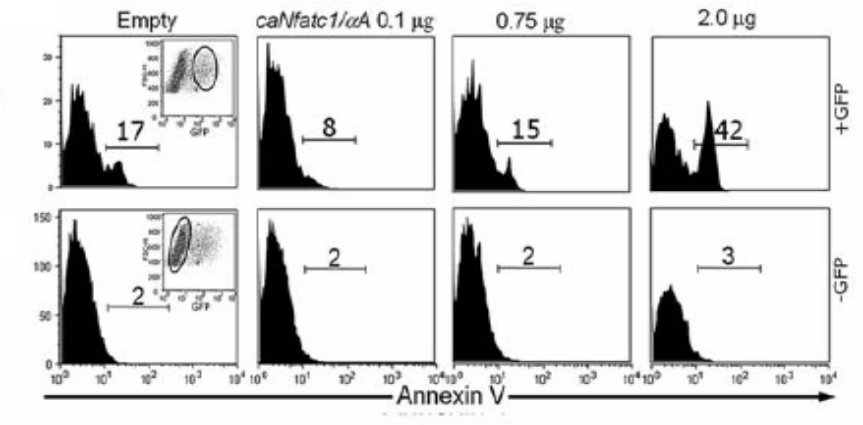


Figure 3.18 NFATc1 plays dual role in B cell survival. B cells pre-activated by α-IgM Ab treatment overnight were nucleoporated with increasing amounts of a constitutive active version of NFATc1 (*caNFATc1/αA*) and *EGFP*, and after a second α-IgM Ab+LPS stimulation for 24 hr, their apoptosis was determined by annexin V staining. Data represents one typical example out of three such experiments.

3.7 NFATc1 deficiency leads to defective immune response upon T cell-independent type-II antigenic challenge

To asses the functional aspects of *Cd23-cre x Nfatc1*$^{flx/flx}$ B cells, we first measured the normal serum titer in naïve non-immunized mice. Our data indicated presence of somewhat higher amounts of IgM antibody in serum from mice bearing NFATc1$^{-/-}$ B cells compared to WT B cells. In contrast, no considerable differences in the titer of other antibodies were found (Fig. 3.19).

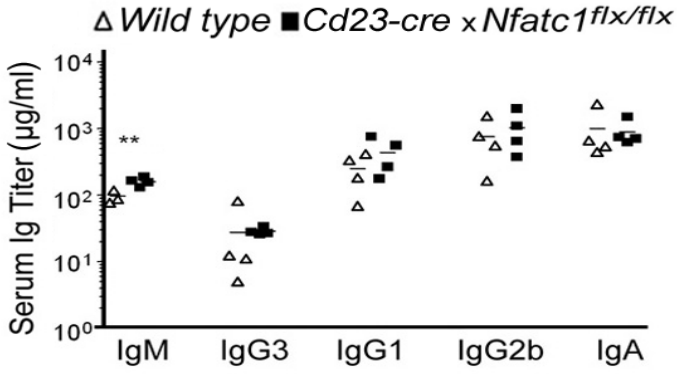


Figure 3.19 NFATc1-deficient mice demonstrate a significantly high IgM serum titer in basal level. ELISA data showing Ab serum titer in non-immunized WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice. Each symbol represents one mouse.

To investigate T cell-independent or dependent antigen responses, next we immunized mice with either NP-Ficoll to asses its response against T cell-independent antigens or with NP-KLH to study T cell-dependent antigen responses. We found a somewhat higher IgM titer in WT mice 14 days after primary immunization with NP-Ficoll, whereas the IgG3 titer was already significantly less in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice by day 7 as well as on day 14 after primary immunization (Fig. 3.20).

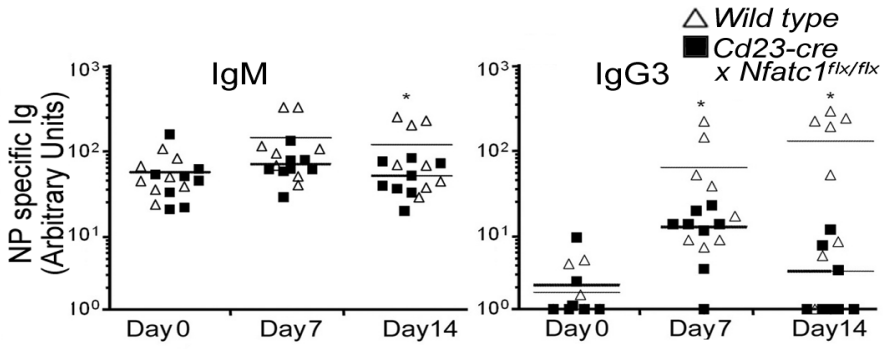


Figure 3.20 NFATc1-deficient mice show a marked less serum IgG3 Ab titer after primary immunization with TI type II antigen. ELISA data showing serum IgM and IgG3 titers in WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice after primary immunization with NP-Ficoll. Each symbol represents one mouse.

From the ELISA assay, it was evident that *Cd23-cre x Nfatc1*$^{flx/flx}$ mice can mould immune responses against TD antigens. *Cd23-cre x Nfatc1*$^{flx/flx}$ mice immunized with NP-KLH had no defect in Ig class switch - except a weak decrease in switch to IgG2a - after primary immunization (Fig 3.21A).

To address the possibility of having altered affinity of anti-NP antibodies in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice, we also performed experiments using high affinity NP-BSA (NP_4-BSA) to coat the ELISA plates and capture more specific antibodies generated upon NP-KLH immunization. Mice were re-challenged on day 14 after primary immunization to assess secondary immune response. However, no considerable differences were found in the affinity of IgG1 antibody after primary or secondary challenge (Fig 3.21B).

In a third approach, we measured Ab titers about 4 months after primary challenge to monitor differences in population of long-lived B cells in bone marrow or the memory B cell response. But again, we did not observe any defect in class switched Ig Abs. However, *Cd23-cre x Nfatc1*$^{flx/flx}$ mice showed higher IgM serum titer compared to the WT control groups (Fig. 3.21C).

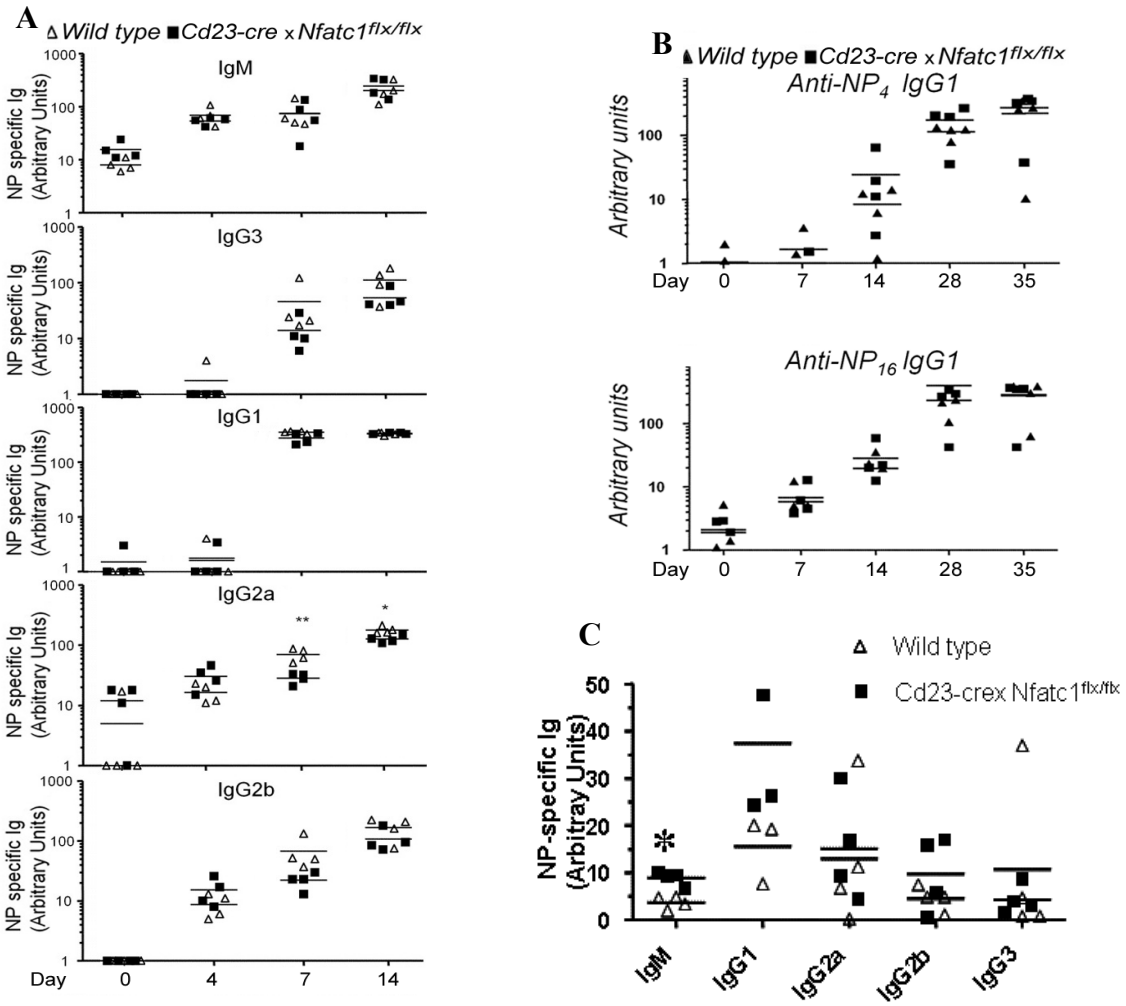


Figure 3.21 NFATc1-deficient mice can mould a sufficient immune response against TD antigen. ELISA data showing serum titers of IgM, IgG1, IgG2a and IgG2b (A) or higher affinity IgG1 (B) in NP-KLH-immunized WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice. ELISA data showing serum titer of IgM, IgG1, IgG2a and IgG2b in WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice 4 months after the primary challenge with NP-KLH (C). Each symbol represents one mouse.

3.8 B cell specific NFATc1$^{-/-}$ mice immunized with NP-Ficoll generate a reduced number of antigen-specific long-lived plasma cells and memory B cells

The defective production of class switched IgG3 antibodies in B cell specific NFATc1$^{-/-}$ mice, after NP-Ficoll immunization, led us to investigate the number of total plasma cells in these mice before and after antigen challenge. We found appearance of GC B cells ($CD19^{+}GL7^{+}PNA^{+}$) after immunization. However, no substantial difference between WT

and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice were observed in day 7 post-immunized mice (Fig. 3.22A). We further analyzed splenic B cells and BM B cells to quantify the percentage of short-lived and long-lived plasma cell (PC) population, respectively. The data suggested a markedly diminished population of splenic short-lived ($CD19^{+}CD138^{+}$) (Fig. 3.22B) and BM long-lived ($CD19^{-}CD138^{+}$) (Fig. 3.22C) plasma cells in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice compared to control group.

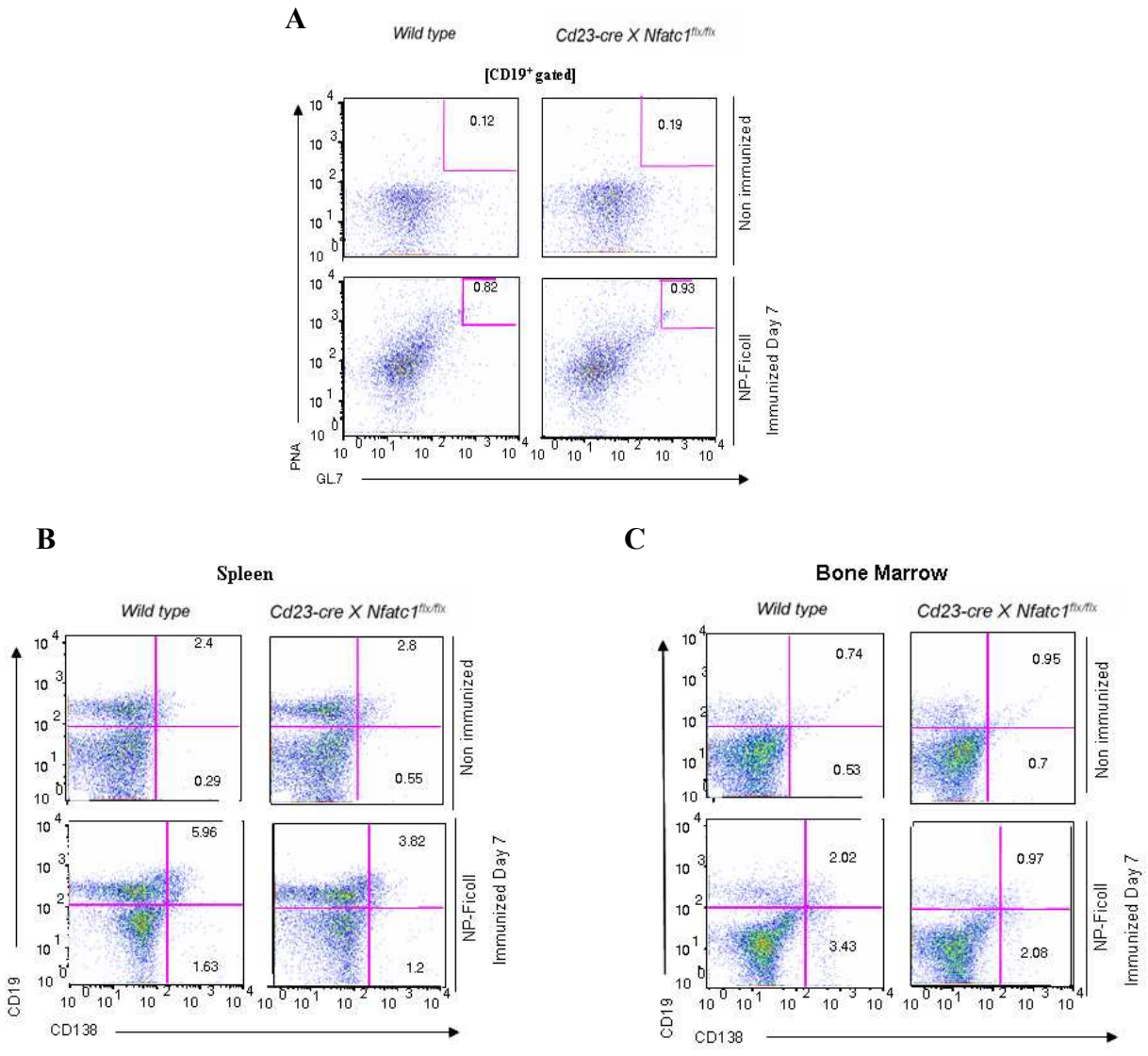


Figure 3.22 NFATc1 deficiency results in reduced plasma cell population after TI-II antigen challenge. Flowcytometric analysis showing percentage of germinal centre B cells (A), short-lived splenic plasma cells (B) and long-lived BM plasma cells (C) in WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice after NP-Ficoll immunization. Experiments were conducted on two mice in each group.

Marginal zone (MZ) B cells are known to be important for modulating first hand response against TI-II antigens (Guinamard, Okigaki et al. 2000). The suppressed immune response of *Cd23-cre x Nfatc1*$^{flx/flx}$ mice against NP-Ficoll directed us to consider substantial developmental defects in MZ B cell compartment. We observed no reduction in the frequency of MZB ($CD19^+CD23^-CD21/35^+$) cells in naïve *Cd23-cre x Nfatc1*$^{flx/flx}$ mice compared to WT. The percentage of follicular (FO) B cells ($CD19^+CD23^-CD21/35^+$) appeared to be quite normal as well (Fig. 3.23).

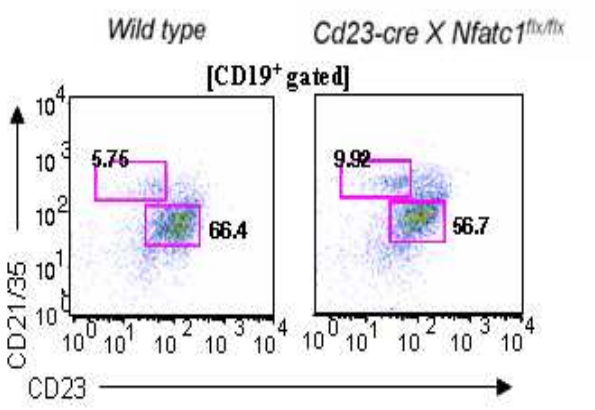


Figure 3.23 NFATc1 ablation does not cause a reduction in the percentage of marginal zone B cells. Data showing percentage of MZB cells in naïve *Cd23-cre x Nfatc1*$^{flx/flx}$ and WT mice. Figure represents one typical example out of more than three such experiments.

We then analyzed cellular responses of *Cd23-cre x Nfatc1*$^{flx/flx}$ and WT mice 21 days after TNP-Ficoll immunization. No significant difference in the frequency of total (Fig. 3.24A) or antigen-specific (TNP^+) (Fig. 3.24B) plasma cells in spleen was detected in contrast with our findings on day 7 (Fig.3.22B). On the other hand, although the numbers of splenic short-lived plasma cells were normal at day 21, the frequency of total (Fig. 3.24C) as well as TNP-specific (Fig. 3.24D) long-lived plasma cells in the BM was distinctly reduced. We further examined the memory B cell pool and detected a decrease in the number of antigen-specific memory B cells in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice (Fig. 3.24F) as compared to controls while the number of total memory B cells remained comparable in both types of animals (Fig. 3.24E). All these findings support our *in-vivo* ELISA data which also showed the impaired immune response of NFATc1-deficient mice towards TI-II antigen, such as NP or TNP-Ficoll.

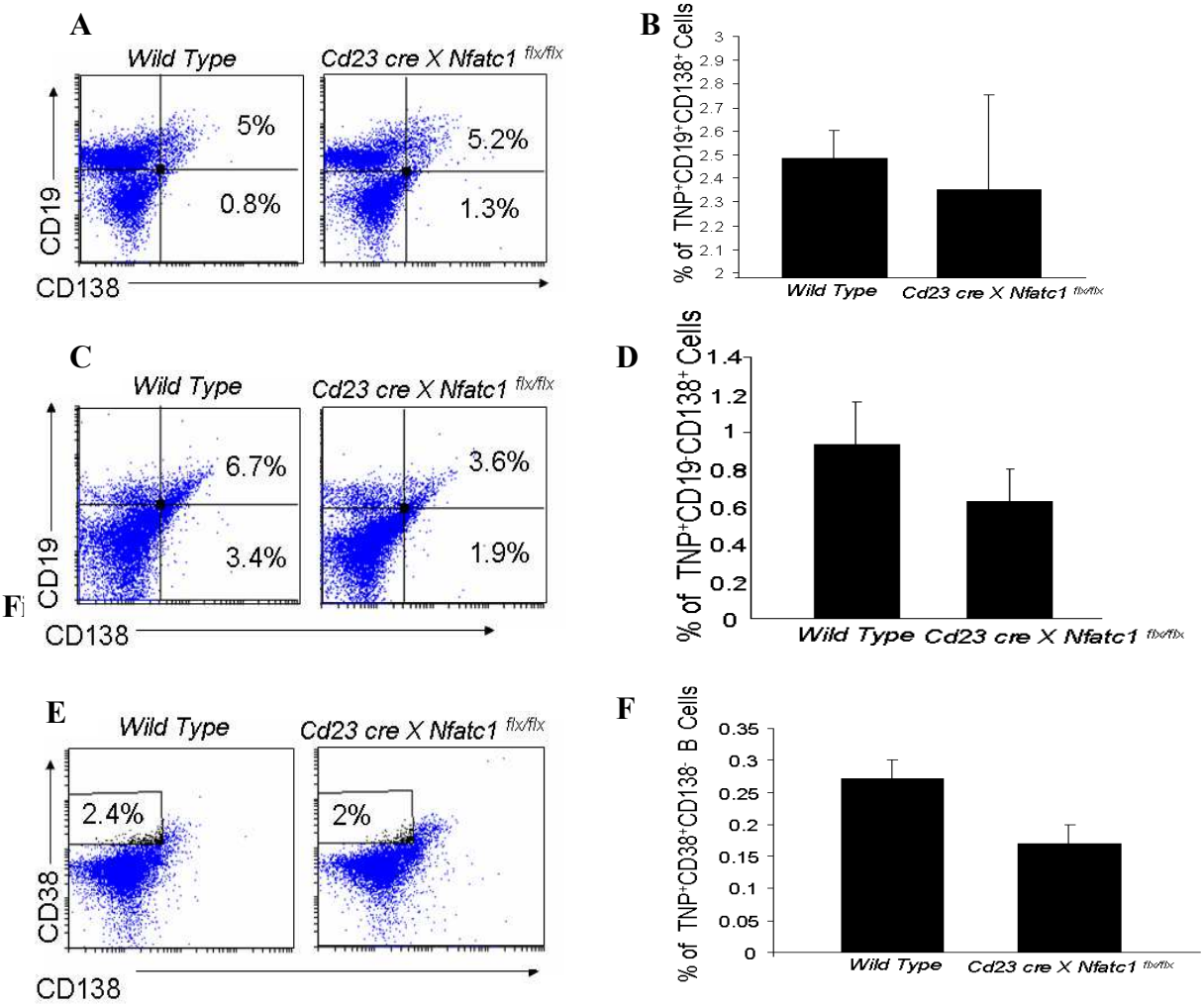


Figure 3.24 Development of antigen-specific long-lived plasma cells and memory B cells are impaired in NFATc1-deficient mice after immunization with TI-II antigen. Flowcytometric analysis showing frequency of total or antigen-specific short-lived and long lived plasma cells in spleen and BM (A,B,C,D) of WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice on day 21 after NP-Ficoll immunization, Percentage of total and antigen specific memory B cells in same mice are shown in E and F. Experiments were conducted on three mice from each group.

3.9 Mice bearing NFATc1$^{-/-}$ B cells fail to evoke immune responses to TI antigen *in-vitro*

In order to critically analyse our *in-vivo* findings that the immune response against TI antigen is diminished in *Cd23-cre x Nfatc1$^{flx/flx}$* mice, we performed a series of *in-vitro* assays. While no defect in IgG1 switch was observed among cultured splenic B cells from WT and *Cd23-cre x Nfatc1$^{flx/flx}$* mice as expected a significantly low frequency of IgG3^{+} plasmablasts were detected in cultures of splenic B cells from *Cd23-cre x Nfatc1$^{flx/flx}$* mice (Fig. 3.25A). These findings were also true when we performed an *in-vitro* ELISA using supernatants from 6 day cultured cells. Approximately 50% reduction in secreted IgG3 Ab level was observed in *Cd23-cre x Nfatc1$^{flx/flx}$* cells compared to the WT (Fig. 3.25B).

Isotype-specific "circle transcripts" (CTs) which are transcribed from excised looped-out circular DNA (CD) during Ig class switch recombination (CSR), are considered to be a hallmark for active CSR (Kinoshita, Harigai et al. 2001). In our study, we detected CTs of IgG1 region in both mice, whereas CTs of IgG3 region were found only in WT but not in *Cd23-cre x Nfatc1$^{flx/flx}$* B cells (Fig. 3.25C). This shows again the defect of CSR to IgG3 upon immunisation with TI type II antigen in NFATc1-deficient B cells

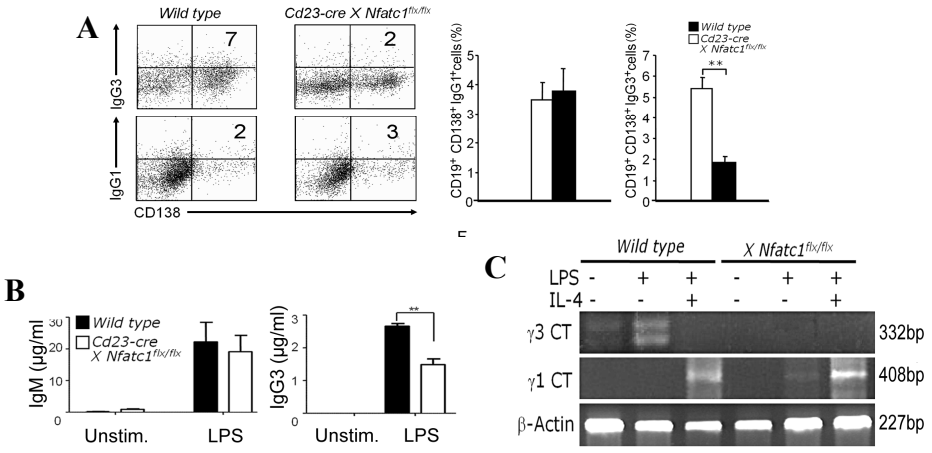


Figure 3.25 B cell specific NFATc1-deficient mice fail to mould an adequate immune response against TI type II antigen *in vitro*. Data showing the generation of IgG3^{+} plasmablasts (A) and IgG3 titer in cultured WT and *Cd23-cre x Nfatc1$^{flx/flx}$* cells (B), experiments were repeated three times. RT-PCR showing, circular IgG3 and IgG1 transcripts in WT and *Cd23-cre x Nfatc1$^{flx/flx}$* splenic B cells after *in vitro* culture for 48 hr in the presence of LPS (C), Figure represents one out of two such experiments.

The incapability of moulding sufficient cellular response against TI antigen could be due to increased cell death, as evidenced by apoptosis assay. Plasmablasts generated *in vitro* failed to proliferate well and showed relatively higher rate of apoptosis in NFATc1-deficient B cells (Fig. 3.26).

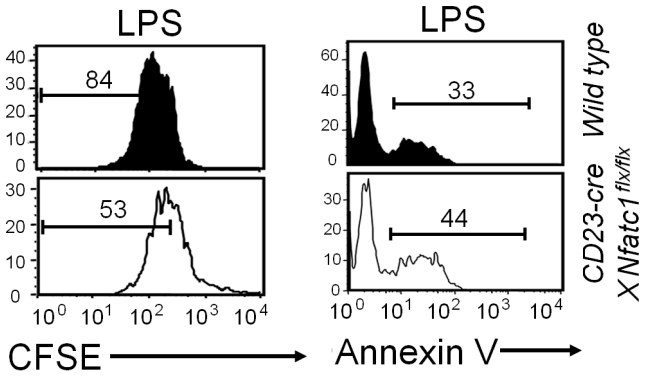


Figure 3.26 NFATc1 deficiency leads to increased rate of plasma cell death *in vitro*. Flowcytometric detection of proliferation (A) and AICD (B) of $CD138^{+}$ plasmablasts generated from splenocytes of WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice upon 3 day culture in presence of LPS. Experiments were repeated at least twice.

3.10 NFATc1-deficient B cells need T cell help for survival

We co-cultured WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ B cells stimulated with Ova protein with or without OT- II T cells bearing an Ova-specific T cell receptors. The addition of Ova protein to the culture - instead of Ova peptide - was to direct the B cells towards antigen processing and presentation, as we know T cells can only recognize peptide and not protein. A higher rate of apoptosis was evident when *Cd23-cre x Nfatc1*$^{flx/flx}$ B cells were cultured in the absence of any T cells compared to the culture condition where T cell help was eminent (Fig 3.27). This data strongly indicates that T cell help subsequent to antigen engagement is crucially important to prevent AICD in NFATc1-deficient B cells.

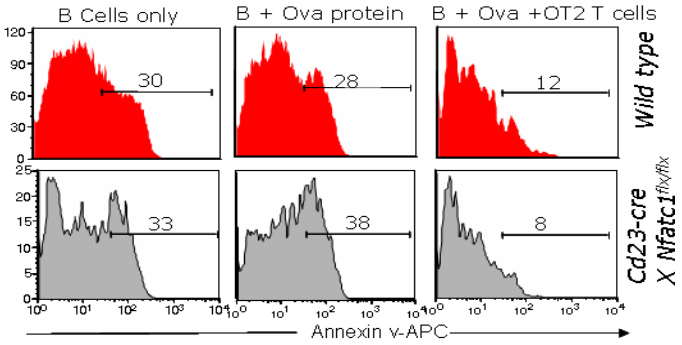


Figure 3.27 NFATc1-deficient B cells need T cell help for their survival. Splenic B cells from WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice treated or not with ova protein were co-cultured with OT-II T cells or left alone for 48 hr. Cells were stained with α-CD19 and Annexin V/PI for flowcytometric detection of AICD. Histogram showing percentages of Annexin V + cells among CD19 gated cells. This experiment was repeated twice.

3.11 NFATc1-deficiency results in higher amount of IL-10 secretion by splenic B cells

In order to mediate immune reactions, B cells play an important role in cytokine production. The anti-inflamatory cytokine IL-10 produced by B cells exerts a suppressive effect on immune system and is known to prevent autoimmune pathologies (Saraiva and O'Garra 2010). In our study we detected IL-10 secreting plasma cells after LPS treatment *in vitro* and found the percentage of such cells higher in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice compared to the WT B cells suggesting a possible role of NFATc1 in autoimmune diseases (Fig. 3.28).

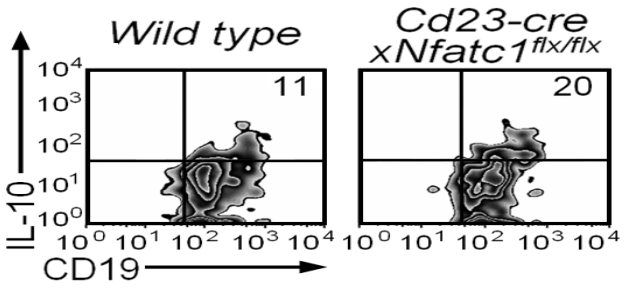


Figure 3.28. High frequency of IL-10-secreting plasma cells in NFATc1-deficient mice. Flowcytometric analysis showing the percentage of IL-10-producing CD138^{+}CD19^{+} plasmablasts after 3 d in culture with LPS (and T+I for the last 6 h) in preparations of splenocytes from WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ B cells. This Experiment was repeated twice.

3.12 Mcl-1 expression is down-regulated in plasma cells from B cell specific NFATc1-deficient mice

One of the most common antigens of choice for immunization of mice is SRBC. Upon immunization with SRBC, the following populations were sorted from WT and B cell specific NFATc1$^{-/-}$ mice after 10 days: GC (GL7^{+}), non-GC (GL7^{-}) B cells, plasma cells (CD138$^{+)}$ and non-plasma cells (CD138^{-}). These cells were sorted in a 'single cell sorting mode' for higher purity. Mcl-1 is an anti-apoptotic member of the Bcl-2 family and has been implicated to play a vital role in B cell survival. We observed slightly higher level of Mcl-1 RNA in sorted GC B cells from NFATc1-deficient mice, while the level was much reduced in NFATc1$^{-/-}$ plasma cells compared to WT mice.

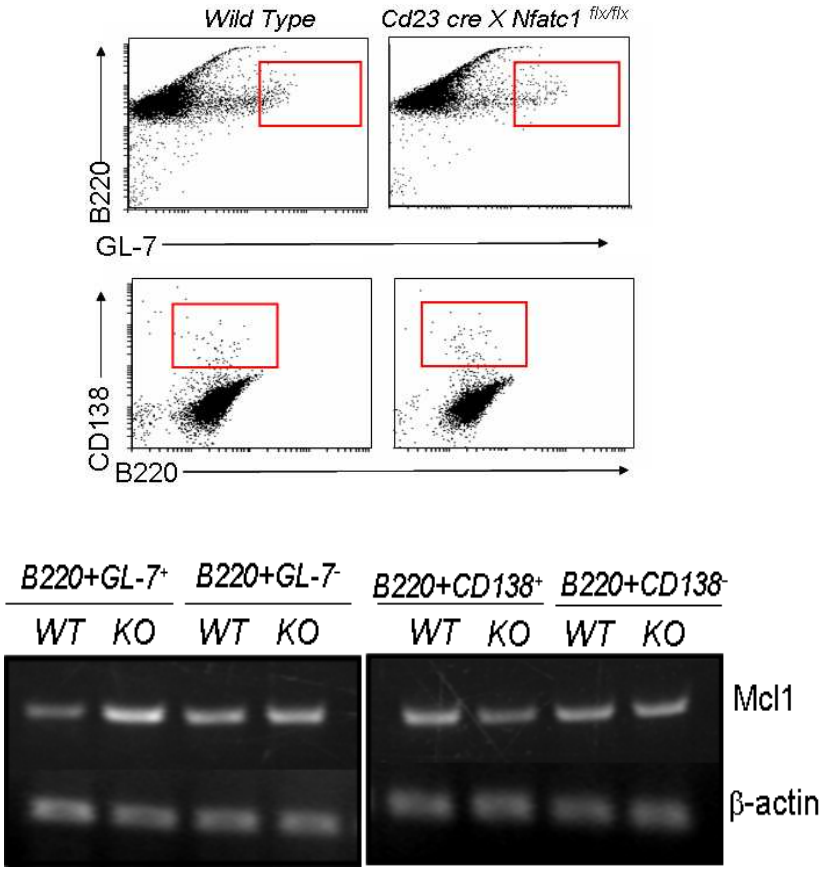


Figure 3.27 Decreased expression of Mcl-1 in NFATc1 deficient plasma cells. Dot plot showing FACS sorted GC or non-GC B cells and PC and non-PC population (A). RT- PCR showing expression of Mcl-1 RNA in wild type or NFATc1$^{-/-}$ GC/non-GC and PC/non-PC population. This experiment was repeated twice.

4. Discussion

Transcription factors play an important role in gene regulation leading to differential cellular responses against biological and environmental stimuli. The importance of NFATc1 transcription factor in early embryonic development (Zhou, Wu et al. 2005), heart and thymus development (Hodge, Ranger et al. 1996; de la Pompa, Timmerman et al. 1998; Ranger, Grusby et al. 1998) and osteoclast formation (Asagiri, Sato et al. 2005) have been known for years. Here we report the role of NFATc1 in B cell development, survival and cellular response against antigenic stimulation.

By using *mb1-cre* x *Nfatc1*$^{flx/flx}$ mice we show that NFATc1 plays no significant role in the development of peripheral B cells, with the exception of $CD5^+$ B1a cells in the peritoneum. During early B cell development the expression level of the *mb-1* gene is much higher than that of the *Cd19* gene (Hobeika, Thiemann et al. 2006). This makes *mb1-cre* mice a better choice to study effects of cre-mediated deletion of *NFATc1*-floxed genes in early B cell stages than in *Cd19-cre* mice which have widely been used for this purpose in former experiments (Rickert, Roes et al. 1997). However, *mb1* is expressed in the pro-B cell stage, and the *mb1-cre* x *Nfatc1*$^{flx/fl}$ mice model can not depict the role of NFATc1 in B cell development prior to pro-B cell stage.

Although peripheral B cell development was not hindered in *mb1-cre* x *Nfatc1*$^{flx/fl}$ mice, *in vitro* proliferation of B cells upon BCR cross-linking and induction of AICD was significantly altered. It is known that sustained level of "store-operated" Ca^{2+} entry is important for the function of lymphocytes (Hogan, Chen et al. 2003). NFATc1-deficient B cells showed a considerable defect in intracellular Ca^{2+} level and extracellular Ca^{2+} influx, which could well justify the impaired proliferation and increased AICD found in these cells. Interestingly, addition of Ca^{2+} to the culture medium could compensate the adverse effect of NFATc1 loss in these cells. In gene expression profiling assays, among the negatively regulated NFATc1 target genes we identified the *Cd22* gene and found an increased CD22 expression in NFATc1$^{-/-}$ B cells. On the other hand, inactivation of *Cd22* gene directed a strong NFATc1 expression in both unstimulated and LPS-stimulated B cells. The Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1)

positively regulates AICD in B cells (Mizuno, Tagawa et al. 2002). Upon BCR-engagement, SHP-1 binds to tyrosine-phosphorylated CD22 (Doody, Justement et al. 1995) leading to inhibition of BCR-induced Ca^{2+} signaling (Nitschke 2009). Therefore, we conclude that NFATc1 affects Ca^{2+} flux in peripheral B cells by suppressing *Cd22* transcription. However, it remains to be shown whether this is a direct or indirect effect of NFATc1 on the *Cd22* gene. Preliminary chromatin immunoprecipitation (ChIP) assays did not reveal a binding of NFATc1 to the 5' (promoter) region of *Cd22* gene (S. Bhattacharyya, personal comm.)

We also detected the three pro-apoptotic genes *Pdcd1* (Ishida, Agata et al. 1992), *Tnfsf14* (Zhai, Guo et al. 1998) and *Fasl* as the most prominent NFATc1 targets whose expression was significantly suppressed in NFATc1$^{-/-}$ B cells. It has been shown in ChIP experiments that NFATc1 can bind to the *Pdcd1*, *FasL* and *Tnfsf14* promoters regulating their expression (Bhattacharyya, Deb et al. 2011). It is suggested that *Pdcd1* (PD-1) activation is involved in the "classical type of programmed cell death" (Ishida, Agata et al. 1992), and down-regulation of CD5 expression is reported in PD-1$^{-/-}$ mice (Nishimura, Minato et al. 1998), similar to situation in *mb1-cre* x *Nfatc1*$^{flx/fl}$ mice. Transcription of the *Tnfsf14* (LIGHT) gene is CsA-sensitive and requires Ca^{2+} signaling (Castellano, Van Lint et al. 2002), and the NFATc1-mediated Ca^{2+} flux could be essential for expression of LIGHT. Studies have also suggested that "LIGHT-mediated cell killing" requires involvement of both LTβR and TR2/HVEM receptors, and cell surface expression of only one of these receptors fails to induce LIGHT-mediated cell death (Zhai, Guo et al. 1998). Moreover, one of the most prominent pathways regulating AICD is the FasL-Fas-mediated pathway. Similar to T cells, B cells are also known to express both Fas (Kavurma and Khachigian 2003) and, if activated, its ligand (FasL) on their surface. Other researchers have also suggested a possible role of Fas-FasL interaction in AICD of B cells (Hahne, Renno et al. 1996),

Our finding that most prominent NFATc1 target genes include pro-apoptotic genes is in stark contrast to the phenotype of NFATc1-deficient B cells which show an increased susceptibility to apoptosis. In order to resolve this apparent contradiction, we further analyzed the DNA microarray date using GSEA. A number of NFAT-regulated cell cycle

genes have been described for T cells (Baksh et al., 2002; Caetano et al., 2002; Carvalho et al., 2007). Our DNA microarray data, however, confirmed only the *Ccnb1*/Cyclin B1 gene – which is known to control the cell cycle - as a putative NFATc1 target in B cells. But the GSEA analysis suggests that although NFATc1 enhances the expression of genes associated with proliferation only to moderate levels, the combined effect of these genes on B cell fate is much more prominent. Our data also imply a dual role for NFATc1 in B cells. By introducing increasing amounts of a constitutively active (ca) version of NFATc1/αA into splenic B cells, we have demonstrated that NFATc1 activity is needed to protect B cells against AICD, whereas at very high concentrations, NFATc1 can also eliminate the majority of B cells by apoptosis.

Followed by antigen ligation, BCR-mediated signals initiate to transmit extracellular signals to a multitude of cellular responses. Several published studies have reported the importance of individual components of BCR signaling cascade in generating accurate cellular responses. Apart from developmental defects and impaired Ca^{2+} flux, one of the most prominent outcome of impaired BCR-signaling in NFATc1-deficient B cells seems to be the impaired immune response against TI-II antigens. NFATc1 deficiency in splenic B cells (from *Cd23-cre x Nfatc1*$^{flx/flx}$ mice) led to impaired Ig class switch to IgG3 upon NP-Ficoll (TI-II) immunization or LPS (TI-I) stimulation (*in vitro*), whereas no defect has been detected in IgG1 Ab production upon NP-KLH (TD) immunization or *in vitro* culture with LPS and IL-4 . A similar phenotype was detected for Btk, Pyk-2, PLC-γ2 and SLP-65/BLNK `knockout (ko) or Ig$\alpha^{Y204F/Y204F}$ `knock in (k.i.) mice. The major Ig isotype in response to TI-I or TI-II immunization is IgG3 (Mond, Lees et al. 1995), and both B-1a and MZ B cells are known to provide a rapid "first line of defense" against bacterial pathogens (Babbe, McMenamin et al. 2009) which belong to the group of TI antigens. It is also known that MZB cells can produce effector responses more quickly than FO B cells (Oliver, Martin et al. 1999). The most likely reason for the severe block in Ig class switch to IgG3 in Btk$^{-/-}$ and PLC-γ2$^{-/-}$ mice is the reduced number of B-1a cells (Hashimoto, Takeda et al. 2000). It is noteworthy that similar to our *Cd23-cre x Nfatc1*$^{flx/flx}$ mice, Pyk-2$^{-/-}$ mice also have a normal number of peritoneal B-1a cells, and the diminished TI-II response in these mice could be due to reduced MZB cell population

observed in these mice (Guinamard, Okigaki et al. 2000). However, we did not notice any reduction in the percentage of MZB cells in *Cd23-cre x Nfatc1*$^{flx/flx}$ mice, but function of MZB cells in these animals were significantly impaired (Bhattacharyya, Deb et al. 2011). It was suggested that the reduced SLP-65/BLNK phosphorylation in Igα$^{Y204F/Y204F}$ `knock in` mice leads to inhibition of T cell-independent B cell proliferation (Patterson, Kraus et al. 2006), whereas SLP-65/BLNK$^{-/-}$ mice exhibit an increased frequency of BM pre-B cells and lack of B-1b cells (Jumaa, Wollscheid et al. 1999), contrary to *Cd23-cre x Nfatc1*$^{flx/flx}$ mice. These observations put forward NFATc1 activity as a vital molecular mechanism which controls BCR signaling essential for the precise extracellar response of B cells.

The defect in class-switched antibody titer upon immunization with TI antigen directed us to explore the plasma cell population in mice bearing NFATc1-deficient B cells. Generally, it is known that binding of B cells to protein-based antigens - which are known to be T-dependent - directs B cells to find their way to the T-cell zone. T cells are main players in GC formation as they typically provide CD40L to engage CD40 on B cells. This interaction is crucial for the development of fully maturated B cells (Klein and Dalla-Favera 2008). Generation of GC-derived long-lived plasma cells and memory B cells are vital for the maintenance of serum concentration of specific antibodies and also for an immediate recall response upon secondary challenge by the same antigen. Since upon primary and secondary challenge with TD antigen we observed comparable amounts of serum Ig Abs between WT and mice bearing NFATc1$^{-/-}$ B cells, we suggest that the frequency of GC B cells and GC-derived plasma cells as well as memory B cells remained unaffected in those mice.

Surprisingly, surface expression of CD40L has also been reported in B cells (Wykes, Poudrier et al. 1998) which makes T cells dispensable for the initiation of GC reaction in certain situation (Wykes 2003) . Upon NP-Ficoll immunization, we have detected GL7^{+} and PNA^{+} B cells that resemble the GC B cell phenotype in WT and mice bearing NFATc1-deficient B cells. This observation finds support by studies that have reported GC formation upon NP-Ficoll immunization following spontaneous abortion at the time

of high-affinity B cell selection (de Vinuesa, Cook et al. 2000). It was also documented that memory B cells can be generated against TI-II antigenic stimulation (Obukhanych and Nussenzweig 2006). We observed a reduced number of GC-derived long-lived plasma cells and antigen-specific memory B cells in mice bearing NFATc1-deficient B cells upon immunization with NP-Ficoll. *In vitro* stimulation of B cells with LPS, a TI-I antigen, failed to produce IgG3 Ab and $IgG3^{+}$ plasmablasts. These plasmablasts showed an impaired proliferation and enhanced death, suggesting a possible explanation for the diminished cellular response against TI antigens in *Cd23-cre x Nfatc1$^{flx/flx}$* mice.

The main difference between TD and TI antigen in mounting an immune response is the involvement of T cells followed by BCR ligation. Apart from CD40-CD40L ligation, B cell-T cell interaction via CD86-CD28 is also vital for T-cell mediated synthesis of pro-survival cytokines. These cytokines are essential for B cell survival and/or proliferation (Goodnow, Vinuesa et al. 2010). It is notable that $NFATc1^{-/-}$ B cells show increased apoptosis following stimulation with α-IgM, which acts as a TI antigen (Wortis, Teutsch et al. 1995). On the contrary, stimulation with α-CD40, which would act as a CD40L provided by T cells, enhanced the survival of NFATc1-deficient B cell *in vitro*. To confirm our hypothesis that the involvement of T cells is vital for the survival of $NFATc1^{-/-}$ B cells, we co-cultured WT and $NFATc1^{-/-}$ B cells with or without OT-II T cells and supplemented the culture media with ova-protein. OT-II mice are ova-specific transgenic mice (Barnden, Allison et al. 1998), their T cells can specifically recognize ova-protein. Interestingly, we observed a higher rate of apoptosis in NFATc1-deficient B cells cultured in the absence of OT-II. Alternatively, $NFATc1^{-/-}$ B cells that received T cell help in culture showed a better survival. These findings imply that NFATc1-deficiency in splenic B cells does not hinder antigen internalization and antigen presentation by B cells to T cells, which is also reported for $Ig\alpha^{Y204F/Y204F}$ `knock in (k.i.) mice (Patterson, Kraus et al. 2006). Therefore, we conclude that T cell help subsequent to antigen engagement is vital to prevent B cells from AICD.

BCL-2 family members are well known for their pro or anti-apoptotic role in controlling the survival of immune cells. *Mcl-1*, an early-induction gene from the BCL-2 family, has

a role in cell viability (Yang, Buchan et al. 1996) and short-term survival (Zhou, Qian et al. 1997) of otherwise dying cells. One of the most striking features of NFATc1-deficient B cells is increased apoptosis followed by BCR-engagement. As Mcl-1 has been suggested to be important in the generation of antigen-specific IgG B cells (Vikstrom, Carotta et al. 2010), we checked Mcl-1 RNA expression in sorted GC and plasma cells from SRBC-immunized WT and *Cd23-cre x Nfatc1*$^{flx/flx}$ mice. In NFATc1$^{-/-}$ plasma cells, we detected a marked reduction in Mcl-1 RNA level compared to WT plasma cells. On the contrary, Mcl-1 expression was not reduced in NFATc1$^{-/-}$ GC B cells. To exert its anti-apoptotic effect in mature lymphocytes, Mcl-1 selectively inhibits pro-apoptotic members of Bcl-2 family, such as the activity of Bax, Bak and Bim proteins (van Delft and Huang 2006) ; (Opferman, Letai et al. 2003). In addition, degradation of Mcl-1 would account for functioning of these proteins in the induction of apoptosis.

One possible explanation behind the relatively unchanged level of Mcl-1 in NFATc1$^{-/-}$ GC B cells could be related to the fact that in GC, B and T cells are in close physical contact that provides essential survival signals to otherwise vulnerable NFATc1 deficient B cells. A higher Mcl 1 expression in NFATc1-deficient GC B cells could be an indication of inherently stressed conditions of these cells as Mcl1 expression is known to be upregulated in cells that are stressed (Germain, Nguyen et al. 2011). On the other hand, diminished expression of Mcl 1 in NFATc1-deficient plasma cells could indicate the fact that plasma cells generally do not interact with T cells once they exit the GC compartment. The regulation of Mcl-1 expression is known to be mediated by the Serum Response Factor (SRF) (Townsend, Zhou et al. 1999). One potential mechanism by which NFATs might regulate Mcl-1 activation could be via direct physical interactions between the Serum Response Factor (SRF) and NFAT proteins (Gonzalez Bosc, Layne et al. 2005).

The anti-inflammatory cytokine IL-10 which is produced by B cells and numerous other cells of the immune system has a suppressive role on immune cells and is known to prevent autoimmune pathologies (Saraiva and O'Garra 2010) , including the generation of an EAE (Fillatreau et al., 2002). Experimental autoimmune encephalomyelitis (EAE)

(Hur et al., 2007) is an established mouse model of human multiple sclerosis (MS) and has been extensively used by the researchers to study autoimmune pathologies of MS. It has recently been published that $CD19^{+}CD138^{+}$ plasmablasts, but not $CD138^{-}$ cells, secrete IL-10 after *Salmonella* infection which specifically inhibits the $CD4^{+}$ T cell response against the invading foreign antigen (Neves, Lampropoulou et al. 2010). In our study, we detected increased percentage of IL-10 producing splenic B cells and plasmablasts in cultured $NFATc1^{-/-}$ B cells. The functional relevance of increased IL-10 production by $NFATc1^{-/-}$ B cells has recently been reported by our group (Bhattacharyya, Deb et al. 2011). These studies have shown a much milder induction of EAE in mice deficient for NFATc1 in BM B cells than WT controls and a probable role for IL-10 in suppressing EAE by inhibiting T cell-mediated IFN-γ production. Apart from IL-10 involvement, a role of IFN-γ and Osteopontin has also been suggested by previous studies (Hur, Youssef et al. 2007). Interestingly, we have reported *Il10* (IL-10), *Il10R*, *Ifng* (IFN-γ) and *Spp1* (Osteopontin) as NFATc1 target genes based on DNA microarray analysis.

In summary, our study shows that BCR-mediated induction of NFATc1 controls (i) proliferation and AICD of splenic B cells and plasmablasts, (ii) intracellular Ca^{2+} release and extracellular Ca^{2+} influx by negatively regulating the *Cd22* gene expression and, probably, by controlling further signaling molecules of BCR signaling pathway, (iii) Ig class switch to IgG3, the development of long-lived plasma cells and antigen-specific memory B cells upon TI-II antigenic stimulation, and (iv) IL-10 production by splenic B cells.

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Abbreviations

Ab	Antibody
Ag	Antigen
AICD	Activation-induced cell death
BAC	Bacterial artificial chromosome
BCR	B cell receptor
BM	Bone marrow
Btk	Bruton's tyrosine kinase
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
Cn	Calcineurin
CsA	Cyclosporine A
CSR	Class switch recombination
CT	Circle transcript
EAE	experimental autoimmune encephalomyelitis
ELISA	Enzyme likend immunosorbent assay
FCS	Fetal calf serum
FO	Follicular B cell
GC	Germinal center
GFP	Green fluorescence protein
GSEA	Gene set enrichment analysis
IFN	Interferon
IgG	Immunoglobuling G
KI	Knock in
KO	Knockout
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MZB	Marginal zone B cell

NFAT	Nuclear factor of activated of T cells
NP	4-Hydroxy-3-nitrophenylacetyl
PC	Plasma cell
PCR	Polymerase chain reaction
PI	Propidium iodide
PLC- γ2	Phospholipase Cγ2
SRBC	Sheep red blood cell
T+I	TPA (12-O-tetradecanoylphorbol-13-acetate) + ionomycin
TCR	T cell receptor
TD	T cell dependent
TI	T cell independent
TNP	2,4,6-Trinitrophenyl
WT	Wild type

Curriculum Vitae

Personal Profile:

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Academic Profile:

- **MS** Biomedical Science 2007-2008
  University of Wolverhampton, Wolverhampton, West Midlands, UK

- **M.Sc**. Applied Genetics 2004-2006
  Indian Academy, Bangalore University, Bangalore, India

- **B.Sc**. Genetics 2001-2004
  Gardencity College, Bangalore University, Bangalore, India

Oral and Poster presentations:

- Oral presentation in the 13th Joint Meeting of the Signal Transduction Society (STS), Weimer, Germany-2009

- Poster Presentation in the 2nd European Congress of Immunology, Berlin, Germany-2009

List of Publications:

Sankar Bhattacharyya[1], **Jolly Deb[1]** , Amiya K. Patra[1] , Duong Anh Thuy Pham, Wen Chen, Thomas Benkert, Martin Vaeth, Friederike Berberich-Siebelt, Stefan Klein-Hessling, Edward D. Lamperti, Julia Jellusova, Astrid Schweizer, Lars Nitschke, Ellen Leich, Andreas Rosenwald, Swen Engelmann, Ursula Bommhardt, Andris Avots, Martin R. Müller, Eisaku Kondo & Edgar Serfling. (2011) NFATc1 affects mouse splenic B-cell function by controlling the calcineurin-NFAT signalling network. J Exp Med **208**(4): 823-839.

Jolly Deb[1], Harpreet Dibra, Sujith Rajan, Song Shan, Chris J. Perry and Iain D. Nicholl (2010) Antitumour activity of Aspirin analogues and Vanillin toward a Human Colorectal Cell Line [in communication]

Sign……. Date……..