UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA MOLECULAR

Tesis Doctoral

The proto-oncogene *c-myc* in terminal B lymphocyte differentiation: its role in plasma cell and memory B cell generation

Maitane Ortiz Virumbrales

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1. Introduction

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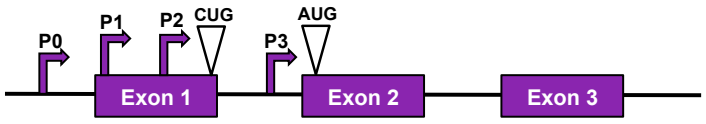
1. Introduction

1.1 The c-Myc transcription factor

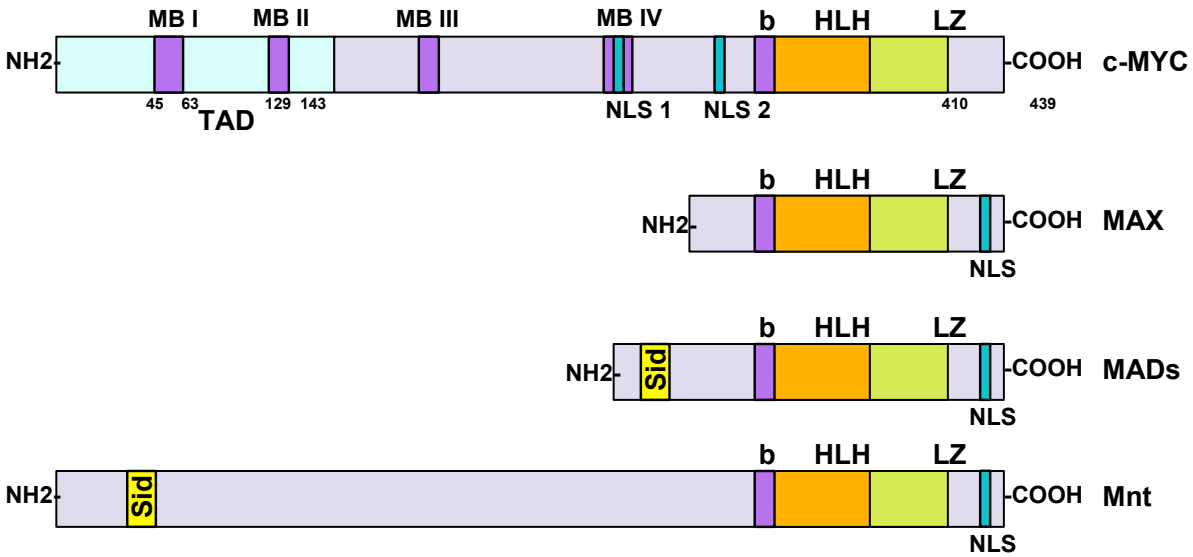
c-myc was identified as the cellular homolog of the oncogene *v-myc* of the avian myelocytomatosis virus (Vennstrom *et al.*, 1982). The avian retrovirus MC29 induces a wide spectrum of neoplastic diseases in chickens, including renal and hepatic carcinomas, sarcomas, and leukemias (Graf *et al.*, 1978). It was subsequently found that the *c-myc* proto-oncogene is activated in various animal and human tumors (Cole *et al.*, 1986). Only in the USA, *c-myc* deregulation is involved in 70,000 cases of human cancer each year that result in death (Dang, 1999; Nesbit *et al.*, 1999). For this reason, efforts to study this proto-oncogene and understanding its function have been

Figure I1

a. *c-myc* gene



b. Structure of the MYC/MAX/MAD transfription factors



significant in recent years, with the hope that therapeutic insights will emerge.

c-Myc belongs to the myc family of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors, together with N-Myc, L-Myc, B-Myc and s-Myc; however, only cMyc, L-Myc, and N-Myc have neoplastic potential (Henriksson *et al.*, 1993). *N-myc* and *lmyc* were also identified by their homology with *v-myc* in amplified sequences of neuroblastoma and lung small cell tumors, respectively (Kohl *et al.*, 1986; Nau *et al.*, 1986).

1.2 The c-Myc protein

The *c-myc* gene, located on human chromosome 8, is comprised by 3 exons and has four different promoters. The *c-myc* gene encodes a 64 kDa protein if starting at the canonical AUG start codon in exon 2, or a 67 kDa polypeptide if initiated 15 codons upstream of this AUG, in exon 1 (Figure I1a). There are no clear functional differences between them (Hann *et al.*, 1984; Spotts *et al.*, 1990).

The c-Myc protein has several functional domains. The C-terminal domain (CTD) harbors the basic helix-loop-helix leucine zipper (bHLH-LZ) motif for dimerization with its partner, Max, and subsequent DNA binding of Myc-Max heterodimers. The basic region is necessary to bind to DNA consensus sequences, called E-boxes (canonical : 5'-CACGTG-3', and noncanonical: 5'-CANNTG-3'), whereas the HLH-LZ domain is required for protein binding to Max. The N-terminal domain (NTD) harbors two highly conserved 'Myc boxes' I and II (MBI and MBII), essential for transactivation of c-Myc target genes (Dang, 1999; Pelengaris *et al.*, 2002). MBII is critical for the majority of c-Myc functions (Oster *et al.*, 2002). In the central region, we find the conserved Myc boxes III and IV, and nuclear localization signals 1 and 2 (NLS1 and NLS2) (Figure I1b). In addition, the central region of the Myc protein has a highly acidic stretch containing multiple CKII phosphorylation sites important for its activity (Dang *et al.*, 1988; Henriksson *et al.*, 1993).

1.3 Controlling c-Myc

c-Myc nuclear protein is expressed ubiquitously during embryogenesis and in those adult tissue compartments with high proliferative capacity. Resting or terminally differentiated cells have nearly undetectable c-Myc mRNA and protein levels, but expression is activated rapidly following exposure to a variety of mitogens, and promotes G0/G1 transition (Campisi *et al.*, 1984; Kelly *et al.*, 1983; Eilers, 1991). c-Myc activity is normally controlled precisely by external signals including growth factors, mitogens and β-catenin, which promote proliferation, and factors such as TGF-β, which inhibit it. c-Myc is also a target gene of the c-Myb transcription factor. In dividing cells,

c-Myc levels are maintained at a relatively constant intermediate level throughout the cell cycle (Pelengaris *et al.*, 2002).

Once c-Myc is activated, its half life is very short (20-30 minutes) (Hann *et al.*, 1984), and its degradation is mediated by the ubiquitin/26S proteasome pathway (Gross-Mesilaty *et al.*, 1998). Phosphorylation of c-Myc is also involved in the stability of the protein. Although there are at least ten major phosphorylation sites in the protein (Henriksson *et al.*, 1993), two are mainly responsible for c-Myc stabilization and degradation: Ser62 (S62) and Thr58 (T58). Phosphorylation of S62 stabilizes c-Myc protein, whereas phosporylation on T58 leads to degradation (Hann, 2006). Point mutation of these residues results in c-Myc transformation activity (Nasi *et al.*, 2001).

1.4 Myc/Max/Mad network

The search for a Myc binding partner protein resulted in the discovery of an HLH-LZ human Max protein (Blackwood *et al.*, 1991). Myc interacts specifically *in vivo* with a nuclear protein called Max, to form a complex with sequence-specific DNA-binding activity (Blackwood *et al.*, 1992).

Max also has a bHLZ-LZ region, and disruption of the HLH-LZ structure prevents its association with Myc. Several forms of Max protein have been described, presumably derived from alternative splicing. The 22 kDa form (p22Max) differs from the 21 kDa form (p21Max) by the insertion of nine amino acids upstream of the basic region. Both are the major Max protein forms detected in a wide variety of cell types and species, and both heterodimerize with Myc (Blackwood *et al.*, 1992).

In vitro-translated Myc-Max complexes appear to bind to E-boxes with a higher affinity than does either homodimer alone. The E-box sequence is sensitive to CpG methylation (Prendergast *et al.*, 1991). In contrast to tightly regulated Myc expression, Max levels are unaffected by changes in the cell cycle. Both *max* mRNA and the encoded protein are readily detected in quiescent cells at levels comparable to those in a cycling cell. Moreover, Max expression is not influenced by Myc levels, and it appears to be highly stable, with a half life of over 12 hours (Blackwood *et al.*, 1992).

In vitro binding studies show that Max can form homodimers in addition to its ability to heterodimerize with Myc family proteins. In quiescent cells, Max-Max dimers are predominant; in mitogen-activated cells, the presence of Myc causes a shift to Myc-Max heterodimers, because Max has higher affinity for Myc than for itself (Spencer *et al.*, 1991). Max/Max homodimers do not activate transcription of target genes, as they lack an activation domain (Kato *et al.*, 1992); the activation activity of Myc-Max dimers is therefore due only to the c-Myc transactivation domain.

Other proteins in this network are the

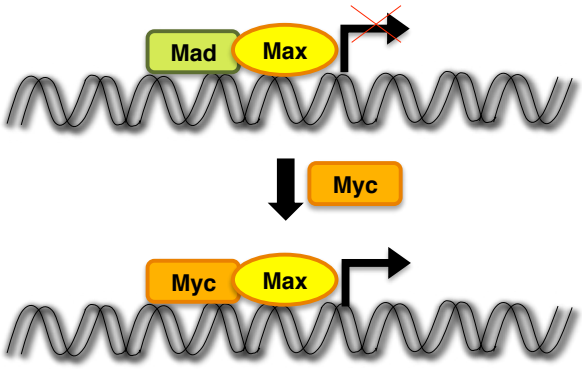
Mad proteins. Mad was discovered through its ability to bind Max (Hurlin *et al.*, 1995). The Mad protein family, including Mxi-1, Mad1, Mad3, Mad4, bear the Sind3-interacting domain motif, which recruits Sin3, N-Cor and other proteins with deacetylase activity, which is the main mode of transcriptional silencing by Mad. The expression of different Mad/Mxi-1 protein family members coincides with downregulation of c-Myc expression, and cells begin to exit the cell cycle. Mad proteins are restricted to post-mitotic differentiated cells (Chin *et al.*, 1995). Mad/Max dimers act as transcriptional repressors and bind to the same consensus sequences as Myc/Max, the E-boxes (Amati *et al.*, 2001); there is thus a balance between Myc/Max and Mad/Max heterodimers that determines the switch between cell states and transcriptional activation or repression of their common target genes (Amati *et al.*, 2001) (Figure I2a).

1.5 Mechanisms of c-Myc transcriptional activation

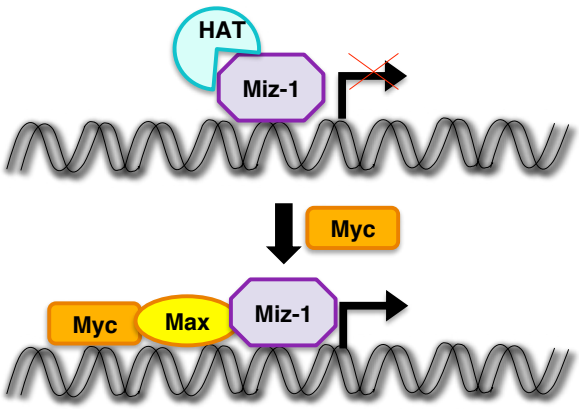
Myc/Max heterodimers bind the E-box sequences and c-Myc interacts with the TATAbinding protein (TBP) and the transcriptional machinery to regulate expression of their target genes. The first level of DNA packaging in eukaryotes is its

Figure I2

a. MYC/MAX/MAD activation of target genes expression



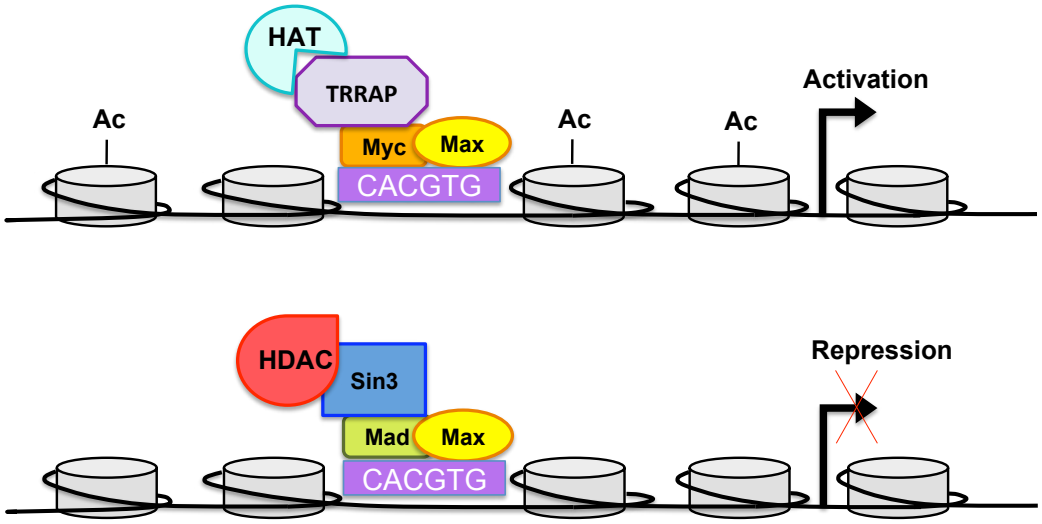
b. Repression of target genes expression



wrapping around the core histones H2A, H2B, H3, and H4 to form the nucleosome (Luger *et al.*, 1997). The promoter regions would thus be inaccessible to transcription factors that

Figure I3

Chromatin remodelling mechanisms



modulate gene expression. Two different chromatin remodeling processes have been reported for c-Myc, histone acetylation and ATP-dependent remodeling of nucleosomes.

Acetylation levels are controlled by a variety of histone acetyltransferases (HAT) and deacetylases (HDAC), which are recruited to promoters by sequence-specific activators and repressors, and mediate their transcriptional activities (Kingston *et al.*, 1999). c-Myc recruits, and binds by its N-terminal transactivation domain, a coactivator multisubunit complex containing transformation/transcription domain-associated protein (TRAPP) and HAT such as GNC5 (McMahon *et al.*, 1998, 2000), important for modulating the acetylation status of core histones (Figure I3). Activation of a MycER chimera (Littlewood *et al.*, 1995) in quiescent cells rapidly induced H4 acetylation. In the second process, Myc/Max binds to a component of the SWI/SNF complex with ATPase activity involved in nucleosome remodeling (Park *et al.*, 2001). These two processes allow an open chromatin conformation and accessibility to RNA polymerase II (polII) to activate c-Myc target genes, translating mitogenic stimuli into chromatin modification.

1.6 Transcriptonal repression by c-Myc

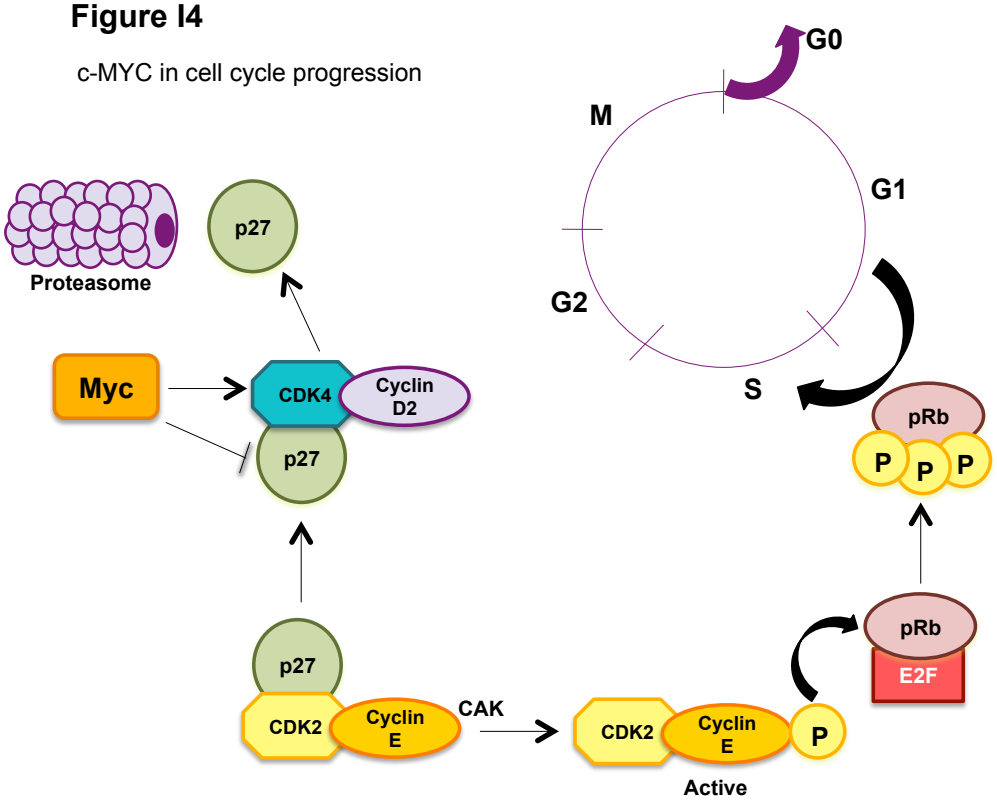
Transcriptional repression mechanism by c-Myc is not as well understood as c-Myc dependent activation. Most c-Myc-repressed genes show a TATA-less promoter with an

Initiator element within (Inr), but c-Myc-dependent repression was also reported in promoters lacking Inr (Gartel *et al.*, 2003). The Inr element is defined as a weak consensus YYCAYYYYY, where Y is a pyrimidine base.

c-Myc represses transcription of its target genes by two distinct mechanisms. One is dependent on a transcriptional initiator (Inr) element in TATA-less promoters, and requires interaction with Ying Yang (YY1) and TFII-I activator proteins (Shrivastava *et al.*, 1993; Roy *et al.*, 1993); the other is via activity of Miz-1, a zinc-finger protein identified as a c-Myc partner (Staller *et al.*, 2001) (Figure I2b). TFII-I binds and stimulates basal transcription from the Inr element (Roy *et al.*, 1991) by sequential addition of other general factors, providing a mechanism for initiation of transcription from TATA-less promoters. C-Myc can bind TFII-I and form a complex associated with DNA containing an Inr sequence, which resists further binding of basal transcriptional machinery, preventing transcriptional activation from the Inr (Roy *et al.*, 1993). The Inr-independent mechanism involves inhibition of Smad-mediated activation of the Sp1 transcription factor by c-Myc (Feng *et al.*, 2002).

Figure I4

c-MYC in cell cycle progression



1.7 c-Myc biological functions

1.7.1 c-Myc and the cell cycle

c-Myc can promote cell cycle progression. A rat fibroblast cell line in which both *c-myc* alleles were deleted shows very reduced proliferation rates and

deficiency in global mRNA and protein synthesis (Mateyak *et al*., 1997), whereas ectopic induction of Myc activity is sufficient to drive quiescent growth factor-deprived fibroblasts into the cell cycle (Eilers *et al*., 1991). This demonstrates that Myc regulates genes that mediate the mitogenic response.

Cell cycle progression is promoted by cyclin-dependent kinases (CDK) and their cyclin regulatory subunits, and is repressed by cell cycle inhibitors such as p27 or p21. c-Myc promotes G1-S transition through gene activation and repression; it activates cyclin A and D1, and also induces cyclin E-CDK2 activity early in the G1 phase (Steiner *et al*., 1995). CCND2 and CDK4 are also direct c-Myc targets, and their expression leads to sequestration of the CDK inhibitor KIP1 (p27) in cyclin D2-CDK4 complexes (Bouchard *et al*., 1999). This prevents p27 binding to cyclin E-CDK2 complexes. Moreover, KIP1 is degraded by two c Myc target genes, CUL-1 and CKS (Hermeking *et al*., 2000). Cyclin E-CDK2 complexes are now accessible to phosphorylation by cyclin activating kinase (CAK), resulting in increased activity of CDK2 and CDK4, which in turn hyperphosphorylate Retinoblastoma protein (Rb) to allow its release from E2F (Figure I4). *cdc25* is also a direct target of c-Myc, which is responsible for CDK2 and CDK4 activation (Galaktionov *et al*., 1996). c-Myc can repress other CDK inhibitors such as p15 and p21, which mediate cell cycle arrest via Miz-1 and SP1 (Dang, 1999; Grandori *et al*., 2000).

1.7.2 The role of c-Myc in cell growth

c-Myc involvement in metabolism is further suggested by the roles of some of its target genes that encode translational regulatory factors, such as eIF-2α and eIF-4E, as well as ECA39, which is implicated in amino acid transport, and RNA helicase MrDb, IRP-2 and Hferritin (Jones *et al*., 1996; Rosenwald *et al*., 1993; Grandori *et al*., 2000).

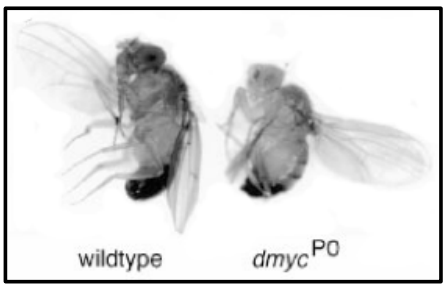
RNA polymerase III (polIII) is involved in the generation of transfer RNA and 5S ribosomal RNA is required for protein synthesis in growing cells. PolIII is activated via cMyc binding to TFIIIB (Gomez-Roman *et al*., 2003).

If c-Myc activates metabolism and protein synthesis, it is plausible that it is also linked to cell growth, and studies of c-Myc function in cell size and body size demonstrate this implication. In Drosophila melanogaster, downregulation of *dmyc* (*c-myc* ortholog) gives rise to smaller flies as a result of a decrease in cell size and number (Figure I5); *dmyc* overexpression yielded larger cells with a normal division rate (Johnston *et al*., 1999). c-Myc is therefore an integrator of cell proliferation and metabolism. Another example is the Eμ-myc transgenic mouse model, in which *c-myc* overexpression in B lymphocytes causes an increase in cell size in the absence of cell division (Iritani *et al*., 1999). B lymphocytes proliferate *in vitro* after activation with anti-CD40 plus interleukin-4 (IL-4), and this

proliferation is accompanied by a cell size increase. c-Myc is indispensable for B cell growth after antigen stimulation (de Alborán *et al*., 2001). In addition, *c-myc* inactivation in liver leads to a decrease in hepatocyte size (Baena *et al*., 2005) and regulates cell ploidy (Conner *et al*., 2003). c-Myc also regulates body size; hypomorphic mice expressing reduced c-Myc levels show smaller body size due to a decrease in cell number in many organs, without affecting cell size (Trumpp *et al*., 2001).

Figure I5

c-MYC function in body size



Johnston et al, 1999

1.7.3 Role in induced apoptosis

c-Myc-induced apoptosis was first recognized in studies of the 32D.3 myeloid progenitor cell line. Enforced c-Myc expression had no effect on 32D.3 cells in normal growth conditions; in the absence of growth factors (IL-3), however, c-Myc overexpression accelerated cell death (Askew *et al*., 1991).

Growth arrest is accompanied by rapid downregulation of c-Myc expression, leading to study of the ability of cells constitutively expressing c-Myc to arrest when deprived of mitogens. In a fibroblast cell line (Rat-1), which expresses human c-Myc under a constitutive promoter, serum deprivation conditions lead to complete growth arrest and cells enter a G0/G1-like state. The authors also observed that cells with higher c-Myc levels are more prone to enter apoptosis. The first cell deaths were observed within 30 min of serum withdrawal and continued at a more or less constant rate (Evan *et al*., 1992). This demonstrates that c-Myc has a role in cell death induced by the absence of necessary growth factors.

Mutations in different *c-myc* regions demonstrated complete concordance between those regions required for apoptosis and those necessary for cotransformation and proliferation. These regions include the bHLH-LZ at the C terminus and part of the N-terminal region (Evan *et al*., 1992).

c-Myc expression sensitizes cells to a wide range of pro-apoptotic stimuli such as hypoxia, DNA damage and depleted

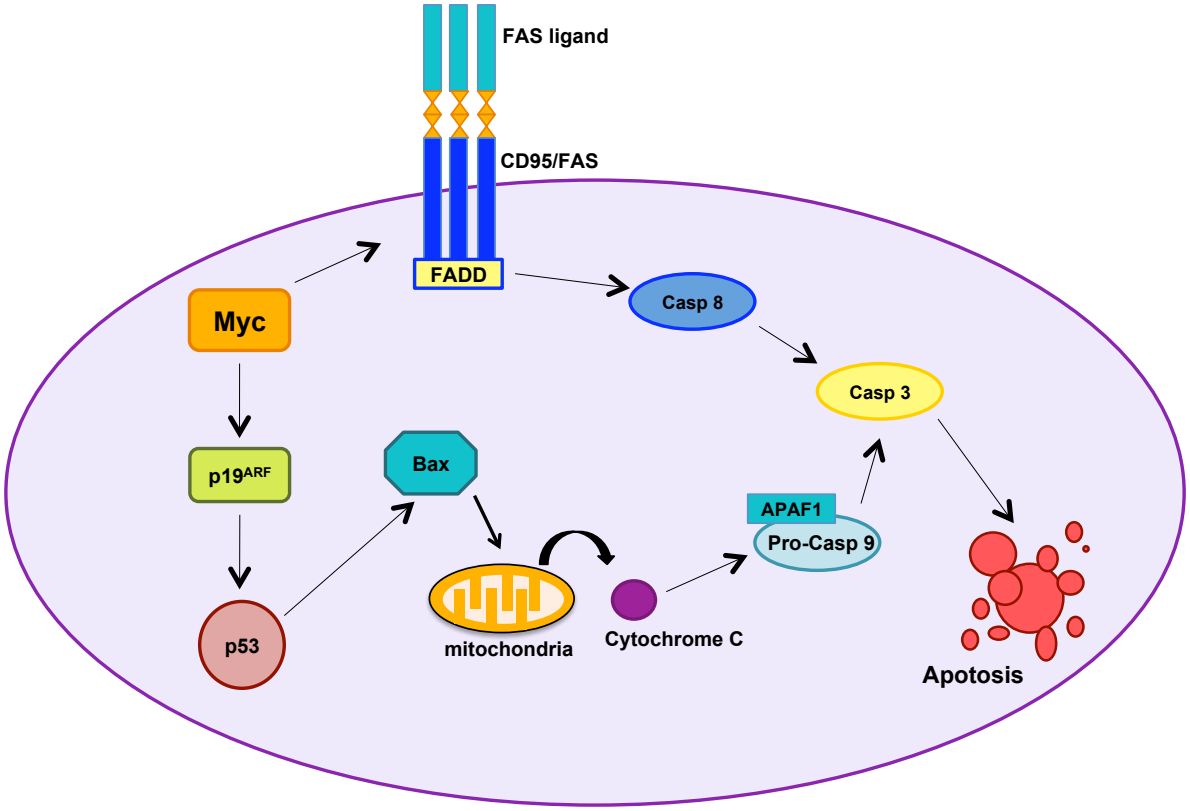
survival factors, as well as enhancing sensitivity to signaling through CD95 (Fas receptor) (Hueber *et al.*, 1997), TNF (Klefstrom *et al.*, 1994) and TRAIL (Lutz *et al.*, 1998). Various pathways are implicated in c-Myc-induced apoptosis; cMyc induces cytochrome c release from the mitochondria into cytosol by direct activation of the pro-apoptotic molecule Bax (Juin *et al.*, 2002; Soucie *et al.*, 2001). Bax creates pores in the mitochondrial membrane, resulting in mitochondria outer membrane permeabilization (MOMP). Cytocrome c associates with a protein termed apoptotic protease-activating factor 1 (APAF-1) to create the apoptosome, which acts as a scaffold for activating procaspase-9. Caspase 9 is activated in the presence of ATP, leading to activation of the downstream caspase cascade, including caspase-3, which leads to protein degradation, cell disassembly and death (Figure I6).

Another apoptotic pathway is mediated by CD95/Fas signaling, which leads to recruitment of the Fas-associated death domain (FADD). FADD binds procaspase-8, which auto-activates and triggers caspase cascade activation. TNF receptor ligation results in activation of RIP and signaling via FADD. c-Myc can also activate apoptosis through indirect activation of p53 via ARF (Zindy *et al.*, 1998).

Anti-apoptotic signals such as Bcl-2 and Bcl-xL reside in the outer mitochondrial membrane and block

Figure I6

Pathways of c-MYC induced apoptosis



MOMP through sequestration of BAX or binding to APAF-1 to prevent caspase 9 activation (Pelengaris *et al.*, 2003).

The complex interrelationship between c-Myc and apoptosis is determined by several factors, including cell type, tissue location and the presence or absence of additional mutations in other pro- and anti-apoptotic genes (Pelengaris *et al.*, 2003).

1.8 c-Myc in B cell differentiation

c-Myc functions at a critical decision point of cell growth to promote proliferation and block terminal differentiation. c-Myc is present in dividing cells, and its donwregulation is reported to accelerate terminal cell differentiation (Chang *et al.*, 2000; Henriksson *et al.*, 1996), whereas ectopic c-Myc expression has the opposite effect in several cell types, both *in vivo* and *in vitro* (Lachman *et al.*, 1986; Iritani *et al.*, 1999).

C/EBPα, which is necessary for myoblast commitment to the granulocytic lineage, forms a complex with E2F/pRB that represses *c-myc* expression (Johansen *et al.*, 2001). Another example of c-Myc downregulation in terminally differentiated cells is found in plasma cells (PC). c-Myc transcription in PC is repressed by the plasmacytoma repressor factor Blimp-1 (B lymphocyte induced maturation protein). Blimp-1 overexpression in a pro-monocytic cell line results in repression of *c-myc*; this drives these cells to macrophage and B cell generation (Lin *et al.*, 1997; Chang *et al.*, 2000). Repression of c-Myc is thus a component of the Blimp-1 program of terminal B cell differentiation. These data indicate that *c-myc* is involved in the programs for differentiation to specific cell fates.

As the focus of this thesis is the B cell differentiation process, we will review some work in this area. c-Myc is expressed throughout the B cell differentiation process at various levels. c-Myc expression begins at the pro-B cell stage after IL-7 stimulation (Morrow *et al.*, 1992). It is also expressed, with N-myc, in large pre-B cells during maturation and expansion to the small pre-B stage (Zimmerman *et al.*, 1990). These findings prompted researchers to study c-Myc function in B cell development using murine models.

Gain-of-function studies in a mouse lymphomagenesis model (*E*μ-*c-myc* mouse) have been a useful tool to reveal *c-myc* function in B cells. In this mouse, the *c-myc* proto-oncogene is expressed under the control of the immunoglobulin heavy chain enhancer (Eμ); these mice show a blockade in pro/pre-B (IgM^-) to immature B cell (IgM^+) transition in bone marrow, causing a reduction in mature B cells in spleen (Iritani *et al.*, 1999).

Loss-of-function experiments were also used to study the role of c-Myc in B cell differentiation. Germline *c-myc* inactivation causes embryonic lethality before gestation day E10.5, due to abnormalities in growth and in cardiac and neural development, among other defects (Davis *et al.*, 1993).

N-myc germline deficiency is also embryonic lethal (Charron *et al.*, 1992). c-Myc-deficient cells are nonetheless able to proliferate and embryos are viable until E10.5, probably due to rescue of c-Myc functions by other myc family members such as N-Myc. Insertion of the *N-myc* coding sequence in the *c-myc* locus, replacing the *c-myc* gene, rescued embryonic viability, so N-Myc can replace c-Myc function *in vivo* (Malynn *et al.*, 2000). To study the effect of c-Myc deficiency in adult tissues, the use of conditional knockout (KO) mice became necessary. The *c-myc*^{flox} model in which Cre/LoxP technology allows *c-myc* deletion in adult mice was generated in 2001 (de Alborán *et al.*, 2001). In these mice, B lymphocyte proliferation in response to anti-CD40 + IL-4 is severely impaired; B cells accumulate in G0/G1 phase, and show similar size after activation to that of non-activated cells. These *c-myc*-deficient B cells show upregulation of p27 (de Alborán *et al.*, 2001) and are resistant to CD95-induced cell death because they express lower surface levels of CD95 and CD95L after mitogenic stimulus (de Alborán *et al.*, 2003). The important role of c-Myc in B cell differentiation was also observed in the double *N-myc/c-myc* KO, in which the B cell lineage is blocked at the pro- to pre-B transition (Habib *et al.*, 2007). Our laboratory recently reported that c-Myc regulates early B cell differentiation through the transcription factor EBF1, by direct binding of the c-Myc protein to the promoter region of *ebf1* gene (Vallespinós *et al.* 2011, *in press*).

All these findings connect *c-myc* with early B cell differentiation. c-Myc expression nonetheless remains constant until the mature B cell stage, and is not downregulated until mature B cells fully differentiate to PC. The aim of this thesis is to study the function of c-Myc in terminal B cell differentiation, as it might have an role beyond cell proliferation in early stages of this process, before repression by Blimp-1. There are no *in vivo* studies that connect c-Myc with an important function in plasmacytic differentiation other than proliferation. There is only one previous study using a mature B cell lymphoma line (BCL-1) *in vitro*, which reported that repression of *c-myc* is necessary but not sufficient for PC differentiation, demonstrating the existence of additional Blimp-1 target genes essential for this process. Constitutive expression of c-Myc blocked plasma cell differentiation. Furthermore, ectopic expression of cyclin E impaired terminal differentiation of BCL-1 cells in response to cytokine, and mimicked the effects of c-Myc in proliferation, indicating that abrogation of proliferation is indispensable for BCL-1 differentiation. Inhibition of c-Myc by a dominant negative form was nonetheless insufficient to drive BCL-1 differentiation (Lin *et al.*, 2000).

Nevertheless this work leaves an open door to the requirement for c-Myc expression at early stages of terminal B cell development, necessary for activating an indispensable pathway for full PC or memory B cell generation. c-Myc might

act as a key transcription factor, not only as a protein to be eliminated at the termination of B lymphocyte differentiation.

1.8.1 Early B cell differentiation

Early B cell differentiation is a tightly regulated process that occurs in fetal liver or adult bone marrow (BM), from common lymphoid progenitors (CLP) to mature B cells. It is linked to the V(D)J rearrangement state of the immunoglubulin heavy (IgH) and light (IgL) chain genes. This process is accompanied by expression of several surface molecules, which allow identification of different B cell subsets through maturation from pre-pro-B to mature recirculating B cells (Hardy *et al*., 2001). Early development depends on several transcription factors, including early B cell factor (EBF), E2A and paired box protein (PAX5) (Busslinger *et al*., 2004). Successful rearrangement of the IgH gene segments in pro-B cells leads to the pre-B cell stage, in which pre-B cell receptor (pre-BCR) expression starts. This is a checkpoint for expansion, and allows rearrangement of IgL gene segments, which in turn triggers surface IgM expression. The cell becomes a naïve mature B cell which, after surviving negative selection, exits the bone marrow and further differentiates in secondary lymphoid organs such as spleen. These recent BM emigrants in the periphery are called transitional B cells, which are divided into transitional stages T1 and T2 (Chung *et al*., 2003). A small fraction of these transitional B cells home to the splenic marginal zone (MZ) and do not recirculate, but most T2 cells mature into follicular B cells, a B cell subset that recirculates between the follicles, lymph nodes and BM until they die, or encounter antigen and mature completely (Shapiro-Shelef *et al*., 2005).

B lymphocytes become antibody-secreting cells (ASC), the mediators of the humoral response. A productive antigen encounter will result, among other things, in multiple rounds of division and secretion of antigen-specific antibody to the serum and interstitial fluids. After antigen stimulation, B cells will acquire the ability to become terminally differentiated cells: plasmablasts, short and long-lived PC or memory B cells (Manz *et al*., 2005). Which B cell subset is generated depends on the nature of the antigen, dose and location of encounter (Shapiro-Shelef *et al*., 2005).

1.8.2 Terminal B cell differentiation

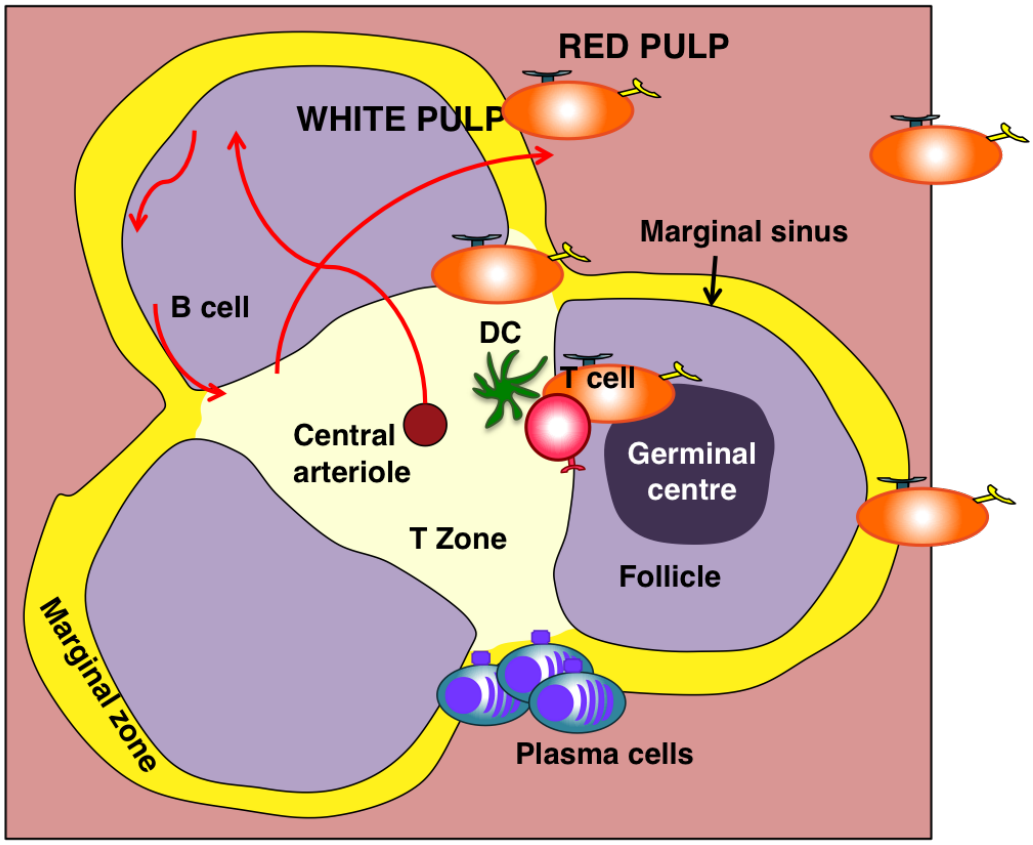
Terminal B cell differentiation takes place in secondary lymphoid organs, which include lymph nodes, Peyer's patches, tonsils and adenoids, mucosa-associated lymphoid tissue, and the white pulp of spleen. These organs provide points of intersection between naïve and antigen-experienced lymphocytes, accessory cells, molecules from the innate immune system and antigens.

We will focus our study of terminal

B cell differentiation in the murine spleen. The spleen is divided into red pulp, where macrophages remove senescent red blood cells from the circulation, and the white pulp, comprising follicles, T cell zones (periarteriolar lymphoid sheaths or PALS) and MZ. B and T cells segregate into separate regions of this organ.

Figure I7

Spleen architecture



B cells migrate from the BM to the spleen, where they enter via the blood. They are called transitional B cells, identified by the surface phenotype $IgM^{hi}IgD^{+}CD23^{int}CD21i^{int-hi}$. These T1 and T2 cells can either be recruited into the MZ, located between the white and red pulp, or organize into follicles, which are spheroid aggregates located in the surroundings of the PALS, where we also find follicular dendritic cells (FDC) and a few T cells (Figure I7). The MZ also contains macrophages and a small population of memory B cells in rodents.

Antigens are classified as T-dependent (TD) or T-independent (TI), based on their capacity to induce a response in nude (athymic) mice. TI antigens are subdivided into TI-1, such as LPS or endotoxin, and TI-2, such as bacterial polysaccharides.

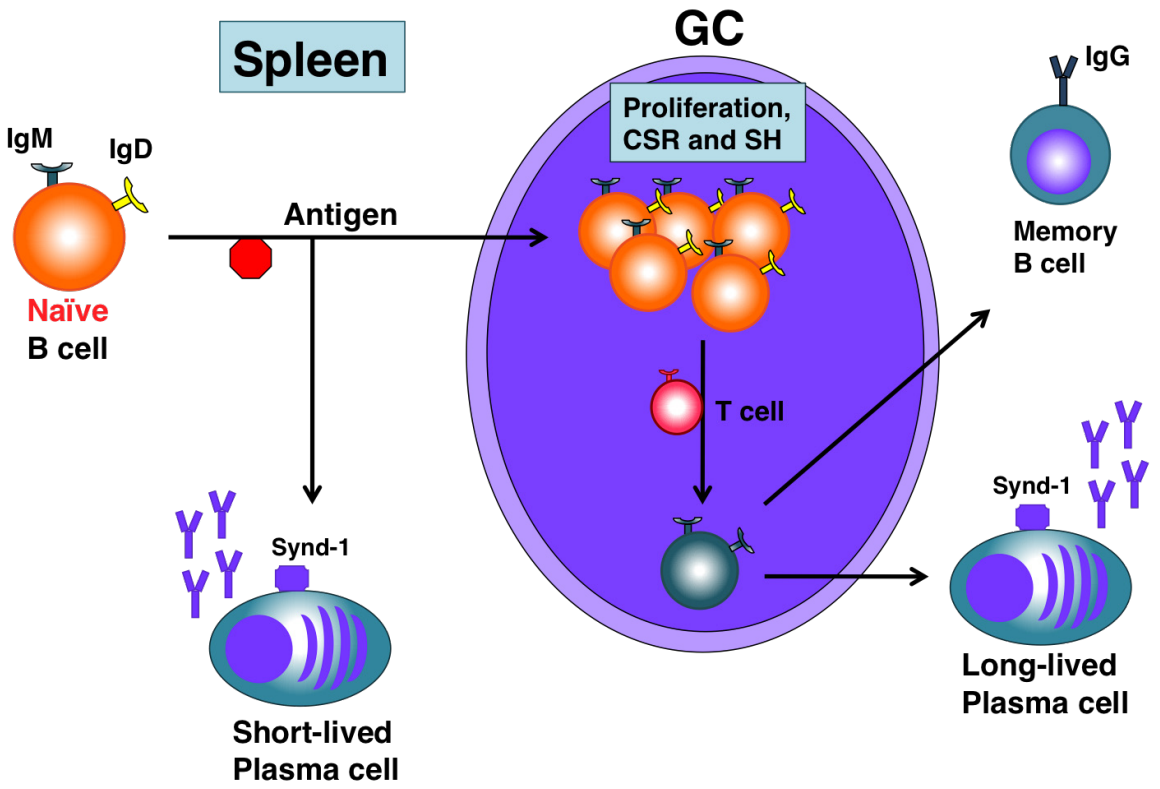
MZ B cells ($IgM^{hi}IgD^{lo}Cd21^{hi}CD21^{hi}CD23^{lo}$) surround the marginal metallophilic macrophage ring

identified by the MOMA-1 antibody (Kraal *et al.*, 1986), and provide an early response to bacteria and mainly to TI-2 antigens. This triggers a burst of proliferation and generation of plasmablasts or short-lived PC foci in extrafollicular regions along the periphery of the PALS. Circulating mature follicular B cells activated after antigen stimulation can also form extrafollicular foci and give rise to plasmablasts and short-lived PC. These short-lived PC have not undergone somatic hypermutation (SH) or class switch recombination (CSR), and die by apoptosis *in situ*.

When follicular B cells ($IgM^{lo}IgD^{hi}CD21^{lo}Cd23^{hi}$) encounter antigen and receive T cell help, they are able to form germinal centers (GC). The choice between the formation of extrafollicular foci or to enter a germinal center seems to be based on the strength of antigen recognition by the BCR (Paus *et al.*, 2006). Germinal centers are specialized follicular areas that arise from a small number of precursors derived from B cells activated in the T zone by TD antigens, which undergo massive proliferation. They give rise to centroblasts that populate the dark zone, proximal to the T cell area. Centroblasts exit the cell cycle to become centrocytes, which are located close to mature FDC in the light zone, in the opposite

Figure I8

From Naïve to plasma and memory B cells



pole or the GC. In the GC, B cells undergo several rounds of SH and CSR. CSR consists of DNA rearrangement events in which deletion replaces one Ig heavy constant region gene segment (typically μ) with a more 3' gene segment (γ, ε or α) to change the effector properties of the Ig secreted. SH is a unique mutation mechanism targeted to the variable regions of Ig genes that leads to affinity maturation, hence the specificity for the recognized antigen is increased.

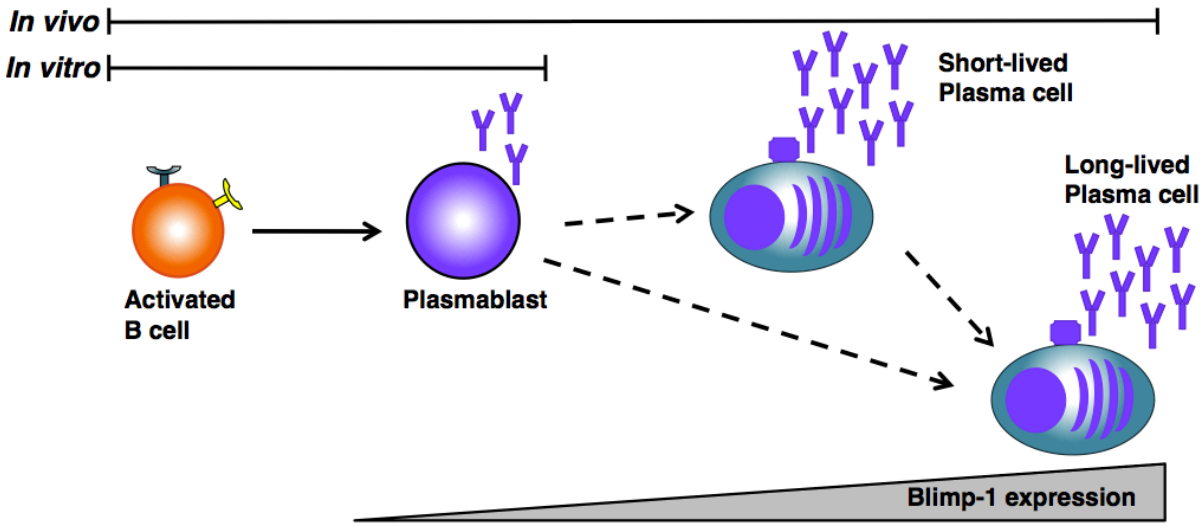
The GC response peaks between day 10 and 14 and then diminishes. This reaction gives rise to long-lived PC and memory B cells which have undergone SH and CSR (McHeyzer-Williams *et al.*, 2001; Vinuesa *et al.*, 2001; Shapiro-Shelef *et al.*, 2005) (Figure I8).

1.8.3 Subpopulations

1.8.3.1 Plasmablasts

Plasmablasts are short-lived cycling ASC that are found in extrafollicular foci and resemble ASC generated after *in vitro* antigen stimulation. They are an intermediate stage between activated B cells and PC, with morphology similar

Figure I9 Plasma cell differentiation markers



Location	Spleen	Spleen	Spleen	BM
Proliferation	++	++	-	-
Ig secretion	-	++	+++	+++
B220	+++	+	+/-	-
Synd-1	-	+/-	+/-	+/-
CD19	+++	++		
Pax5	+++	+		
CIITA	+++	+		
IRF4	+	+++		

to proliferating blast cells coupled to the ability to secrete low amounts of antibody, retaining some B cell characteristics such as B220 and CD19 expression (Figure I9).

1.8.3.2 Plasma cells

Plasma cells are fully differentiated cells of the B cell lineage that have acquired the ability to secrete large amounts of antigen-specific antibody. They can be classified as short-lived PC, formed in extrafollicular areas such as the MZ of the spleen in response to TD and TI antigens, with a life span of a few days or weeks, and long-lived PC, generated after T-dependent stimulation in follicular regions, with a half life of months to years in the absence of antigen or cell division. If these long-lived cells have been generated in GC, they undergo the processes of SH and CSR, and will hence secrete high-affinity switched antibody. The mechanism by which PC are selected to become a long-lived population could be stochastic selection by their ability to inhabit the limited number of environmental niches that support their survival.

Terminally differentiated PC do not proliferate and they express the proteoglycan Syndecan-1 (Synd-1 or CD138) on their surface, which is a heparan sulfate proteoglycan that participates in cell adhesion and is used as a cell surface marker to identify PC populations (Sanderson *et al.*, 1989), accompanied by downregulation of B220 in membrane, so we can identify the PC subset by the surface phenotype $B220^{lo/-}$ $Synd\text{-}1^{+}$ (Figure I9).

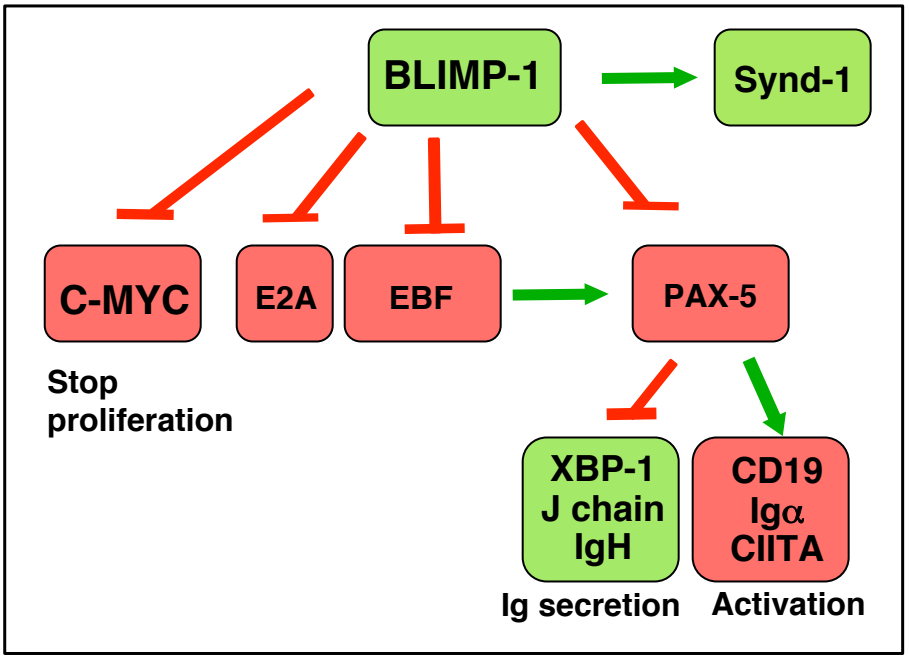
1.8.3.3 Memory B cells

Memory B cells are formed after T-dependent antigenic stimulation and express class-switched immunoglobulin (Ig) on their surface, but do not secrete antibody. Memory B cells provide rapid recall by quickly becoming PC that secrete high-affinity Ig after secondary antigenic challenge. They provide defense to antigens for years or even the organism's entire life, because they remain in their survival niches even without the presence of antigen. These cells are generated in the spleen, among other secondary lymphoid organs. Most models of post-germinal center B cell development posit that cells destined to become PC and memory B cells develop separately. An alternative possibility is that there is a single developmental pathway, beginning with memory cells and progressing to plasmablasts and PC. There is no firm evidence in this controversy, but the fact that Blimp-1 is dispensable for memory B cell generation, acting only in the memory-to-plasma cell step, suggests different pathways for plasma and memory B cell differentiation.

1.8.4 Transcriptional control of terminal B cell differentiation

PC are high producers of soluble antibody, hence they are crucial for an efficient humoral response. Activation of mature B cells, differentiation to secretory cells and their survival must be tightly regulated. The transcription program controlling gene expression during B cell differentiation and activation versus PC differentiation are mutually exclusive. This exclusion is achieved by the participation of stage-specific factors that repress other factors needed for the alternative developmental state (Calame *et al.*, 2003), ensuring a solid unidirectional pathway once B cells are committed towards fully differentiated ASC. This network is composed of a few transcription regulators with a complex regulation between them, and some hierarchical relationships still remain unclear. The most relevant factors in this regulatory pathway are Blimp-1, Xbp-1, Irf4, Bcl-6 and Pax5.

Figure I10 Blimp-1 regulation



1.8.4.1 Blimp-1

The 856-amino-acid Blimp-1 (B lymphocyte induced maturation protein) was first identified as a repressor of the human interferon-β promoter (Keller *et al.*, 1991) and subsequently described in 1994 when the B cell lymphoma cell line BCL1 was activated with IL-2 and IL-5 to promote Ig secretion. The authors found only 1% of genes whose expression changed after comparison with non-activated BCL1 cells, and Blimp-1 was one of about 20 novel cDNAs isolated (Turner *et al.*, 1994). Its mRNA rises five-fold rapidly after activation. The protein Blimp-1 encoded by the gene *prdm1*, is a transcriptional repressor that has five Krüppel-type zinc motifs, proline-rich (PEST) sequences that increase sensitivity to intracellular degradation, and acidic regions. This nuclear protein is not present

in fibroblasts, pre-T, T cells, pro-B or pre-B cells. Expression in B cells is either absent or at very low levels compared to those of PC. Transfection of Blimp-1 into B cell lymphoma lines leads to plasma B cell differentiation, accompanied by induction of J chain mRNA, Ig secretion, Synd-1 upregulation and increased cell size (Turner *et al.*, 1994).

Blimp-1 was considered the 'master regulator' of PC generation because its expression was reported to be necessary and sufficient to initiate and establish PC differentiation (Shapiro-Shelef *et al.*, 2003). Blimp-1 is induced by antigen-driven PC differentiation, and its overexpression can drive mature B cells to PC (Turner *et al.*, 1994). It was nonetheless reported that commitment and initiation of ASC differentiation is independent of this transcription factor, although it is indeed essential for subsequent high Ig production and full PC differentiation. The first event that triggers ASC differentiation is the downregulation of *pax5*, allowing the derepression of PC-specific genes such as *xbp1*, *flt3*, *embigin* and *IgJ*, to proceed to a state called the pre-plasma cell. These cells are Synd-1$^-$ but secrete low levels of Ig in the absence of Blimp-1, suggesting that these cells had indeed initiated ASC differentiation; however, they failed to upregulate *irf4*. Transduction of these cells with Irf4 did not restore normal *igJ*, *xbp1* or endogenous *irf4* levels, suggesting that Blimp-1 controls factors other than *irf4* to promote ASC differentiation (Kallies *et al.*, 2007).

Blimp-1 directly represses *c-myc* transcription (Lin *et al.*, 1997), which explains cell cycle cesation in PC. Blimp-1 also inhibits *pax5*, allowing Xbp-1, J chain and Ig heavy chain transcription, which induces Ig secretion (Shaffer *et al.*, 2004). Synd-1 is induced after Blimp-1 expression (Turner *et al.*, 1994), Blimp-1 participates in generation of both long- and short-lived ASC and its expression is required for memory-to-PC transition after re-exposure to antigen, but it is dispensable for memory B cell generation (Angelin-Duclos *et al.*, 2000).

The *prdm1* gene has two intronic regions that confer transcriptional repression mediated by Bcl-6 binding (Tunyaplin *et al.*, 2004). Bcl6 must be downregulated after GC reaction to allow the initiation of the Blimp-1 differentiation program.

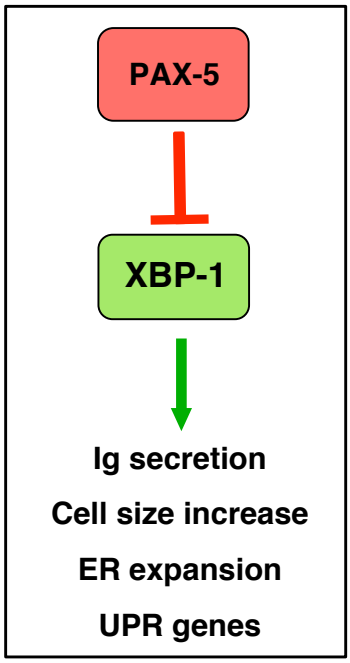
1.8.4.2 Xbp-1

Xbp1 is a basic-region leucine zipper protein positively-acting transcription factor of the CREB/ATF family (cyclic AMP response element binding protein/ activating transcription factor) which is ubiquitously expressed (Liou *et al.*, 1990). Overexpression of Xbp1 in BCL1-3B3 cells triggers Synd-1 expression and CD44 downregulation, identical to the effect caused by Blimp-1 overexpression in these cells (Reimold *et al.*, 2001).

Xbp1 germline deletion is embryonic lethal due to severe liver hypoplasia that

results in fatal anemia (Reimold *et al.*, 2000). Analysis of *xbp1*$^{-/-}$;*rag2*$^{-/-}$ chimeric mice showed normal B cell proliferation, GC formation and Blimp-1 expression, but they were devoid of PC. They showed a severe Ig secretion defect, demonstrating that this factor is necessary for PC differentiation and mediates increase in cell size, lysosome content, mitochondrial mass and function, as well as protein synthesis, to establish the phenotype of a secretory cell. Xbp1 is also implicated in the function and survival of secretory cells other than PC such as exocrine gland acinar cells and Paneth

Figure I11 XBP1 regulation



that Blimp-1 does not compensate for the lack of Xbp1 (Reimold *et al.*, 2001). A similar phenotype was described in *xbp1*$^{fl/fl}$;*cd19*$^{cre/+}$ mice, which present very low Ig levels even in response to stimuli due to a lack of μS upregulation. However, the genetic program of PC development is unaltered, and IRF4, Blimp-1 and Pax5 mRNA levels as well as Synd-1 expression are normal. Xbp1 was nonetheless shown to be dispensable for memory B cell generation (Todd *et al.*, 2009), suggesting later Xbp1 intervention after PC commitment to allow the changes in secretory apparatus and cell morphology needed for large-scale Ig secretion. Xbp1 cells (Kaser *et al.*, 2008), and is associated with the UPR (unfolded protein response).

UPR is elicited by overexpression or misfolding of endoplasmic reticulum (ER) proteins (Harding *et al.*, 2000). This ER stress is transduced by IRE1, a transmembrane ER protein that is autophosphorylated when BiP chaperone binds to misfolded proteins and releases IRE1 to allow its multimerization and kinase activity. IRE1 also has endoribonuclease activity, which mediates postranscriptional processing of Xbp1 mRNA, giving a as result the active form of Xbp1. Xbp1 in turn activates transcription of

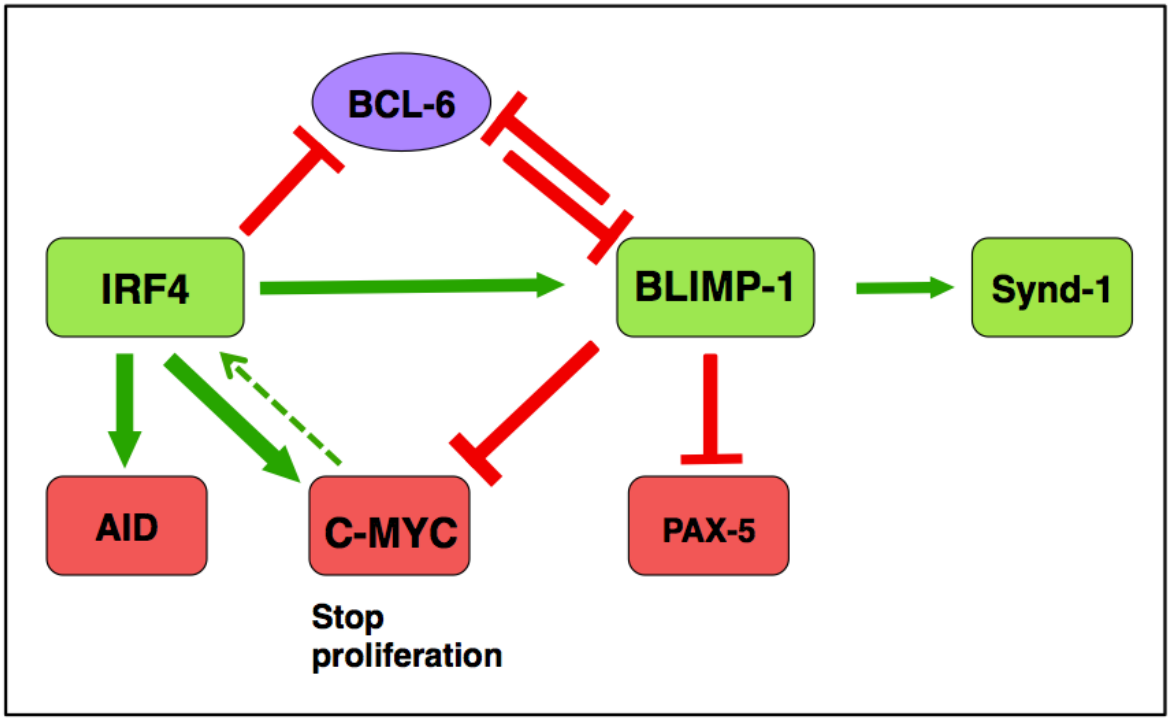
UPR genes, involved in protein entry in the ER, folding, glycosylation, ER-associated degradation (ERAD) and vesicular trafficking (*edem*, *dnaJc3*, *dnaJb9*, *hspa5*, *cope*) (Cox *et al.*, 1996; Travers *et al.*, 2000; Shaffer *et al.*, 2004). UPR is also mediated by two other signaling pathways via activation of PERK and ATF6. PERK normally interacts with BiP, but this association is broken by ER stress; PERK then phosphorylates the translation initiator eIF2α, which mediates global protein synthesis inhibition and activates ATF4 transcription factor. ATF4 in turn activates other UPR genes such as *chop* and *BiP* (Harding *et al.*, 2001). ATF6 is also released by BiP and exported to the Golgi, where it is processed and upregulates expression of ER chaperones and Xbp1 (Yoshida *et al.*, 2000).

Xbp1 expression is strongly inhibited by Pax5; this accounts for high Xbp-1 expression levels in PC once Pax5 is no longer expressed (Reimold *et al.*, 1996). Its expression is induced by IL-4 during B cell activation, whereas production of the spliced form is dependent on Ig synthesis levels during PC differentiation (Iwakoshi *et al.*, 2003).

1.8.4.3 Irf4

Irf4 (also known as LSIRF/ICSAT/ Pip/MUM1) is a member of the interferon regulatory family of transcription factors containing a DNA binding domain with five conserved tryptophan repeats (Taniguchi *et al.*, 2001). In contrast to other IRF family members, Irf4 expression is not induced by interferons. Its expression is limited

Figure I12 Irf4 regulation



to lymphocytes, dendritic cells (DC) and macrophages (Eisenbeis *et al.*, 1995). It is induced by antigen receptor or mitogen stimulation and its expression is maximum in PC (Matsuyama *et al.*, 1995). Irf4 interacts with other partners such as PU.1, SpiB, Irf8, Stat6 and E2A to modulate its transcriptional activity (Eisenbeis *et al.*, 1995).

Irf4 is first expressed in immature B cells together with Irf8, and then is re-expressed in some centrocytes and in PC (Falini *et al.*, 2000). Irf4 controls processes of PC differentiation and isotype switching by induction of Blimp-1 and activation-induced deaminase (AID), respectively. Irf4 KO mice show low serum Ig, although the mature B cell subset is unaffected, GC center formation is normal, and they show normal proliferation levels and induction of activation markers CD25, CD69, CD80 and CD86. Irf4-deficient B cells are compromised for CSR and Ig secretion, they lack post-GC PC and are unable to differentiate from memory B cells into PC (Mittrucker et al, 1997; Sciammas et al, 2006; Klein et al, 2006). Irf4 coordinates PC differentiation and CSR in a Irf4 graded expression manner; low Irf4 levels are sufficient to activate its target AID, whereas only PC with very high Irf4 expression are able to induce Blimp-1 and fully differentiate into Synd-1$^+$ ASC. Restoration of AID in Irf4-deficient cells induced CSR, although rescue was incomplete, suggesting that Irf4 might regulate other genes implicated in isotype switching. Irf4 transduction, even with a mutant that is unable to interact with PU.1 or SpiB, restored Ig secretion and CSR, indicating that these Irf4 functions are independent of its transcription partners. Irf4 is not reported to bind directly to the *aicda* gene (encoding AID), but binds to a region between exons 5 and 6 of the *prdm1* gene (Klein *et al.*, 2006; Sciammas *et al.*, 2006). Irf4 is reported to act downstream of Blimp-1, as it is expressed in a GC B cell population that already expresses Blimp-1; Irf4 is also activated by ectopic Blimp-1 expression in a B lymphoma (M12) (Sciammas *et al.*, 2004), suggesting an autoregulatory loop between Irf4 and Blimp-1.

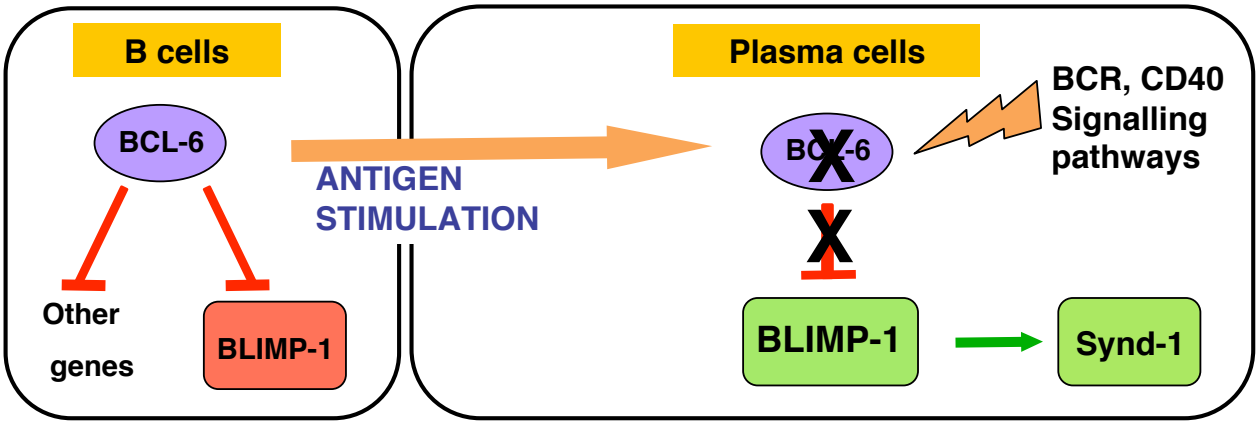
Irf4 binds to enhancers in the IgLκ and λ loci, and thus might induce high transcription of Ig genes. It is not required for memory B cell generation, however (Klein *et al.*, 2006). Irf4 direct targets act in metabolic control, membrane biogenesis, cell cycle progression, cell death and PC differentiation.

It is not clear whether Irf4 alone is sufficient to drive Blimp-1 expression and trigger PC differentiation. In the absence of Irf4, Blimp-1 expression is insufficient for PC differentiation. The two transcription factors are thus non-redundant, essential for ASC formation and critical upstream activators of Xbp1. Irf4 binds directly to the promoter of Bcl6 and downregulates its transcription (Saito *et al.*, 2007), contributing to establishment of the PC transcription program.

Elevated Irf4 expression has been described in multiple myeloma, a malignancy of PC, and Irf4 knockdown was toxic to

these cells, resulting in cell death. *c-myc* downregulation was reported after treatment with Irf4 small haipin RNA (shRNA), and chromatin immunoprecipitation (ChiP) demonstrated cMyc binding to a region of the *Irf4* first intron, revealing a positive regulatory loop in which Irf4 and c-Myc mutually reinforce their expression (Shaffer *et al.*, 2008).

Figure I13 Bcl-6 regulation



1.8.4.4 Bcl-6

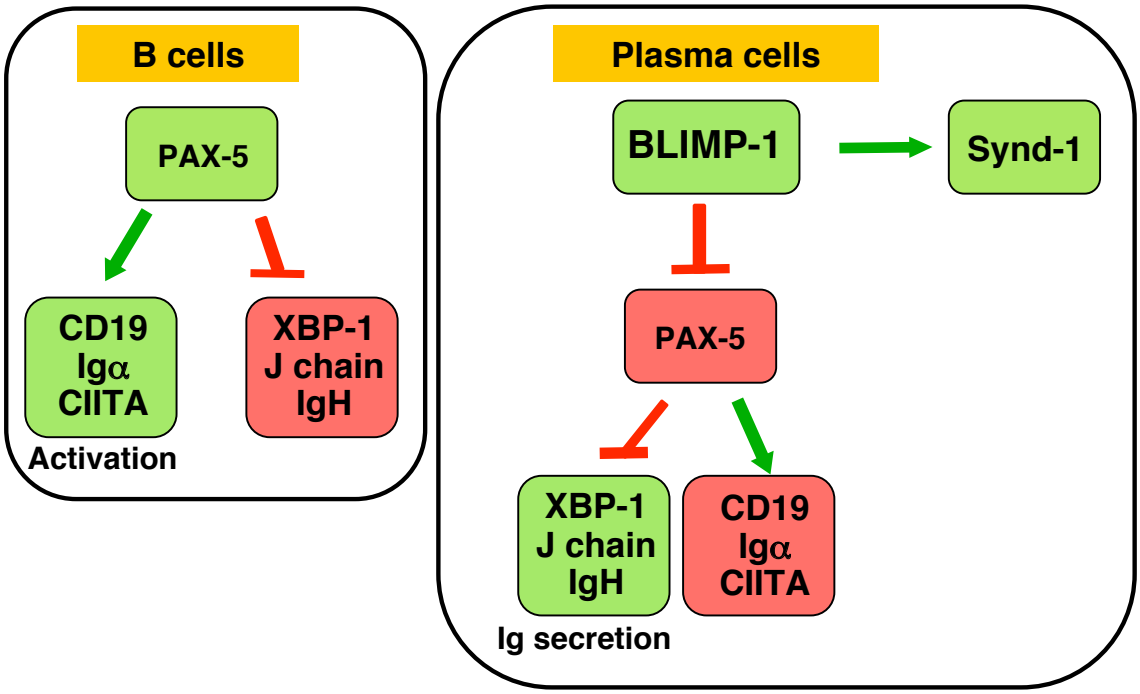
Bcl-6 (B Cell Lymphoma protein 6) was identified by its implication in chromosome translocations affecting chromosome 3q27 in diffuse large cell lymphoma (DLCL) (Baron *et al.*, 1993). One-sixth of all B cell non-Hodgkin's lymphomas have translocations of the *bcl-6* gene, which is the most frequently translocated in these cancers (Dalla-Favera *et al.*, 1999). Bcl-6 is a six *Krüppe*l-type zinc-finger-containing transcriptional repressor (Chang *et al.*, 1996).

It is expressed mainly by lymphocytes, with highest expression in GC, including all centroblasts and centrocytes (Cattoretti *et al.*, 1995). Nascent pre-GC cells upregulate Bcl6, migrate to the follicular area and form GC, while cells that do not upregulate Bcl-6 after antigen encounter differentiate into plasmablasts in the PALS (Fukuda *et al.*, 1997). Bcl-6 has at least two functions in GC, promoting cell cycle progression and inhibiting differentiation. It also suppresses the cell's intrinsic apoptotic response to DNA damage to allow GC B cells to sustain the DNA damage associated to AID-dependent CSR and V gene SHM (Phan *et al.*, 2007).

Plasmacytic differentiation, both pre- and post-GC, occurs only in the absence of Bcl-6, since *bcl-6* must be downregulated to allow Blimp-1 expression. Other Bcl-6 target genes are *CD44*, *CD69*, *cyclin D2*, *p27kip1* and *id2*, although only *cyclin D2* and *blimp-1* are direct Bcl-6 targets (Shaffer *et al.*, 2000). Bcl-6 levels are maintained in GC by STAT (Signal Transducers and Activators of Transcription) activation and absence of BCR and CD40 signalling. The ASC program starts when Bcl-6 levels are downregulated

by several mechanisms: by MAPK (Mitogen-Activated Protein Kinases) phosphorylation and subsequent degradation after BCR stimulation (Moriyama *et al.*, 1997) and Irf4-dependent inhibition of *bcl-6* transcription via NFκB (Nuclear Factor-κB) activation following CD40 stimulation (Saito *et al.*, 2007).

Figure I14 Pax5 regulation



Bcl-6 mutant mice fail to form GC during a T cell-dependent immune response, have increased PC formation after immunization, and develop a fatal inflammatory disease (Dent *et al.*, 1997). Their B cells have an activated surface phenotype (in the absence of immunization), with downregulation of surface IgM, CD69 and CD44 expression, although they fail to complete plasmacytic differentiation (no Synd-1 expression or downregulation of MHC (Major Histocompatibility Complex) class II) (Shaffer *et al.*, 2000).

1.8.4.5 Pax5

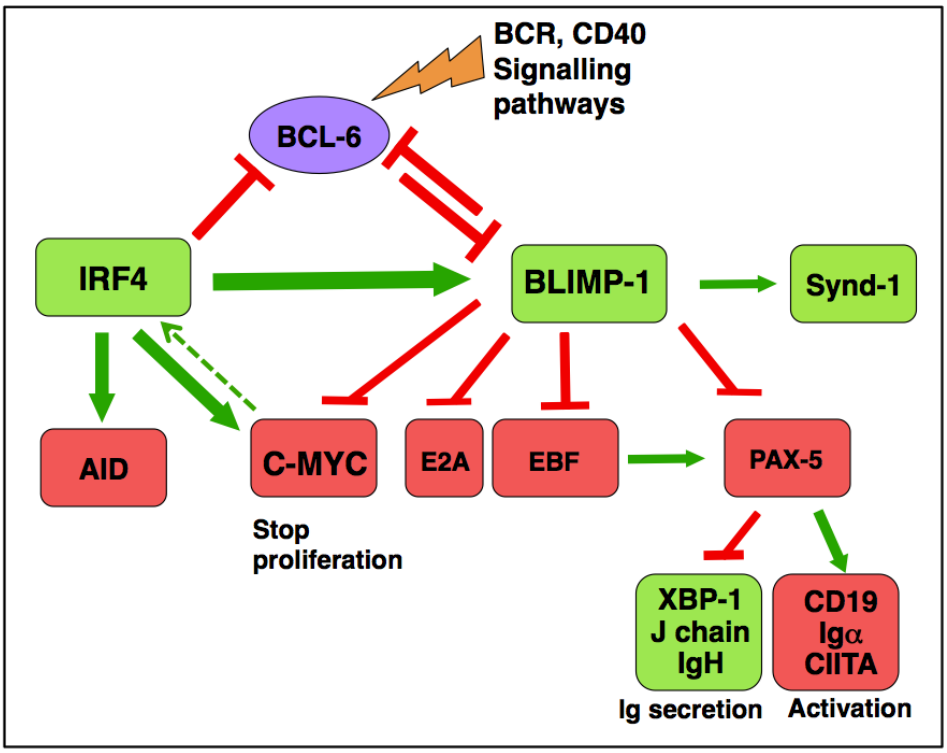
Pax5 (PAired Box protein 5) was first identified as a homolog of the sea urchin protein TSAP (Tissue-Specific Activator Protein) and was originally named BSAP (B cell lineage-Specific Activator Protein). It binds DNA by a bipartite 128–amino-acid paired domain, which shows similarity with some homeobox-containing proteins from *D. melanogaster*, and can function as transcriptional activator or repressor. It has a role in B cell differentiation and also in neural development and spermatogenesis (Adams *et al.*, 1992).

Pax5 is expressed throughout B cell development and activates target genes

important for early B cell differentiation and activation, including Igα, CD19 and B-cell linker (BLNK) (Nutt *et al.*, 2001; Horcher *et al.*, 2001). It also induces AID expression, necessary for CSR and SH in the GC (Gonda *et al.*, 2003). Pax5 represses *xbp1*, *Igh*, *IgL* and *j chain* expression, blocking the PC differentiation program (Reimold *et al.*, 1996; Rinkenberger *et al.*, 1996; Singh *et al.*, 1993). Experiments by

Blimp-1 directly represses *pax5* by binding to a site in its promoter. Blimp-1 is necessary and sufficient for Pax5 repression during the PC differentiation program. Enforced BSAP expression inhibits formation of IgM-secreting cells, although Blimp-1 is induced normally (Lin *et al.*, 2002). Pax5 repression is thus the first event necessary to shift from the early to late B cell transcription

Figure I15 PC transcription program



Kallies *et al.*. (Kallies *et al.*, 2007) led to a model in which the PC program begins by inhibition of *pax5* expression, allowing the change to new genetic programs and derepressing genes necessary for Ig secretion, although full ASC differentiation needs the expression of other transcription factors such as Blimp-1. The factor(s) that modulate Pax5 levels nonetheless remain unknown.

program, although other transcription factors must be expressed for further progress in differentiation. A summary of the transcription pathway controlling PC differentiation versus B cell activation is shown in Figure I15.

2. Aims

2. Aims

1) To characterize the role of c-Myc in terminal B cell differentiation using a loss-of-function murine model: generation of plasma cells and immunoglobulin secretion *in vitro*.

2) To characterize the immune response in c-Myc-deficient mice after immunization with T-dependent and T-independent antigens.

3) To analyze the gene expression levels of the major transcription factors involved in terminal B cell differentiation in the absence of c-Myc.

4) To rescue the wildtype phenotype by gain-of-function experiments overexpressing potential cMyc targets.

3. Materials & Methods

3.1 Generation of *c-myc* $^{flox/flox}$;*cd19*$^{Cre/+}$;*rosa26*$^{egfp/egfp}$ Mice

The generation of the *c-myc*^{flox};*cd19*^{cre} mice has been described previously (de Alborán *et al.*, 2001; Rickert *et al.*, 1997). Briefly, *c-myc*^{flox};*cd19*^{cre} mice were bred with *rosa26*^{egfp} mice (Mao *et al.*, 2001) and progeny bred to yield homozygous *c-myc*$^{flox/flox}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$ mice (*c-myc*$^{fl/fl}$) or heterozygous *c-myc*$^{fl/+}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ mice (*c-myc*$^{fl/+}$). Mice were genotyped using a PCR-based analysis of tail genomic DNA with specific primers:

Flox 3' (TTTTCTTTAAGATTGCTGAC) and Flox 5' (TAAGAAGTTGCTATTTTGGC) to detect flox alleles in *c-myc* locus

CD19.8 (AATGTTGTGCTGCCATGCCTC) and CD19.9 (GTCTGAAGCATTCCACCGGAA) for CD19 wt allele

Cre3 (CAATTTACTGACCGTACA) and Cre4 (CATCGCCATCTTCCAGCA) to detect Cre recombinase

GFPI (GGCTTAAAGGCTAACCTGTG), GFPIII (GGAGCGGGAGAAATGGATATG), Lacz1 (GTGGTGGTTATGCCGATCG) and Lazc2 (TACCACAGCGGATGGTTCGG) to amplify EGFP or wildtype (WT) alleles *in rosa26* locus.

3.2 Immunizations

For the study of T-dependent response in *c-myc*$^{fl/fl}$ and littermate control mice, 6- to 8-week-old, were immunized intraperitoneally (ip) with 200 μg of 2,4,6, Trinitrophenyl-Keyhole Limpet Hemocyanin (TNP-KLH) (Biosearch Technologies) in Alum adjuvant (Pierce) at ratio 1:1 in 0.2 ml of phosphate-buffered saline (PBS). For memory response, the same dose of TNP-KLH was given 4 weeks after the initial dose. Mice were analyzed 12 days after secondary immunization for FACS and histology. Mice were bled weekly for serum ELISA.

For T-independent response mice were injected intravenously (iv) with 3μg of LPS (Sigma). Mice were analyzed 3 or 7 days later

3.3 Flow Cytomery

Single cell suspensions were incubated in NH_4Cl buffer for erythrocyte lysis and stained with a suitable dilution of surface anti-mouse antibodies in PBS with 2% fetal bovine serum (FBS). Antibodies were from eBioscience (B220-Pecy7, GL-7-biotin, IgM-APC, CD38-Alexa700 and F4/80-APC); Pharmingen (CD23-PE, CD21-APC, CD69-biotin and Gr1-APC); BD (CD138-PE, IgG1-PE); Biolegend (B220-Pacific Blue and IgD-Alexa647); Southern Biotech (IgD-biotin, IgE-biotin). Biotynilated antibodies were

revealed with streptavidin-PE or streptavidin-Pecy7 (Pharmingen). To detect TNP-specific memory B cells by FACS TNP-BSA-biotin was used (Biosearch Technologies). Ant-human CD2-APC was from IOTest.

For cell cycle analysis cells were stained with propidium iodide using Coulter DNA PREP kit (Beckman Coulter) and following manufacturer's instructions.

A FACS Coulter flow cytometer was used for analysis and an EPICS-ALTRA (Beckman Coulter) or FACS Aria (BD) were used for cell sorting

3.4 B Cell Purification

Freshly isolated splenocytes were stained with anti-B220-Pecy7 in PBS with 2% FBS. $B220^+$ GFP^+ cells were purified (purity > 95%) for B cells culture with a Beckman Coulter sorter. For gene expression analysis spleen cells were separated into $B220^+CD138^-GFP^+$ and $B220^{lo}CD138^+GFP^+$ 3 days after activation.

3.5 B Cell Culture

Splenocytes were suspended in NH_4Cl buffer to lyse red blood cells, were stained with the corresponding antibodies and purified by sorting. Purified spleen cells were plated at 10^6 cells/ml in RPMI with 15% FCS, β-mercaptoethanol, and penicillin/streptomycin. T-independent stimulation was mimicked with 30 μg/ml of lipopolysaccharide (LPS) (Sigma) and T-dependent stimulation with 20 ng/ml recombinant mouse IL-4 (R&D) and 10 ng/μl anti-CD40 antibody (Pharmingen). Activated cells and supernatants were harvested after 1, 3 or 4 days in culture.

3.6 ELISA

To detect total IgM or IgG1 in culture supernatants 96 Immunosorp flat-bottomed plates were coated with purified polyclonal goat anti-mouse IgM (Southern Biotech) or rat anti-mouse IgG1 (clone A85-1, Pharmingen). Samples were plated in triplicates of 3-fold serial dilutions after blocking wells with PBS with 1% BSA. Secondary antibody was anti-IgK-HRP (clone 187.1, Southern Biotech), it was developed with Sigmafast OPD (Sigma) and reaction was stopped with H_2SO_4 3M. Plates were read at 495nm. For IgE plates were coated with purified anti-IgE and developed with biotinylated anti-IgE (Southern Biotech). Finally, plates were incubated with streptavidin-HRP (Pharmingen) and developed as above.

To measure TNP-specific antibodies in mice sera, plates were coated with TNP-BSA(19) (Biosearch Technologies) 10mg/ml in PBS. Plates were blocked with PBS with 1% BSA and 5-fold serially diluted samples were plated. Biotynilated anti-IgM, IgG2a (Southern Biotech) or IgG1 (Pharmingen)

were used as secondary antibodies. For TNP-specific IgE plates were coated with purified anti-IgE, plated samples and incubated with TNP-BSA-biotin (Biosearch Technologies), and developed as indicated above.

3.7 ELISpot

96 Immunosorp flat-bottomed plates were coated with purified polyclonal goat anti-mouse IgM (Pharmingen). Plates were washed and triplicates of 3-fold serially diluted $B220^{+}GFP^{+}$ spleen cells were plated in RPMI with 1% FBS. They were incubated at 37ºC in a CO_2 incubator with 95% humidity and 5% CO_2 from 5 hours to over night to allow the formation of spots. Then cells were lysated with H_2O containing 0.05% Tween 20 (Tw) and washed in PBS 0.05% Tw. Plates were incubated with anti-IgK-AP (Pharmingen) secondary antibody and developed with BCPI dissolved in AMP buffer (Sigma) added to 3% low melt agarose. Plates were incubated 30 minutes at 37ºC to allow spots appear. They were counted and spots surface measured in an AID Elispot Reader using AID software.

3.8 Immunoprecipitation and Western Blot

The supernatant corresponding to 5 wells (1 mililiter of medium) was harvested 3 or 4 days after anti-CD40 plus IL-4 activation, and total secreted IgM was bound with polyclonal goat anti-mouse IgM (Pharmingen). The remaining supernatant was immunoprecipitated again as a control. Afterwards, IgM bound to antibody was precipitated with protein A/G Plus Agarose beads (Santa Cruz). Beads were washed in PBS with proteinase inhibitors and bound protein was eluted at 95º C. Supernatants were subjected to SDS-PAGE in 10% Acrylamide/Bisacrilamide gel and semidried transfered to PVDF membrane. Blots were incubated with Policlonal goat anti-mouse IgM bound to HRP (Sigma) and developed with Chemiluminiscent Reagent Plus (Perkin Elmer).

3.9 Lentivirus production and infection

EF.CMV.hCD2t vector was obtained by replacing the RFP of the EF.CMV.RFP vector (Addgene) by the human CD2 truncated sequence (hCD2t) preceded by an IRES sequence (obtained from MSCV-IRES-hCD2t vector), to get bicistronic mRNA and detect hCD2t expression by flow cytometry in infected cells. Afterwards the Blimp-1 coding sequence was cloned upstream the IRES of the EF.CMV.IRES-hCD2t 'empty' vector to get the EF.CMV.Blimp-1-IRES-hCD2t full vector.

293T cells were seeded in 10 cm plates to reach about 80% of confluence the following day. 18 hours later they were cotransfected with EF.CMV.Blimp-1-IRES-hCD2t or EF.CMV.IRES-hCD2t (empty) plasmids, pCMVdR8.74 and pMD2G (Vesicular stomatitis virus G protein or VsVg pseudotyping vector, that allows

high range of mammalian cells tropism and allows concentration of the viral particles by ultracentrifugation (Burns *et al.*, 1993)), at ratios 5:5:2 μg. The DNA transfection was performed with Jet PEI (at ratio 2:1) (Polyplus transfection). Lentitviral supernatants were collected 36 and 60 hours post-transfection and filtered through a 45 μm low protein-binding syringe filter (Pall, Life Sciences). Supernatants were concentrated 50x by ultracentrifugation in polyallomer tubes (Beckman) for 2 hours and 30 minutes at 22.500 rpm in a SW 28 rotor (Beckman).

$B220^{+}GFP^{+}$ spleen cells were sorted and plated at 10^{6} cells/ml in U-bottomed 96-well plates in 100 μl of RPMI medium containing 15% heat-inactivated FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin and 50 mM β-mercaptoethanol. They were infected with 100 μl of lentiviral supernatants, to get a multiplicity of infection (MOI) of about 10 viral particles per cell, and incubated for 2 hours and 30 minutes at 37ºC in a CO_2 incubator. Cells were spinned and medium was replaced by fresh complete RPMI medium and supplemented with anti-mouse CD40 antibody (20 ng/ml) (Pharmingen) plus recombinant mouse IL-4 (10 ng/μl) (R&D). Transduction efficiency was monitored by flow cytometry at 72 or 96 hours post-infection by surface hCD2t expression.

3.10 Immunohistochemistry

For paraffin sections, half spleens were fixated in paraformaldehyde (PFA) 4% in PBS at room temperature (RT) for 2-3 hours and then in ethanol 70% v/v at 4º C over night. They were included in paraffin and 5 μm sections were cutted in a microtome. They were deparaffined before staining with Harry's Hematoxylin/Eosin solution (Sigma).

For Spleen cryosections mice were sacrificed 12 days after second immunization and half spleens were snap-frozen in isopentane prevoiusly cooled in liquid nitrogen. Samples were kept at -80ºC until 10 μm sections were cut in a cryostate. They were fixed in acetone for 10 minutes at RT before staining. Cryosections were blocked for endogenous peroxidase activity in methanol 70% v/v with 0.3% H2O2, for endogenous biotin with Avidin/Biotin blocking kit (Dako), and for inespecific antibody binding in 10% inactivated goat serum before staining.

Sections were stained with rabbit polyclonal anti-GFP (ab290, Abcam) or rabbit anti-Cre polyclonal antibody (Covance) plus monoclonal rat anti-mouse B220 (Southern Biotech) and biotynylated anti-MOMA1 (Dianova) or PNA-biotin (Vector Labs). As secondary antibodies we used goat anti-rabbit IgG-HRP (Santa Cruz) or mouse anti-rat IgG labelled with Cy5. Following secondary antibody incubation, sections were stained with Tyramide-FITC (for HRP development) (Perkin Elmer) and streptavidin-Cyn3 (Jackson). Slides were mounted in Vectashield with DAPI (Vector Labs). Images were taken in a Olympus Confocal microscope or fluorescente microscope.

4. Results

4.1 A mouse model to study B cell differentiation: *cmyc*$^{fl/fl}$;*cd19*$^{Cre/+}$;*rosa26*$^{efgp/egfp}$ mouse generation

To study the role of c-Myc in the generation of primary ASC *in vivo* and *in vitro*, we used our previously reported conditional *c-myc*$^{fl/fl}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$ mouse model (*c-myc*$^{fl/fl}$ hereafter (de Alboran *et al.*, 2001). In the *c-myc*$^{fl/fl}$ mouse, the coding exons of the *c-myc* gene are flanked by loxP sites (floxed), which are normally expressed before Cre-mediated deletion. We crossed these mice with the *cd19*^{cre} strain, which expresses the Cre recombinase under the B lymphocyte lineage-specific *cd19* endogenous promoter. In the progeny, Cre expression mediates *c-myc* deletion in B cells. To distinguish and isolate deleted cells, we crossed *cmyc*$^{fl/fl}$*cd19*$^{cre/+}$ mice with a previously described reporter strain in which the enhanced green fluorescent protein (EGFP) was inserted in the *rosa26* locus, preceded by a floxed stop codon (Mao *et al.*, 2001). We thus obtained *c-myc*$^{fl/fl}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/}$

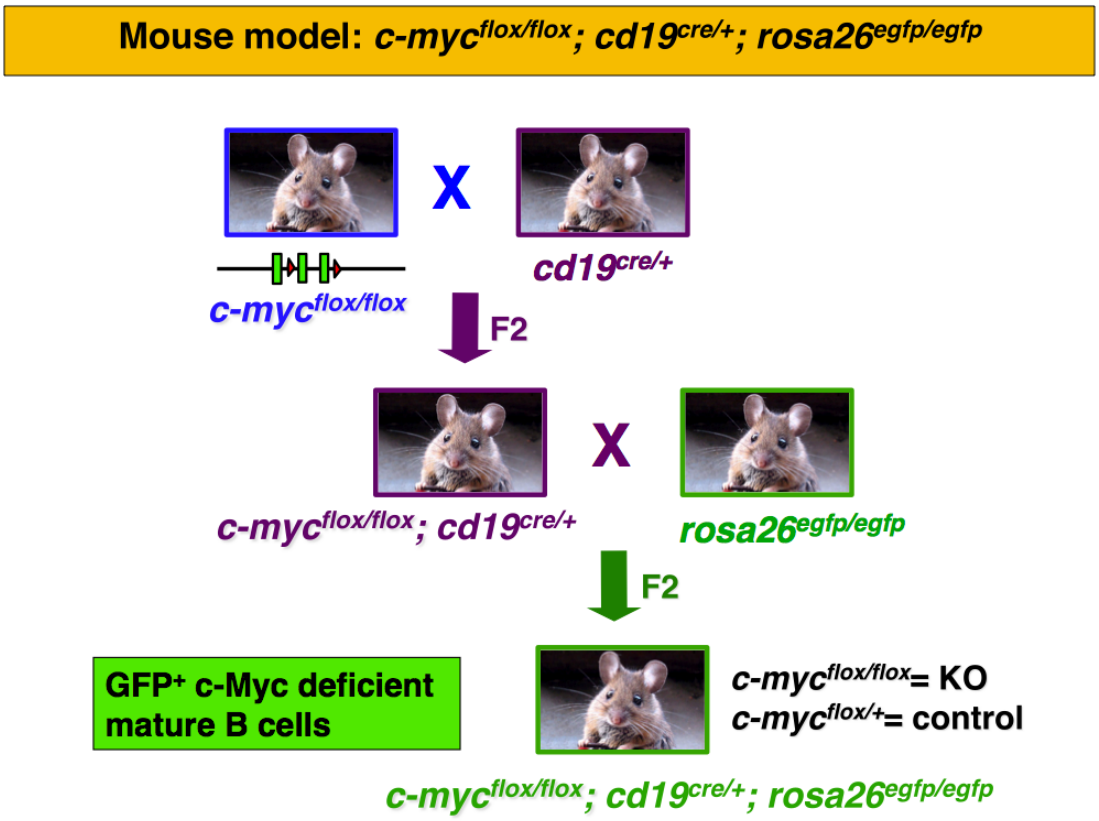


Figure R1. Mouse model for c-Myc deletion in B lymphocytes

^{egfp} mice, in which *c-myc* is deleted and the *egfp* reporter gene is activated specifically in B lymphocytes. *c-myc*$^{fl/fl}$ mice therefore generate cMyc-deficient B lymphocytes that express GFP (Figure R1).

4.2 c-Myc is necessary for differentiation to B220^{lo}syndecan-1^{+} cells *in vitro*

To determine whether c-Myc is necessary for the generation of terminally differentiated B cells *in vitro*, we isolated B220^{+}GFP^{+} cells from spleens of *c-myc*$^{fl/fl}$ or *cmyc*$^{fll+}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$ (*c-myc*$^{fl/+}$) control mice by cell sorting. We first studied *in vitro* generation of plasma cells. Plasma cells downregulate the B220 B cell marker and express the heparan sulfate proteoglycan Synd-1, which recognizes extracellular matrix and groWTh factors and is expressed in ASC (Sanderson *et al.*, 1989). Plasmablasts generated *in vitro* can thus be identified by the surface phenotype B220$^{lo/-}$Synd-1^{+}. Plasma cells can be generated by stimulation with LPS (which mimics a TI response) or anti-CD40 plus IL-4 (TD-like activation). Flow cytometry analysis showed that c-Myc-deficient B lymphocytes did not generate B220^{lo}Synd-1^{+} cells after *in vitro* activation

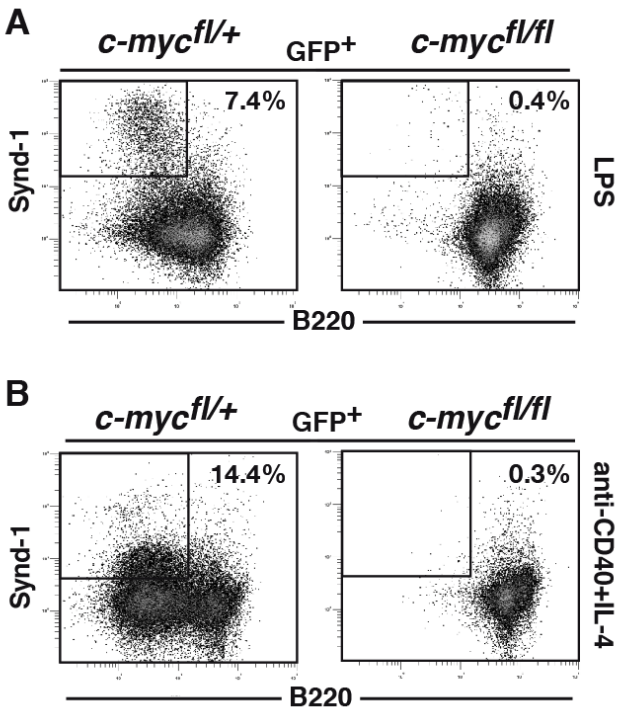


Figure R2. c-Myc is necessary for the generation of B220^{lo}Synd-1^{+} cells *in vitro*. Sorted B220^{+}GFP^{+} spleen cells from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice were activated with LPS (**A**) or anti-CD40 plus IL-4 (**B**) and analyzed by flow cytometry after 4 days. Data are representative of at least three independent experiments.

with LPS (22.2% vs 0.4%) or anti-CD40

plus IL-4 (14.2% vs 0.3%) (Figure R2A, B).

This blockade in plasmablast differentiation could be due to a lack of activation caused by c-Myc deletion. Nonetheless, c-Myc-deficient B lymphocytes (GFP$^+$) in these cultures expressed surface levels of the activation marker CD69 similar to those of control cells (Figure R3 and de Alboran *et al.*, 2001); c-Myc-deficient B cells were therefore able to respond to mitogenic stimuli. We concluded from these experiments that c-Myc-deficient B lymphocytes do not differentiate into B220^{lo}Synd-1$^+$ cells *in vitro*.

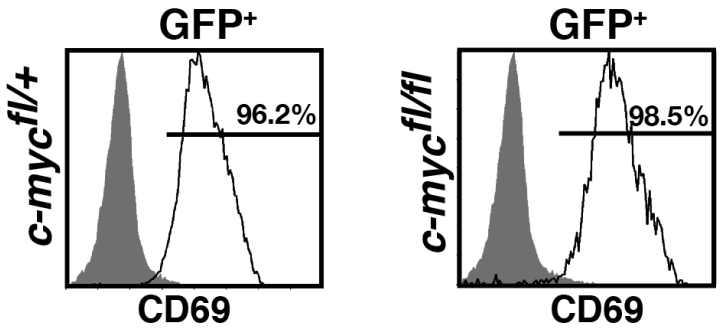


Figure R3. Normal CD69 expression in c-Myc deficient cells. CD69 surface expression in purified B220$^+$GFP$^+$ B cells 3 days after anti-CD40 plus IL-4 activation. Data represents one of three independent experiments.

4.3 c-Myc deficient B cells show greatly reduced proliferation *in vitro*

To analyze the ability of these c-Myc-deficient B cells to proliferate, we counted cells at day 3-4 after *in vitro* activation with T-dependent or -independent antigens. The B cell count after 4 days of activation was approximately 4-fold lower in homozygous wells than in control cultures (Figure R4B) and the B cells blasts formed were notably smaller in homozygous cultures (Figue R4C).

We assayed these cells by propidium iodide (PI) staining and FACS to study the B cell proliferation state. As reported (de Alboran *et al.*, 2001), c-Myc-deficient B lymphocytes showed a notable proliferation impairment after stimulation with either LPS or anti-CD40 plus IL-4 (Figure R4A); only 13.2% of cells were in S-G2/ phase M in KO cultures, compared to 20.1% in control cells after activation with LPS, and 20.1% vs 50.4% when they were activated with anti-CD40 plus IL-4.

4.4 p27 deletion does not rescue proliferation in c-Myc-deficient B cells

The cell cycle inhibitory molecule p27kip is upregulated in these c-Myc-deficient B cells (de Alborán *et al.*, 2001); we therefore tested whether downregulation of p27 expression could restore proliferation rates in KO cells, which would allow us to

study whether, in conditions that normalize proliferation in the absence of c-Myc, plasma cell differentiation would also be normalized.

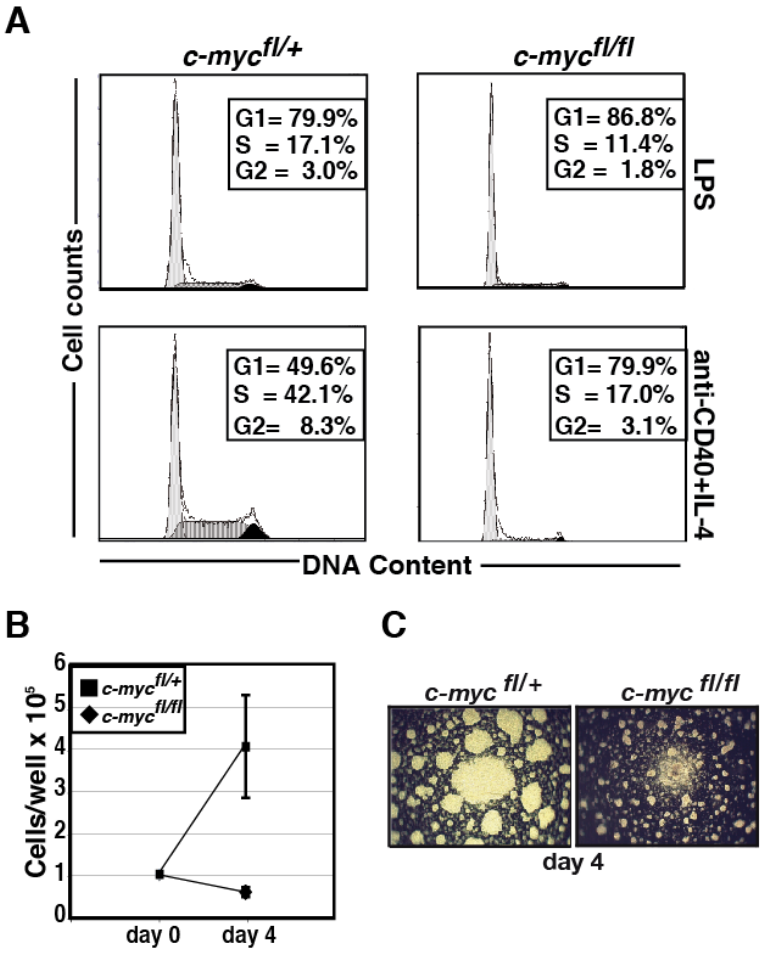


Figure R4. Deficient proliferation of c-Myc KO mature B cells. A) Propidium iodide (PI) staining of sorted B220⁺GFP⁺ splenocytes 4 days after *in vitro* anti-CD40 plus IL-4 stimulation. **B)** Cell counts from the cultures in (**A**), *n = 9*. **C)** B cell blasts formed after activation as in (**A**).

We crossed the *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$ mice with p27knockout mice (Fero *et al.*, 1996) to obtain double homozygous mice and control littermates. Cell cycle analysis in B cells from these mice, when activated *in vitro*, did not show increased proliferation rates, either when measured by PI analysis or in cell counts (Figure R5). p27 deficiency was thus unable to rescue the defective proliferation attributed to the lack of c-Myc.

4.5 c-Myc-deficient B lymphocytes generate ASC

The absence of plasmablasts with the B220l$^{o/-}$Synd-1^{+} surface phenotype in the c-Myc KO cultures indicated that these cells do not express typical surface markers, but

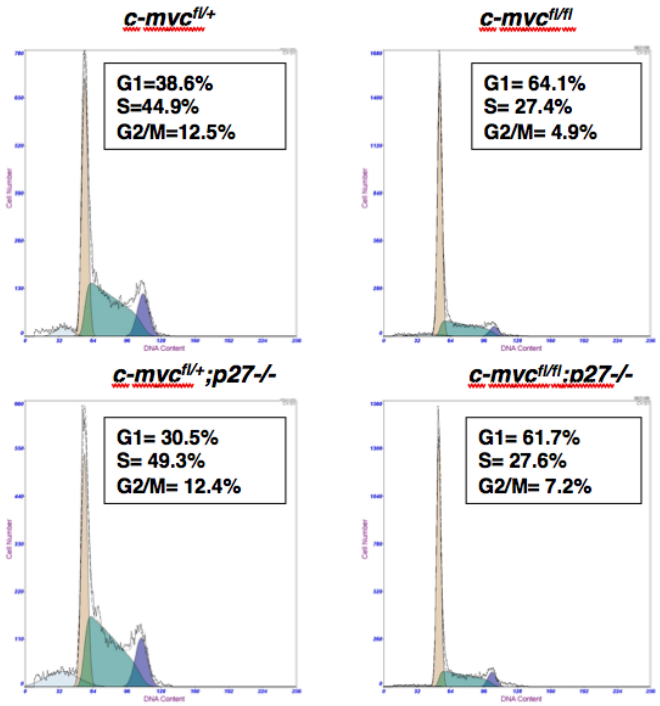


Figure R5. p27 deficiency does not rescue proliferation in c-Myc-deficient B cells. PI staining of sorted $B220^{+}GFP^{+}$ splenocytes 4 days after *in vitro* anti-CD40 plus IL-4 stimulation. Data representative of 4 independent experiments; *n = 8*.

we tested whether functional Ig-secreting cells could be generated in the absence of Myc.. To determine whether c-Myc-deficient B lymphocytes generated ASC, we used ELISA to measure secreted IgM in culture supernatants of cells activated with anti-CD40 plus IL-4 at day 4. We detected comparable concentrations of secreted IgM in supernatants of mutant and control cells (91.7 vs 75.8 μg/ml) (Figure R6A). An additional assay that measures ASC production *in vitro* is ELISpot, which detects the number of functional secreting cells generated from pre-activated B cells in non-proliferating conditions, by the appearance of spots where cells secrete a halo of IgM. In ELISpot, we found an average 6-fold decrease in the total number of ASC per well in cultures of c-Myc-deficient B lymphocytes compared to control cultures (697.9 ± 335.6 vs. 4396.3 ± 1006.2 ASC/well) (Figure R6B). We concluded that c-Myc-deficient B lymphocytes can generate some ASC *in vitro*.

To secrete similar amounts of IgM with a smaller number of ASC, mutant B lymphocytes must either secrete more IgM per cell and/or begin secretion earlier than control cells. To test these possibilities, we used ELISpot to measure the amount of secreted IgM produced at different time points; secretion kinetics was comparable for mutant and control ASC, and reached maximum at day three (Figure R7A). The timing of secretion was therefore not responsible for the amount of IgM observed in the cultures of c-Myc-deficient B lymphocytes.

We analyzed whether mutant ASC secreted more IgM per cell by measuring

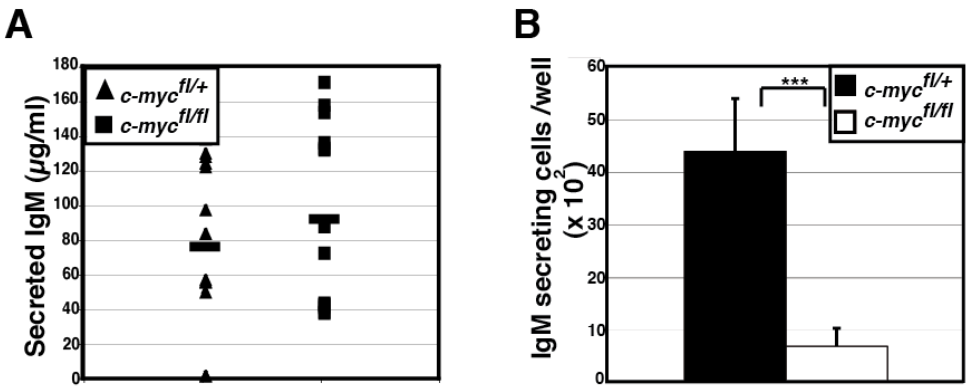


Figure R6. c-Myc-deficient B cells are able to secrete IgM *in vitro*. **A)** Secreted IgM in culture supernatants of activated B220⁺GFP⁺ cells on day 4 after anti-CD40 plus IL-4 stimulation, as measured by ELISA. *c-myc*$^{fl/+}$, $n = 14$; *c-myc*$^{fl/fl}$, $n = 15$, $p = 0.3$ (NS). **B)** Number of IgM ASC/well, quantified by ELISpot. $n = 9$, ***$p<0.001$.

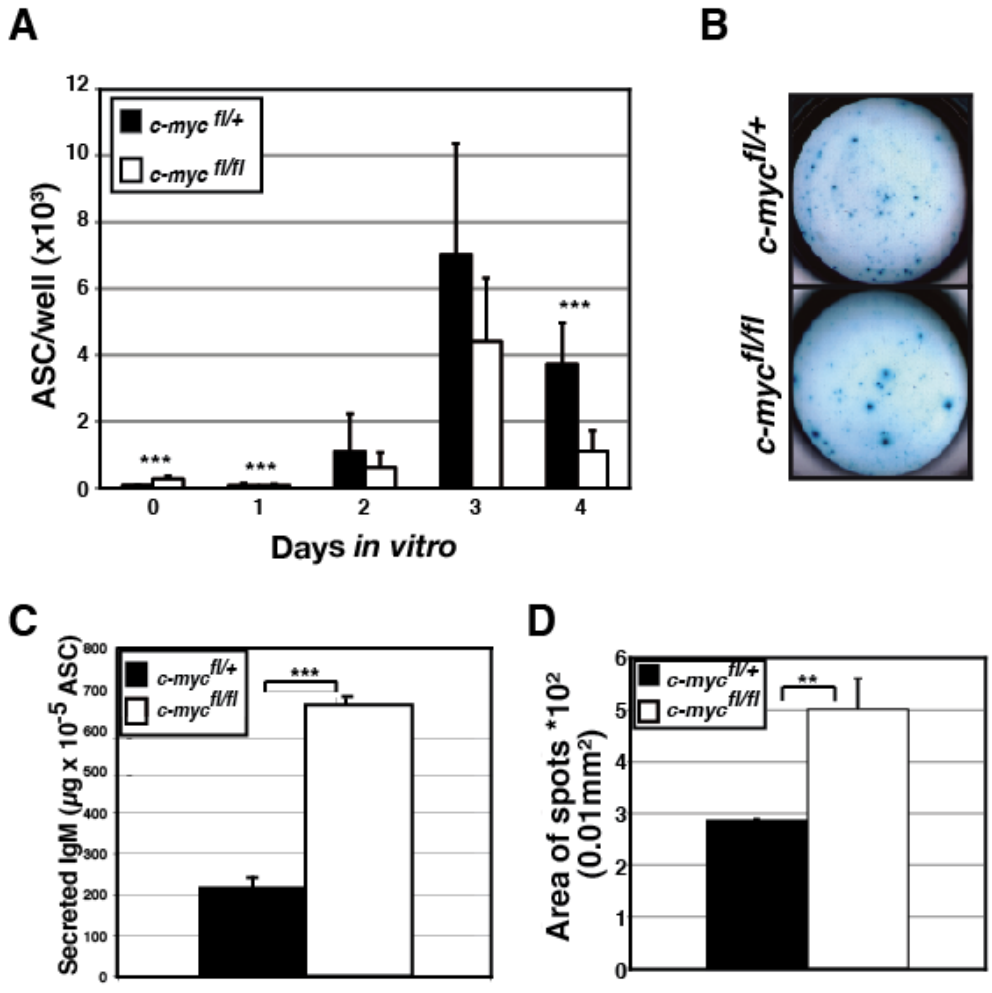


Figure R7. c-Myc-deficient B cells hypersecrete IgM. **A)** Kinetics of IgM ASC generation after anti-CD40 plus IL-4 stimulation of B220⁺GFP⁺ cells, analyzed by ELISpot at different times post-activation; $n = 6$. **B)** IgM ELISpots at day 4 post-activation. **C)** Quantification of IgM antibody secreted per ASC. **D)** Mean spot area at day 4. $n = 3$, **$p<0.01$.

the area of the spots generated in ELISpot assays. We observed a 1.5-fold increase in the spot area generated by c-Myc-deficient cells compared to control ASC (0.0502 ± 0.0058 vs. 0.0286 + 0.0004 mm^2) (Figure R7B, D). Cultures of c-Myc-deficient B lymphocytes showed an average of 6-fold fewer ASC than control cultures. The average of secreted IgM per ASC should be therefore 3-fold higher in mutant than in control cells (661.0 ± 55.9 vs 219.9 ± 19.7 μg/10^5ASC) (Figure R7C) when calculated from the combined ELISA and ELISpot results at day 4 post-activation.

To confirm these results using another technique, we immunoprecipitated secreted IgM from the culture supernatants of mutant and control cells and performed Western blots to detect soluble IgM. c-Myc-deficient B lymphocyte supernatants showed immunoprecipitated IgM amounts

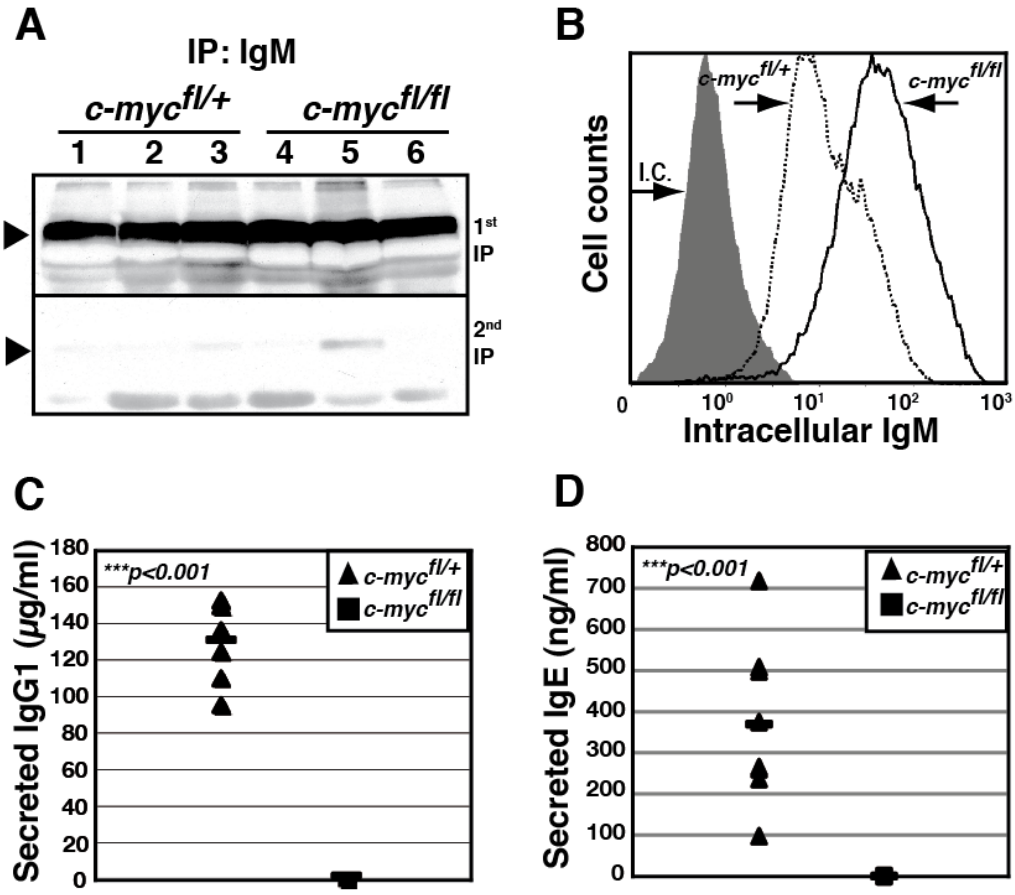


Figure R8. Hypersecretory phenotype of c-Myc-deficient B cells is restricted to the IgM isotype. **A)** IgM immunoblot of total secreted IgM immunoprecipitated with polyclonal anti-mouse IgM antibody from supernatant of anti-CD40 plus IL-4-activated $B220^+GFP^+$ B cells at day 3. A second immunoprecipitation was performed to ensure total precipitation of secreted IgM; blot representative of three independent experiments. **B)** Intracellular IgM staining of purified B lymphocytes activated as in (**A**). Representative data for three independent experiments. Mean peak values were 20.3 ± 0.8 (*c-myc*$^{fl/+}$) and 52.3 ± 8.8 (*c-myc*$^{fl/fl}$ mice), $^{**}p<0.01$. **C,D)** Amount of IgG1 or IgE in supernatants from cultures of B lymphocytes activated as in (**A**), quantified by ELISA; $n = 8$ for IgG1, $n = 7$ for IgE. $^{***}p<0.001$.

comparable to those of controls, despite the reduced ASC numbers in the mutant cell population. We performed a second IgM immunoprecipitation to assure all secreted IgM in supernatant had been precipitated, which confirmed total capture in the first phase (Figure R8A). Finally, intracellular staining showed more intracellular IgM in c-Myc-deficient B than in control cells (Figure R8B).

This hypersecretory effect was restricted to the IgM isotype, since the amounts of secreted IgG1 and IgE in culture supernatants of mutant cells were severely reduced *in vitro* (Figure R8C, D), consistent with the surface staining for IgG1 and IgE in cells activated with anti-CD40 plus IL-4 (not shown).

Based on these data, we concluded that c-Myc deficient B cells are able to generate ASC (although they do not stain for surface Synd-1) and that they secrete more IgM per cell than control cells. Finally, CSR is severely impaired in these cells.

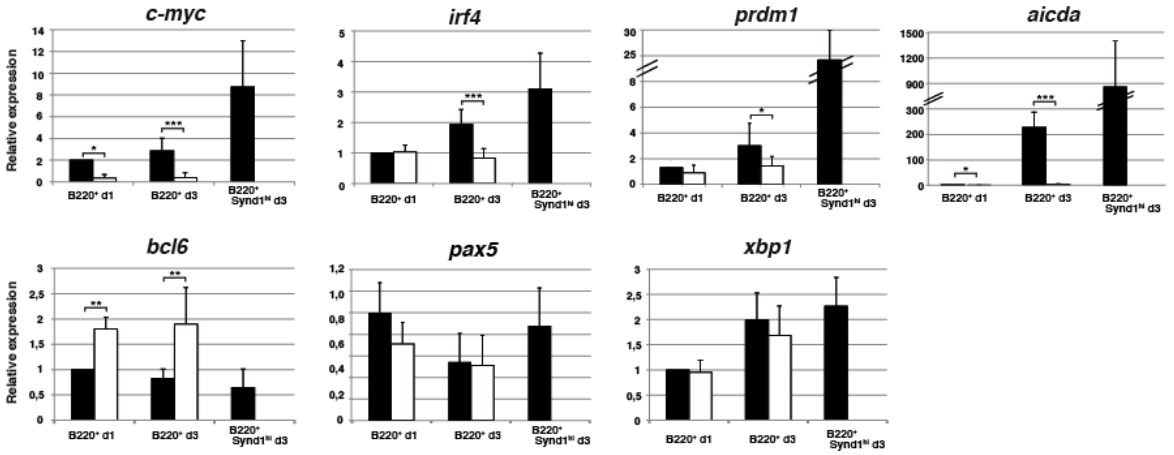
4.6 Gene expression of c-Myc-deficient ASC

Plasma cell generation is orchestrated by a tightly regulated cascade of transcription factors in which the program involved in terminal differentiation is mutually exclusive with the program that activates early B cell differentiation and activation. We examined whether ASC generation by c-Myc-deficient B cells was characterized by activation of the transcription program involved in terminal B lymphocyte differentiation, and studied the expression of several key transcription factors. We used qPCR to monitor gene expression kinetics of $B220^{+}Synd\text{-}1^{-}GFP^{+}$ and/or $B220^{lo}Synd\text{-}1^{+}GFP^{+}$ cells from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice after *in vitro* stimulation with anti-CD40 plus IL-4 at days 1 and day 3 post-activation. $B220^{lo}Synd\text{-}1^{+}GFP^{+}$ cells are not yet generated at day 1, either in *myc*$^{fl/fl}$ or *c-myc*$^{fl/+}$ cultures. This population was generated and sorted in control samples at day 3, and c-Myc-deficient B cells did not generate $Synd\text{-}1^{+}$ cells.

The hallmark of terminal B cell differentiation is the activation of the transcriptional repressor Blimp-1, which is in part inhibited by the action of Bcl-6. We observed a three-fold increase in *bcl-6* levels from early stages activation and a three-fold reduction in *pdrm1* (Blimp1) gene expression in $B220^{+}Synd\text{-}1^{-}GFP^{+}$ c-Myc-deficient cells compared to controls at day 3 (Figure R9). We found no significant changes in the levels of *pax5* and the transcription factor *xbp-1*, which participates in Ig secretion and the UPR pathway (Figure R10). The expression of Xbp1 is compatible with the Ig secretion observed in the c-Myc-deficient ASC. Expression of the *ire-1* gene, responsible for *xbp-1* splicing and activation, was normal in mutant B lymphocytes.

Irf4 gene expression, which is upregulated throughout plasma cell differentiation, controls AID expression and

A



B

Function	Genes	Change
Activation	*stat6, ciita*	NC
UPR and secretion	*ern1, perk, atf6, igj, hspa5, edem1, rbpn1 eif2ak, dnajb9, cope*	NC
Proliferation	*l-myc, p21*	Decreased
Apoptosis	*bcl-2*	NC
Terminal differentiation	*bach-2, oct2, obf1,*	NC
Other genes	*emb, flt3*	Decreased

Figure R9. Gene expression of the main transcription factors involved in terminal B cell differentiation in *in vitro*-activated c-Myc-deficient B cells. A) Sorted B220^{+}GFP^{+} splenocytes from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice were activated with anti-CD40 plus IL-4 and re-sorted at days 1 and 3 to isolate B220^{+}GFP^{+} and/or B220^{lo}Synd-1^{+}GFP^{+} cells. Mutant cells did not generate B220^{lo}Synd1^{+}GFP^{+} cells. cDNA was prepared from the RNA isolated and qPCR performed. Control (black) and mutant cells (white). Results were normalized to β-actin, $n = 6$. **B)** Summary of the gene expression changes of some other genes grouped by functional criteria.

thus SH and CSR, and also activates Blimp-1 expression in normal cells. *Irf4* remained at basal levels in mutant cells even at day 3 of activation, four times lower than that of B220^{+}Synd-1^{+} WT cells on day 3 (Figure R9). This finding would explain the lack of Synd1^{+} cells (as described for the Irf4-deficient mouse model (Sciammas *et al.*, 2006) and the extremely low rates of Ig switched cells. In another similarity to the Irf4 KO model, we found a very remarkable downregulation of the *aicda* gene in the c-Myc KO cells as compared to control cells at day 3 (80-fold reduction) and even at early activation phases (day 1) in B220^{+} cells (six-fold reduction relative to controls) (Figure R9). The lack of appropriate Irf4 activation could in great part explain the phenotype observed.

We analyzed the expression of some genes implicated in the UPR pathway (chaperones, ERAD genes, genes with secretory

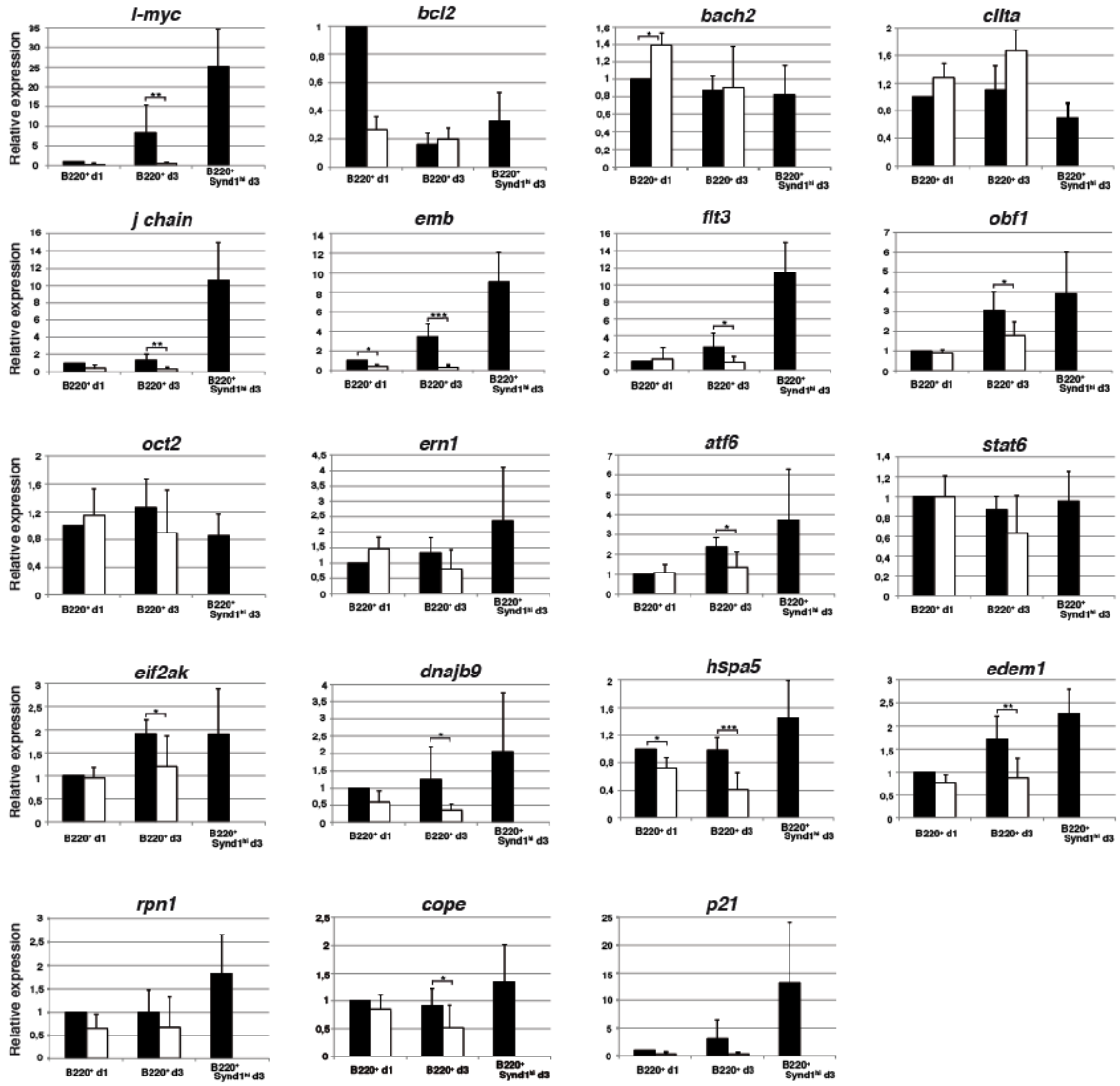


Figure R10. Gene expression profile of c-Myc-deficient B cells. Expression levels of some genes implicated in the UPR pathway, secretion and plasma cell differentiation. Analysis as in Fig. R9; *n = 6.*

function, etc.), including *bip*, *edem1*, *perk*, *cope*, *and hspa5*. We found no clear differences in any of these genes that would account for the hypersecretory phenotype (Figure R10). Moreover, we found no significant differences in the expression of genes from activation pathways such as STAT6 or NF-κB (Figure R10).

In addition, we detected no alteration in the ratio of secreted IgM heavy chain versus membrane heavy chain, as analyzed by RT-PCR (Fig R11); transcription of these Ig forms would thus not be responsible for increased IgM secretion. Therefore we are far unable to identify the mechanism underlying this phenotype.

We conclude from these results that B lymphocytes that lack c-Myc are able to

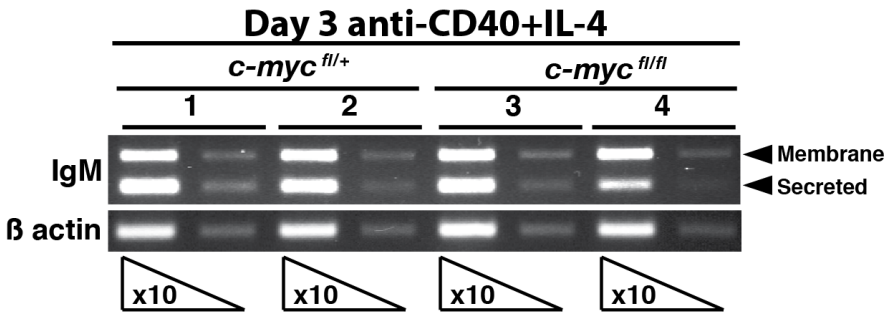


Figure R11. Secreted and membrane IgM forms are transcribed at normal levels in cMyc-deficient B cells. Membrane and secreted IgM forms (μS and μM transcripts) were amplified by RT-PCR from sorted B220^{+}GFP^{+} c-Myc-deficient or control cells activated with anti-CD40 plus IL-4 for 4 days. Representative figure for two independent experiments.

initiate the terminal B cell differentiation transcription program by expressing Xbp1 and low levels of Blimp-1, but fail to reach the final differentiation steps (such as Synd-1 expression and CSR) due to lack of Irf4 expression and high Bcl-6 levels.

4.7 Impaired immune response in *c-myc*$^{fl/fl}$ mice

c-myc gene deletion in spleen B lymphocytes permits study of the immune response in *c-myc*$^{fl/fl}$ mice. GFP expression allowed us to trace c-Myc-deficient cells (GFP^{+}) and distinguish them from non-deleted c-Myc WT cells (GFP^{-}). We immunized mice with TD antigens (TNP-KLH) and TI antigens (LPS) to study the immune response in the context of c-Myc deficiency. Unimmunized *c-myc*$^{fl/fl}$ mice showed four-fold fewer B220^{+} lymphocytes in spleen than control mice (de Alboran *et al.*, 2001) and figure R15B.

To study plasma cell generation in response to T-dependent and -independent antigens, the splenocytes of TNP-KLH or LPS immunized *c-myc*$^{fl/fl}$ and control mice mice were stained and analyzed at different times by flow cytometry. As observed *in vitro* (Figure 1a, c), *c-myc*$^{fl/fl}$ mice did not generate c-Myc-deficient B220^{lo}Synd-1^{+}GFP^{+} plasma cells after LPS challenge at day 3 post-immunization (0.3% vs 1.8%); results were similar when mice were analyzed at day 7. This reduction in the PC population was also very marked when mice were analyzed 12 days after TNP-KLH administration (0.4% vs 1.2%). As a control, GFP^{-} (non-deleted, WT) cells from the same *c-myc*$^{fl/fl}$ mouse generated B220^{lo}Synd-1^{+} plasma cells in response to both Tdependent and -independent antigens (Figure R12). Similar to spleen, long-term plasma cells were nearly absent in the BM of *c-myc*$^{fl/fl}$ mice after TNP-KLH immunization (Figure R13A, B).

To test the ability of c-Myc-deficient mice to generate antigen-specific antibodies in response to *in vivo* immunization, we used a TNP-specific ELISA to measure all the isotypes generated by this stimulation (IgM, IgG1, IgE and IgG2a). As deletion is not

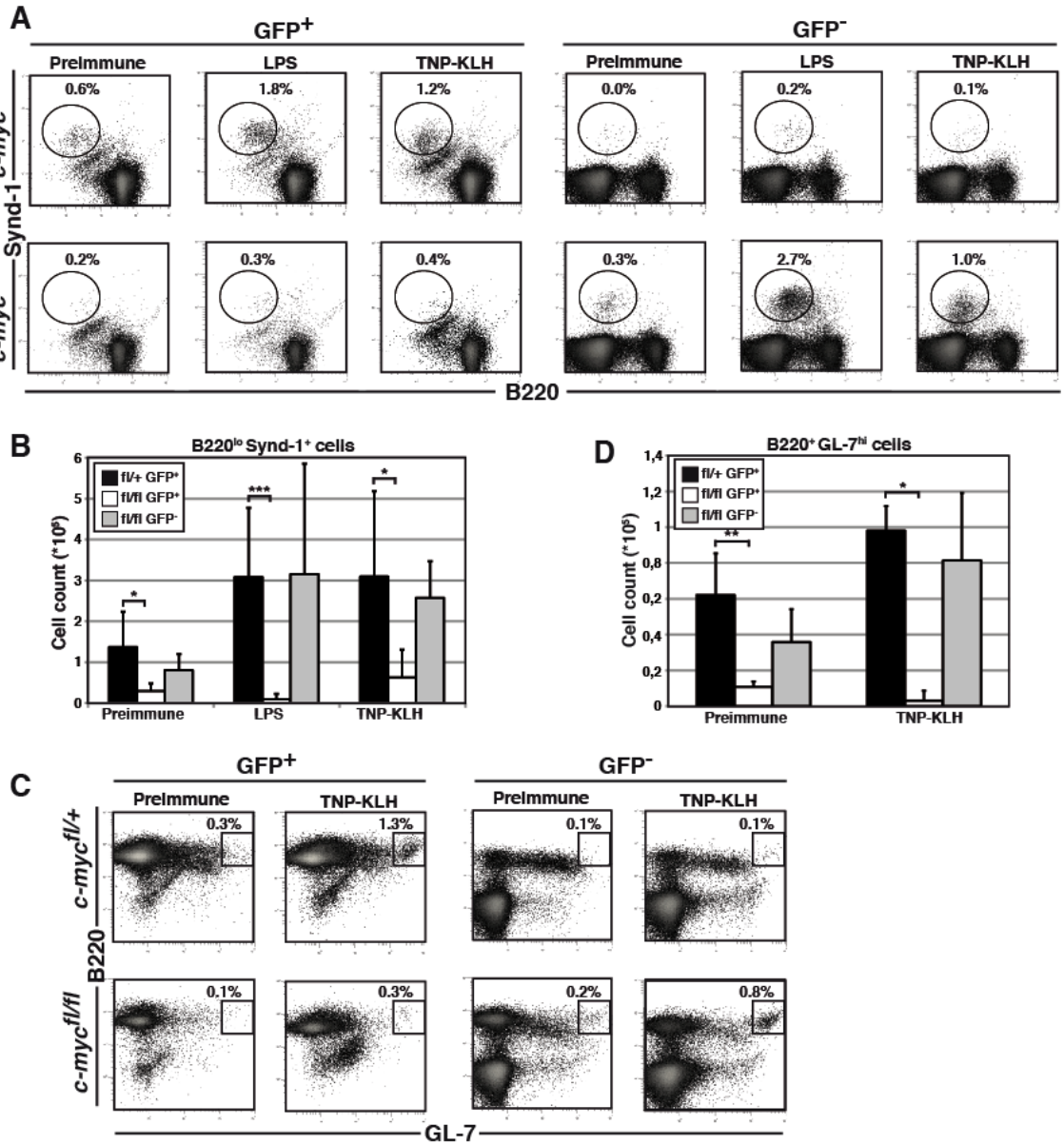


Figure R12. c-Myc-deficient B cell do not generate B220^{lo}Synd-1^{+} cells or GC B cells after immunization. A) Flow cytometry analysis of plasma cells from spleens of *c-myc*$^{fl/fl}$ and control mice immunized with LPS or TNP-KLH. Experiment representative of three independent experiments**. B)** Absolute numbers of B220^{lo}Synd-1^{+} cells in (**A**). Preimmune: *c-myc*$^{fl/+}$(n = 6), *cmyc*$^{fl/fl}$ (n = 4), *p<0.05; LPS: *c-myc*$^{fl/+}$(n = 6), *c-myc*$^{fl/fl}$ (n = 7), ***p<0.001; TNP-KLH: (n = 4), *p<0.05. **C)** Germinal center B cells were identified by GL-7 high expression in B220^{+} cells from spleen by flow cytometry (n mice). **D)** Absolute numbers of GC B cells in (**C**).

complete in the *cmyc*^{flox};*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$ mice, we detected a few wild type cells even in homozygous mice. Nevertheless, TNP-specific IgM was slightly increased in *c-myc*$^{fl/fl}$ mouse serum compared to control mice after a second antigen challenge (Figure R13C). Consistent with our *in vitro* results, other isotypes (IgG1, IgE, IgG2a) were decreased in mutant mice (Figure R13C), although the differences observed

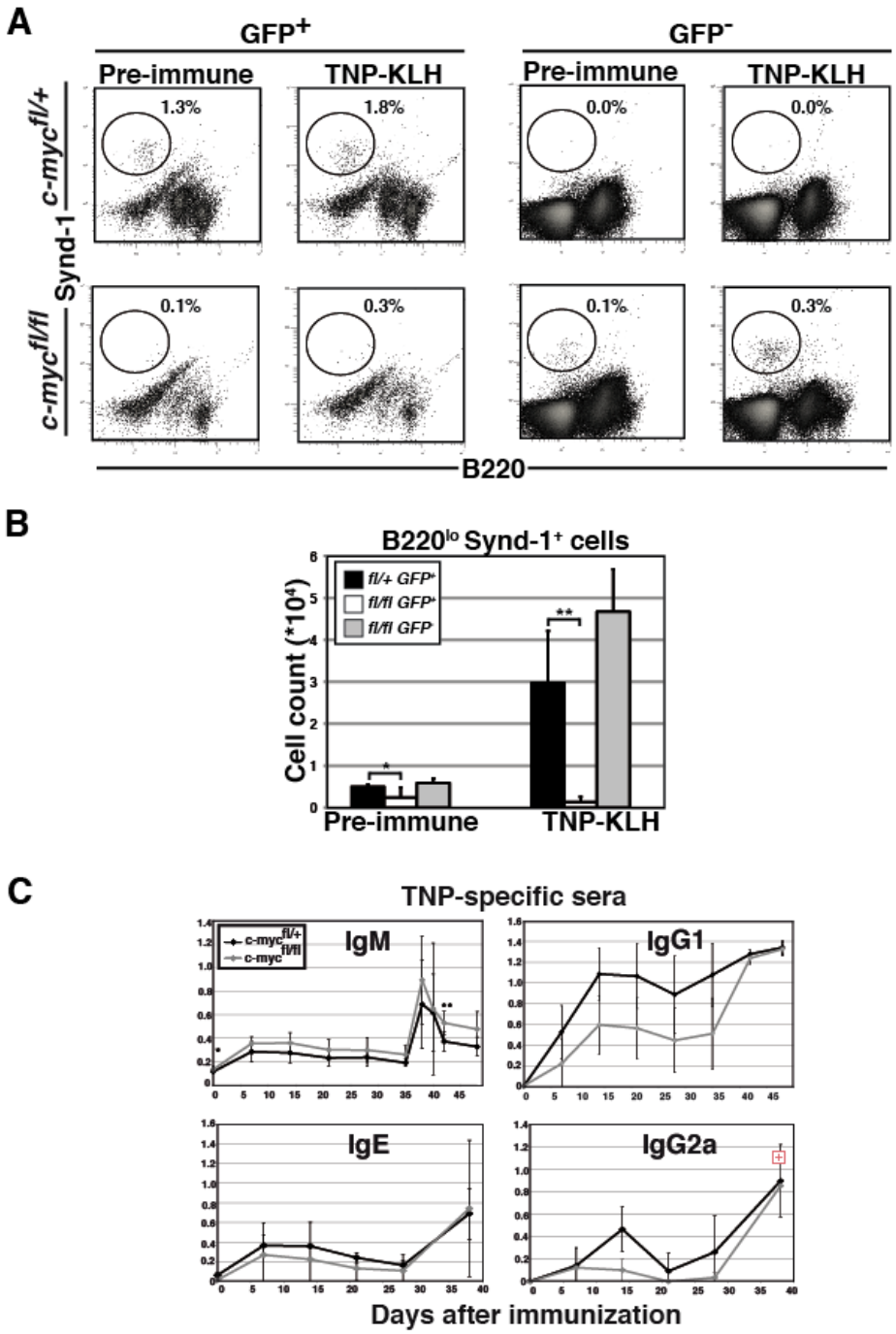


Figure R13. **c-Myc-deficient mice do not generate long-term plasma cells in BM after TD immunization. A)** Lack of long-term plasma cell population in BM of TNP-KLH-immunized *cmyc*$^{fl/fl}$ mice. Single cell suspensions from BM were prepared and stained for the surface markers B220 and Synd-1. **B)** Absolute numbers of B220^{lo}Synd-1^{+} cells in **A.** $n = 3$, $^{*}p < 0.05$ $^{**}p < 0.01$. **C)** ELISA for detection of TNP-specific Ig levels of distinct isotypes in sera of TNP-KLH-immunized mice at different times post-immunization. For IgM and IgE, $n = 9$; for IgG1, $n = 6$; for IgG2a, $n = 4$.

were not significant in any isotype analyzed. This could be explained by the leakiness of the deletion in this mouse model; a large part of the antibody measured in KO mouse serum might be derived from non-deleted WT cells. This would mask the

phenotype, as Ig from deleted and non-deleted cells cannot be distinguished by this method. Based on these data, we cannot rule out that the secretion phenotype observed *in vitro* is found *in vivo* as well.

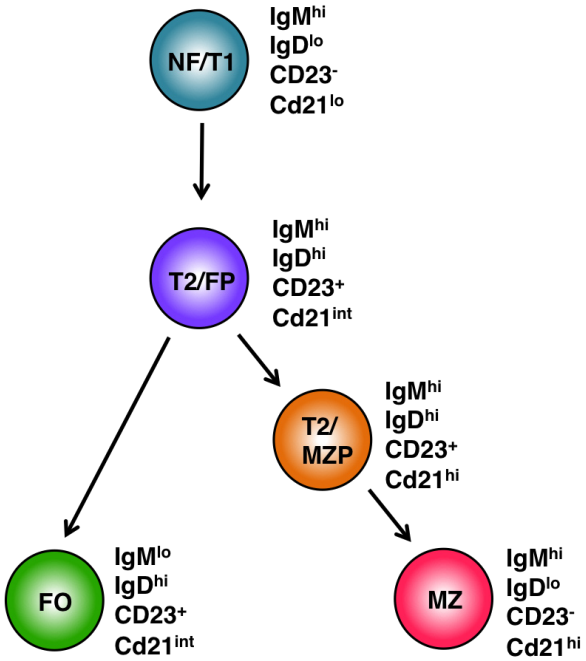


Figure R14. Surface markers to classify B lymphocyte populations in the murine spleen. NF (newly formed)/T1(transitional stage 1), T2-FP (transitional stage 2-follicular precursors), FO (follicular B cells), T2-MZP (transitional stage 2-MZ B cell precursors), MZ (marginal zone B cells). (Based on Pillai *et al.*, 2005)

From the results of the immunization experiments, we concluded that c-Myc is necessary for the generation of long- and short-term plasma cells during a T-dependent or -independent immune response, both in spleen and in BM.

The distribution of spleen B cell populations can be characterized by differential surface expression of CD21, CD23, IgM and IgD (Figure R14). These stainings allow us to separate the differentiation stages: the precursor transitional B cells (T1 and T2), which further differentiate into marginal zone B cells (IgM^{hi}IgD^{lo}CD23^{-}Cd21^{hi}) or follicular cells (IgM^{io}IgD^{hi}CD23^{+}Cd21^{int}).

The reduction in the total number of B lymphocytes observed in the *c-myc*$^{fl/fl}$ mice affected both marginal zone (MZ, CD23^{lo}CD21^{hi}) and follicular B cells (FO, CD23^{hi}CD21^{lo}) (Figure R15). When these populations were analyzed by flow cytometry in non-immunized, in TNP-KLH and in LPS-immunized mice, the MZ/follicular cell ratio was decreased two-fold in mutant mice compared to controls (Figure R15). Nonetheless, hematoxylin/eosin-stained sections showed no apparent alterations in the spleen structure of *c-myc*$^{fl/fl}$ mice (Figure R16C).

To determine the distribution of follicular

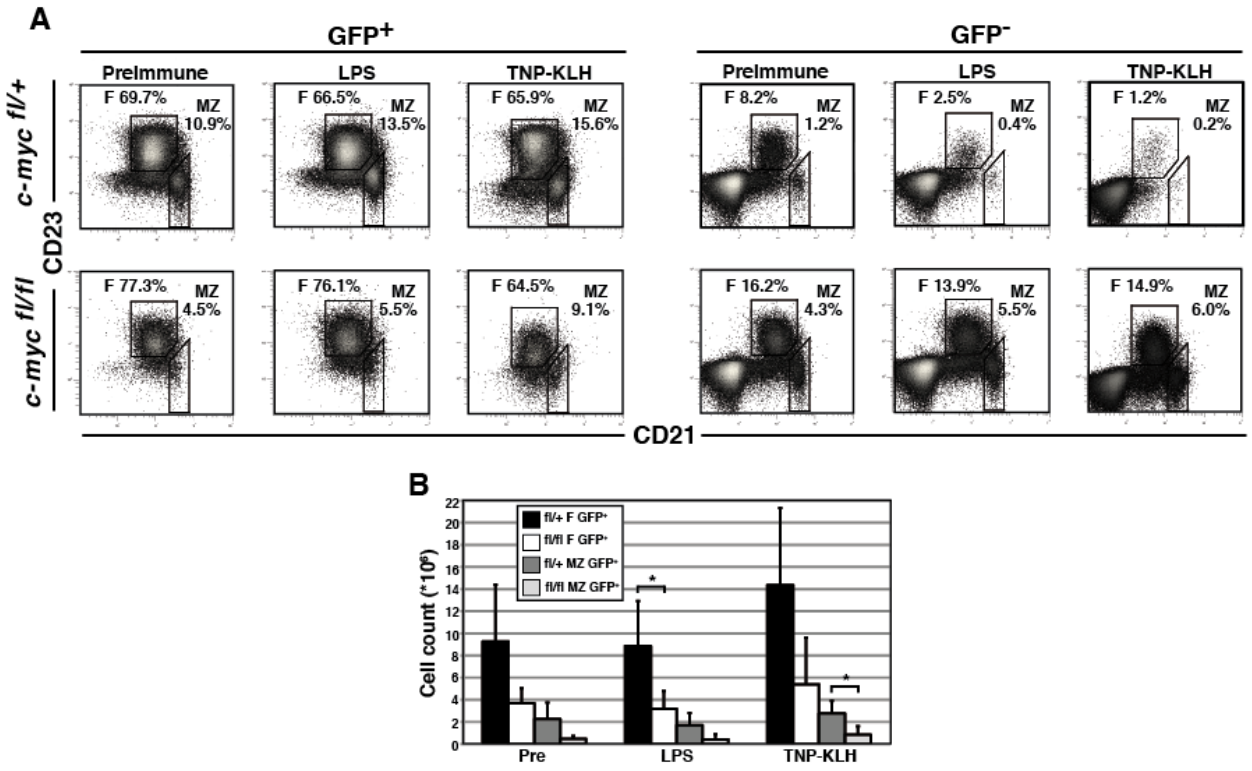


Figure R15. Follicular and MZ B cell populations in c-Myc-deficient mice. **A)** Spleens of *c-myc*$^{fl/fl}$ or control mice were analyzed in preimmune conditiions or after LPS or TNP-KLH immunization. Cells were stained for CD23 and CD21 by flow cytometry to study the MZ and FO populations. **B)** Absolute cell numbers in whole spleen for populations in (**A**). *n = 6*.

and marginal zone populations in the spleen, we performed immunofluorescence in TNP-KLH-immunized *c-myc*$^{fl/fl}$ and control *c-myc*$^{fl/+}$ mice. Consistent with the flow cytometry results, staining with MOMA-1, which binds metallophilic MZ macrophages delimiting the inner ring of the marginal zone, indicated almost complete absence of c-Myc-deficient B lymphocytes (GFP$^+$) in the *c-myc*$^{fl/fl}$ mouse MZ (Figure R16A). Similarly, anti-Cre antibody staining showed the absence of Cre-expressing cells in the MZ of these mice (Figure R17B).

Based on these data, we conclude that total numbers of follicular and MZ B cells are reduced in spleens of c-Myc deficient mice, which also show a severe reduction in the MZ/F ratio before and after antigen stimulation.

4.8 c-Myc-deficient B cells do not generate GC

The generation of GC is one of the hallmarks of a T cell-dependent immune response. These structures contain rapidly proliferating B cells that differentiate into plasma and/or memory B cells, in which SH and CSR events take place. To test whether *c-myc*$^{fl/fl}$ mice generated GC after immunization with TNP-KLH, we analyzed

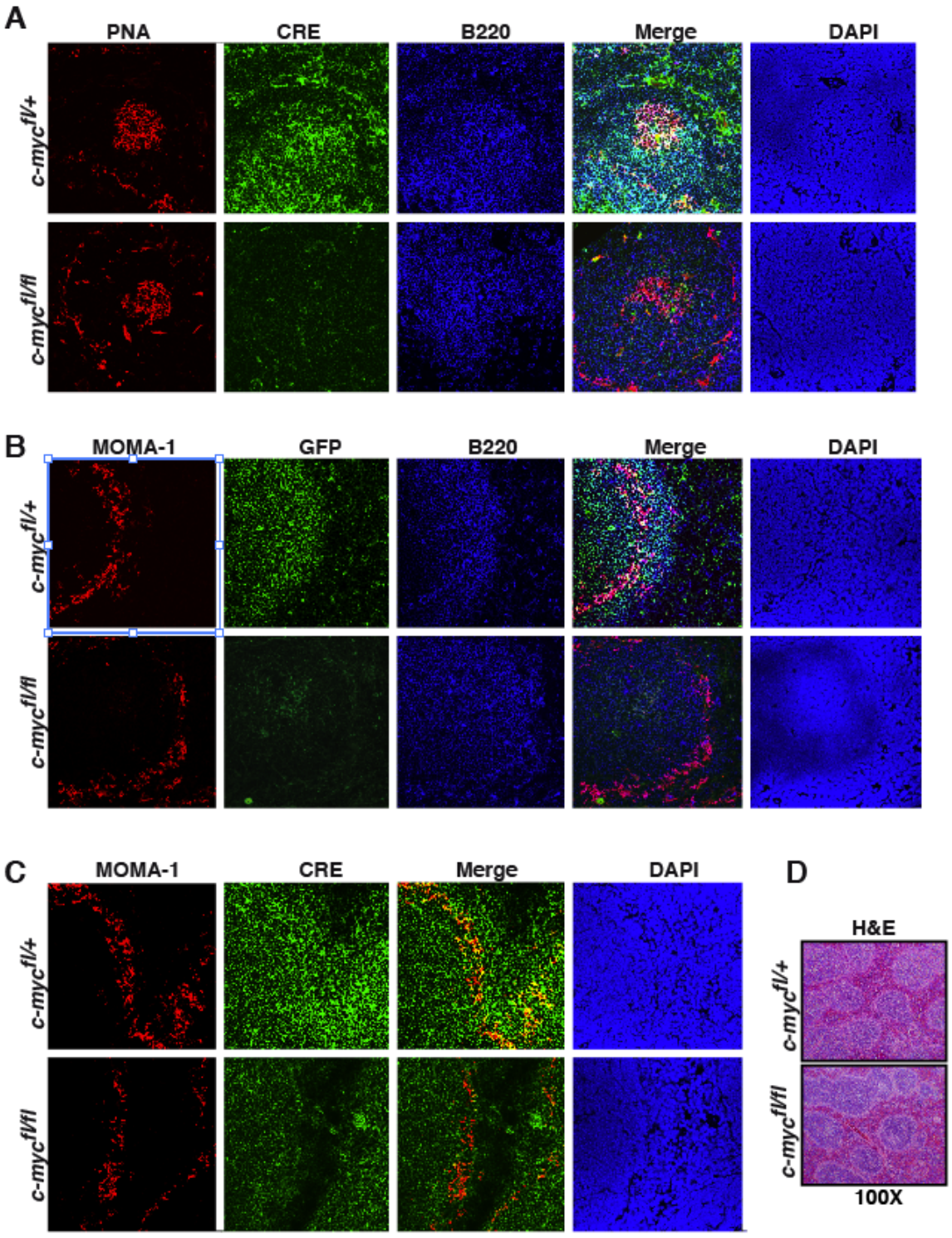


Figure R16. Notable reduction in MZ B cells in c-Myc-deficient mice in MOMA-1 immunofluorescence. Spleen cryosections from TNP-KLH-immunized *c-myc*$^{fl/fl}$ or control mice were stained with anti-MOMA-1, -B220 and -GFP antibodies (**A**) or with anti-MOMA-1 and -Cre (**B**) to identify MZ B cells. DAPI was used for cell nuclei co-staining. **C)** Hematoxylin/eosin (H&E) staining of *c-myc*$^{fl/fl}$ or control mice; *n = 5.*

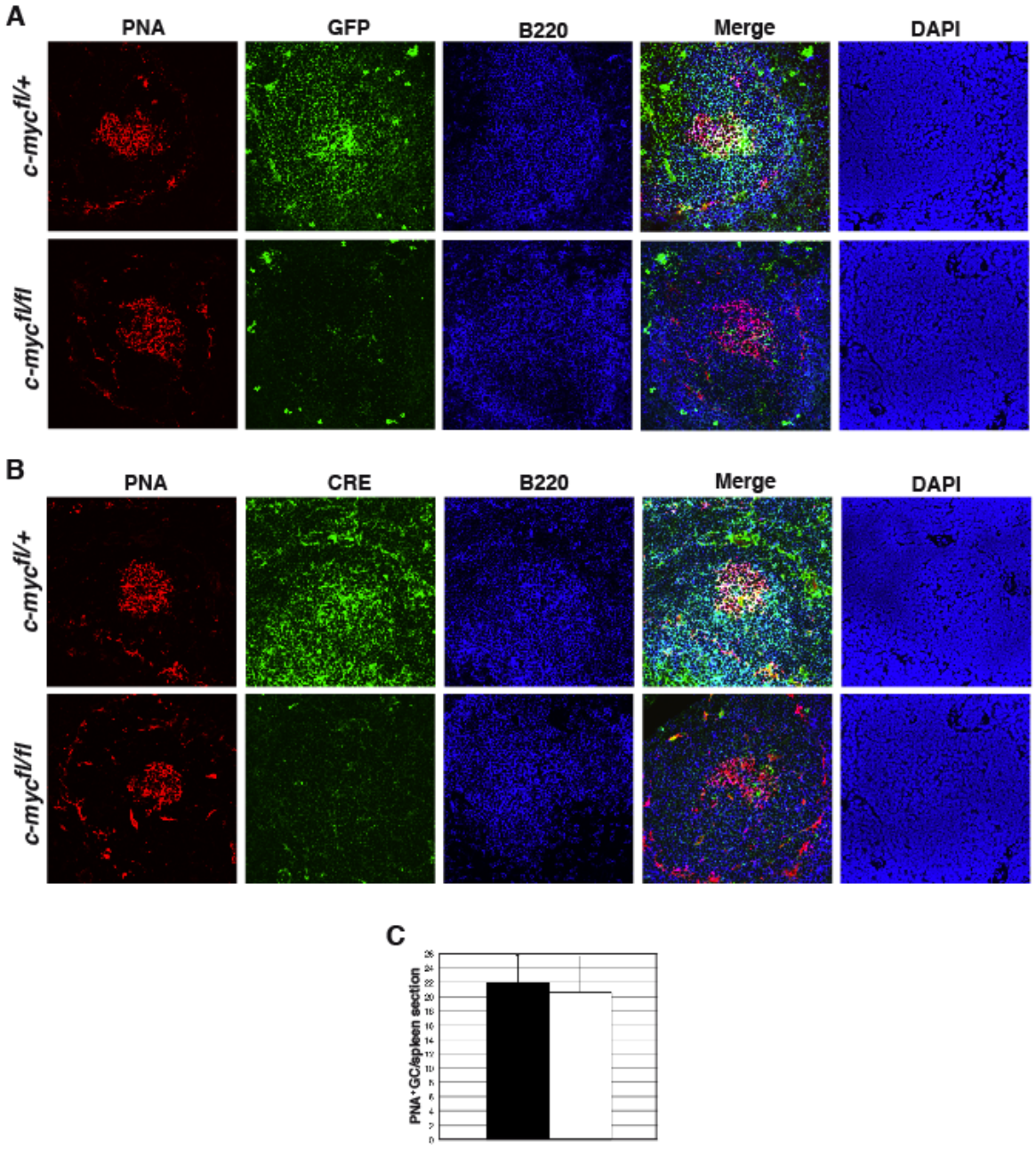


Figure R17. c-Myc-deficient B cells do not generate GC after TD stimulation. Spleen cryosections of TNP-KLH-immunized *c-myc*$^{fl/fl}$ or control mice were stained with PNA lectin, anti-B220 and -GFP antibodies (**A**) or with PNA, anti-B220 and -Cre (**B**) to identify GC B cells. DAPI was used to co-stain cell nuclei. **C)** Number of GC per spleen section; 4-5 sections were counted per mouse; *n = 5*. White bar, *c-myc*$^{fl/fl}$ mice; black bar, controls.

surface expression of GL-7 by flow cytometry. The GC B cells can be identified by high expression of GL-7 on surface (Han *et al.*, 1997). Spleen cells stained with GL-7 showed a marked decrease in the population of B220^{+}GL-7^{hi}GFP^{+} germinal center B cells (1.3% vs 0.3%) in immunized *c-myc*$^{fl/fl}$ mice (Figure R12C and D). In contrast, non-deleted wt B cells (B220^{+}GFP^{-}) from the same mouse expressed normal surface

levels of GL-7 (Figure R12 C and D). GC B cells can be also identified by the binding to peanut agglutinin (PNA) (Molin *et al.*, 1986). We performed immunofluorescence with biotinylated PNA lectin to sduty the generation of GC in spleen sections from c-Myc deficient or control mice. *c-myc*$^{fl/fl}$ mice generated GC after immunization (Figure R17C); however, these GC contained mostly GFP$^-$ or Cre$^-$ cells (Figure R17 A and B). We concluded that c-Myc-deficient GFP$^+$ cells were not responsible for GC generation by wt B cells that escaped Cre-mediated *c-myc* deletion (GFP$^-$) in homozygous mice.

4.9 c-Myc is necessary for the maintenance of memory B lymphocytes

As a result of T-dependent B cell stimulation, short-term and long-term PC are generated. Memory B cells also arise; they have a very long half-life and the capacity to generate PC very quickly after a second exposure to antigen. It remains to be

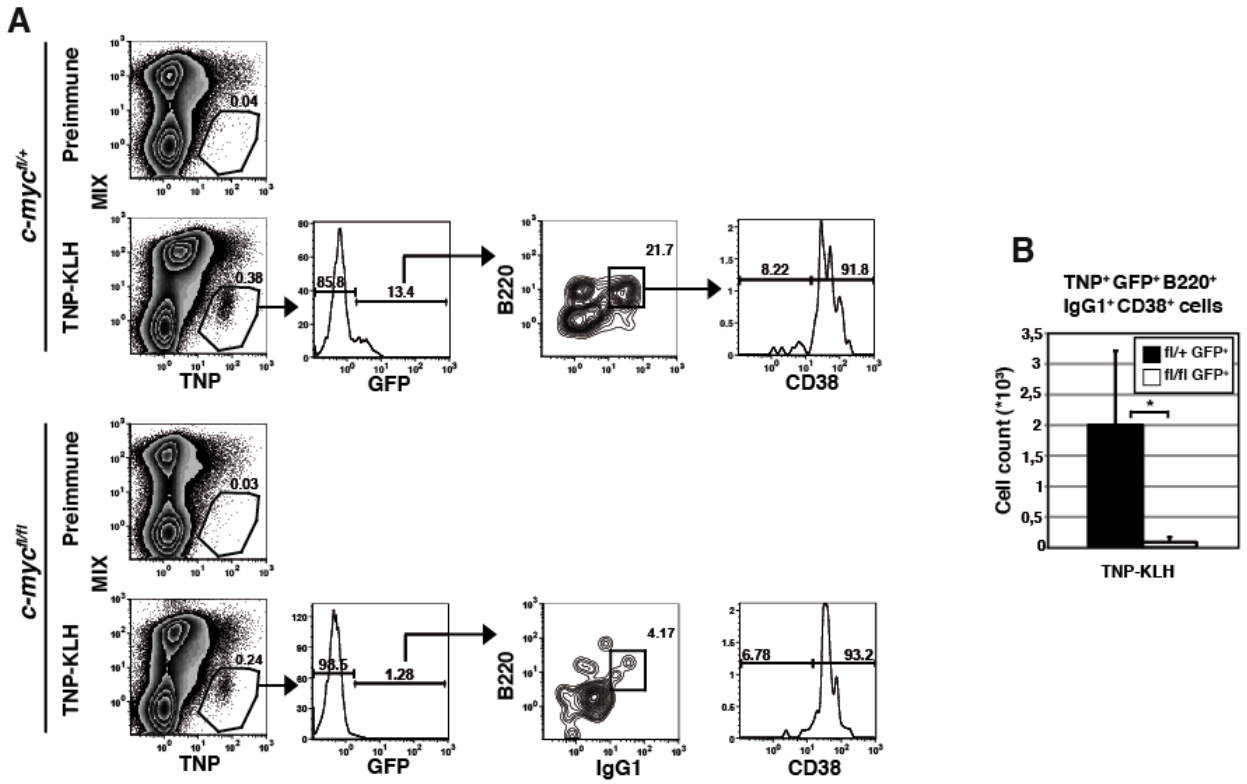


Figure R18. Lack of antigen-specific memory B cells in c-Myc-deficient mice. A) TNP-specific memory B cells were identified by flow cytometry in immunized mice, 12 days after a second TNP-KLH immunization, by excluding IgM$^+$, IgD$^+$, Gr-1$^+$, F4/80$^+$ cells (MIX negative) and staining for TNP-BSA (antigen-specific) and the indicated additional markers. B) Absolute numbers of memory B cells (Mix$^-$GFP$^+$B220$^+$IgG1$^+$TNP$^+$) from (A) (n = 4), *p<0,05.

in these mice, and that this protein was necessary for GC formation. These structures were thus probably generated

elucidated whether differentiation to PC and memory B cells from mature B cells follow the

same pathway. To determine whether c-Myc is necessary for memory B cell generation or maintenance, we analyzed the memory compartment in *c-myc*$^{fl/fl}$ mice immunized with TNP-KLH and challenged with the same TNP-KLH dose 28 days later. Flow cytometry analysis showed a severely reduced percentage of B220^{+}IgG1^{+}CD38+TNP^{+}GFP^{+} cells, gated into IgM, IgD, Mac-1 and F4/80 negative (13.8% vs 11.3%) in the spleen of immunized *c-myc*$^{fl/fl}$ compared to control mice, corresponding to antigen-specific memory B cell that have switched to IgG1 (Figure R18 A). This reduction correlates with absolute numbers of TNP-specific memory B cells in whole spleen (Figure R18 B). These data indicate that c-Myc is necessary for the generation and/or maintenance of memory B cells.

4.10 Lentiviral infection to rescue plasma cell differentiation in c-Myc-deficient B cells

Comparison of Blimp-1 downregulation in c-Myc-deficient and control B cells after anti-CD40 plus IL-4 activation suggested that this expression profile was responsible for the

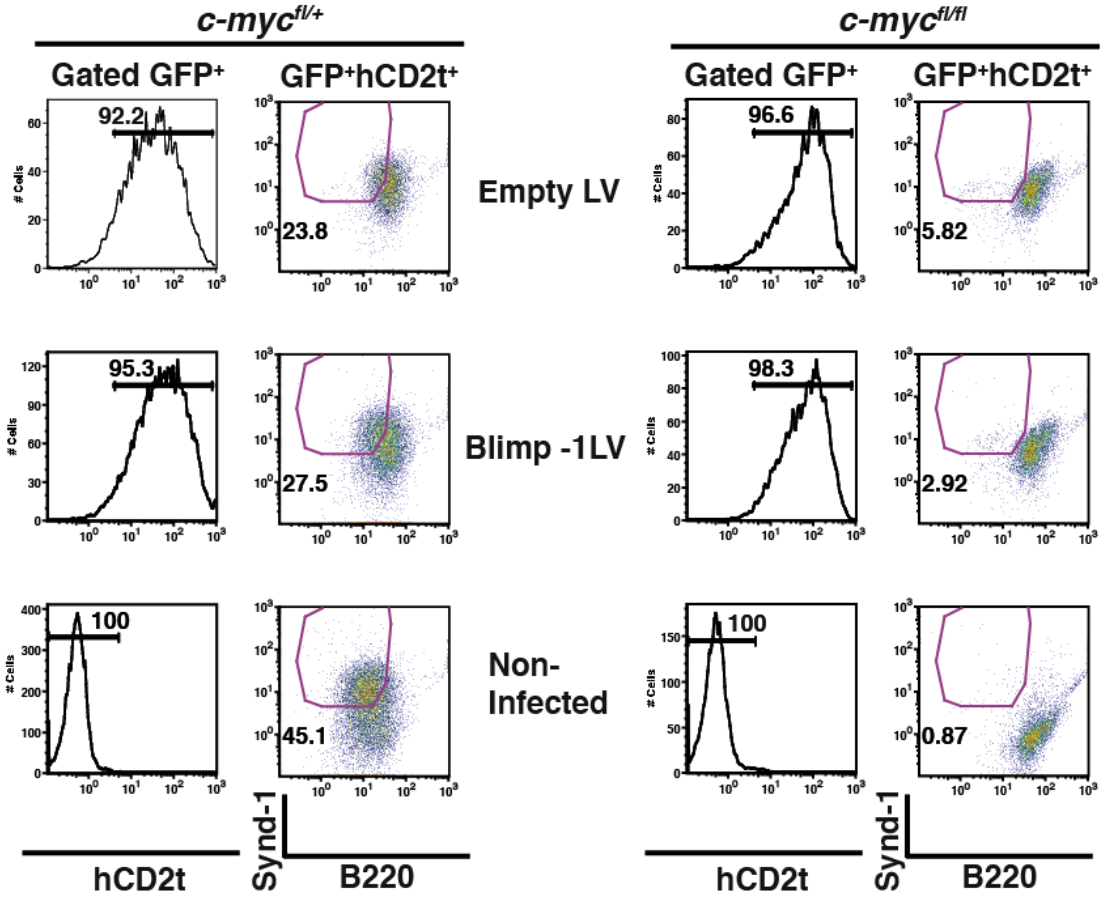


Figure R19. Transduction of c-Myc-deficient B cells with Blimp-1-expressing lentivirus. Sorted B220^{+}GFP^{+} cells from from *c-myc*$^{fl/fl}$ or control mice were infected with Blimp1-expressing, mock LV vectors or not infected (control), followed by activation with anti-CD40 plus IL-4. Cells were stained on day 3.5 post-activation for the indicated markers. Experiment representative of two independen texperiments with three mice (*n = 6*).

PC differentiation blockade. We conjectured that normalization of the expression of these transcription factor involved in plasma cell differentiation might rescue the generation of B220^{lo}Synd-1^{+} cells *in vitro*. We chose a lentiviral vector to overexpress the Blimp-1 gene (*prdm1*); a retroviral vector was not suitable for infection of c-Myc-deficient B lymphocytes, as transduction specificity of retroviruses is restricted to proliferating cells. We used the EF.CMV.RFP vector and specifically the CMV promoter in this lentivirus to overexpress the transgenes, as CMV yields higher expression levels in primary B cells than the EF2alpha promoter (Yu *et al.*, 2003). We replaced the red fluorescent protein (RFP) sequence, which was toxic to our primary B cells, with an IRES, followed by truncated human CD2 (hCD2t). The *prdm1* coding sequence (Chang *et al.*, 2000) was then cloned before the IRES, under the control of the CMV promoter. The transduced B cells were tracked by the simultaneous surface expression of hC2Dt (identified by flow cytometry analysis) and the transgene *prdm1*, mediated by the bicistronic mRNA generated by the IRES sequence.

B220^{+}GFP^{+} cells from *c-myc*$^{fl/fl}$ or control mice were sorted and infected with EF.CMV.Blimp-1-IRES-hCD2t or with a control empty vector, EF.CMV.IRES-hCD2t, at a multiplicity of infection (MOI) of 10 viral particles per cell. Cells were activated with anti-CD40 plus IL-4 for 3.5 days, to allow expression of the transgene and differentiation to plasmablasts. B cells were then analyzed by flow cytometry for hCD2t surface expression and B220/Synd-1 expression. Blimp-1 transduction was unable to rescue the B220^{lo}Synd-1^{+} population (Figure R19). The flow cytometry profile was similar to that of mock-infected cells, although infection slightly altered the brightness of some populations.

From these experiments, we concluded that Blimp-1 overexpression is not sufficient to normalize ASC differentiation of c-Myc-deficient B cells *in vitro*.

5. Discussion

Terminal B cell differentiation is a key process for the immunity of an organism. It provides the immune system with antibody-secreting cells, some of which lasting for the whole life of the organism, which mediate humoral responses that defend against many types of antigens and pathogens; it also generates memory immunity to these antigens.

In addition to *pax5* downregulation, antibody-secreting cell differentiation requires two main events to proceed, Bcl-6 inhibition, which allows Blimp-1 upregulation, and Irf-4 expression to activate Blimp-1, among other targets (Klein *et al.*, 2006; Sciammas *et al.*, 2006). The initiation of two of these events is still unknown, since the factors that inhibit *pax5* expression and activate *irf4* at this level have not been described.

There are many other questions that need to be addressed regarding terminal B cell differentiation, including whether short- and long-lived plasma cells generation follow the same pathway or arise from different populations, and which factors determine the balance between them. Some studies support the idea that both populations are derived from the same precursor, whereas others argue the hypothesis of two differentiated pathways. The very first events that trigger plasma cell initiation, that is, which factors drive the exit from the B cell activation pathway to become ASC, are still undefined.

It is also not known whether the cessation of proliferation, typical of terminally differentiated cells, which occurs in terminal B cell differentiation and is caused by direct inhibition of *c-myc* by Blimp-1, has an additional role in this process. In an *in vitro* study of the BCL1 mature B lymphoma cell line, the authors show that *c-myc* downregulation is necessary but not sufficient to induce terminal B lymphocyte differentiation (Lin *et al.*, 2000). Nonetheless, much remains to be studied regarding this question.

In this thesis, we analyzed the function of the proto-oncogene *c-myc* in terminal B lymphocyte differentiation *in vitro* and *in vivo*, for which we used our previously reported *c-myc*$^{fl/fl}$ model that allows the specific deletion of *c-myc* in mature B lymphocytes. Moreover, it allowed us to isolate or trace c-Myc-deficient B lymphocytes *in vivo*, based on GFP expression after Cre-mediated deletion. We asked specifically whether the contribution of c-Myc to terminal B lymphocyte differentiation was merely the regulation of cell proliferation or if it affected other functions. The data presented here show c-Myc as a key regulator of terminal B cell differentiation, and therefore of the immune response.

Discussion

Terminal B lymphocyte differentiation can be mimicked *in vitro* by activation with T-independent or -dependent stimuli such as LPS or anti-CD40 plus interleukin-4, respectively. In both conditions, c-Myc-deficient B lymphocytes do not generate typical plasmablasts ($B220^{lo}$Syndecan-1^{+}). c-Myc-deficient B cells appear to initiate the differentiation program, but fail to complete it. Gene expression levels of *pdrm-1* (Blimp-1) are decreased in these cells compared to controls. *Pdrm-1*$^{-/-}$ B cells failed to differentiate to plasma cells ($B220^{lo}$Synd-1^{+}), although they initiated the differentiation program. The gene expression profile of these cells shows some similarities to that of c-Myc-deficient B lymphocytes. These Blimp-1 deficient cells induce gene expression of *xbp-1*, *igj* and are able to secrete low amounts of immunoglobulin. We generated a c-Myc-deficient cell population that shows a similar phenotype to that of pre-plasmablasts, in which cells are blocked at a stage at which they secrete antibody, but are between the pre-plasmablast to plasmablast stages.

The blockade we observed is probably due to the low expression levels of *blimp-1*, *irf4*, or both. The abnormally high of Bcl-6 levels observed might also block this pathway. This raises the question as to whether c-Myc controls Bcl-6, Blimp-1 or Irf4 expression. As an alternative to the Blimp-1 and Irf4 expression defect, the blockade could be attributed to a secondary effect due to a general defect in signaling, although normal surface expression of CD69 or CD25 activation markers on c-Myc-deficient B cells argues against this possibility.

Although the generation of Synd-1^{+} cells is blocked in our c-Myc KO model, we detect large amounts of IgM. The intracellular FACS, immunoprecipitation and ELISpot experiments supported the idea that these c-Myc -eficient cells hypersecrete IgM. Several syndromes and mutations are associated with hyper-IgM secretion. One is the hyper-IgM described in AID KO mice (Muramatsu *et al.*, 2000; Kuraoka *et al.*, 2009), in which mice show elevated IgM in serum due to a CSR defect that impairs the switch to isotypes other than IgM; other examples are certain CD40 (Lanzi *et al.*, 2010) and CD40 ligand deficiencies (Heinold *et al.*, 2010; Aghamohammadi *et al.*, 2009), X-linked hyper-IgM (Lee *et al.*, 2005), multiple myeloma, Kawasaki disease in humans, caused by abnormally high IL-6 levels, Lyn-deficient B cell syndrome, and Waldenström macroglobulinemia (Davis *et al.*, 2010; Kelsoe *et al.*, 2010). To attempt to explain the mechanism underlying this hypersecretion, we analyzed expression of UPR pathway-related genes, chaperones, and other transcription factors involved in Ig secretion, but found no significant differences between c-Myc knockout and control cells. Thus far, we can only attribute the hyper-IgM secretion to a defect in CSR caused by deficient AID expression, resembling the hyper-IgM syndrome reported for AID knockout mice.

We also analyzed the immune response

to both TD and TI antigens in our KO model. Correlating with the data obtained *in vitro*, these mice showed a notable reduction in the plasma cell population in spleen and BM compared to control mice, in response to both types of stimuli. These results reinforce the hypothesis that short- and long-term ASC are derived from the same differentiation pathway, as c-Myc deletion results in abrogation of all Synd-1^{+} cell populations *in vivo*.

The generation of GC, whose formation is crucial for a competent secondary response to TD antigens, was highly compromised in our c-Myc KO B cells. This could be due to lack of proliferation or to a still unknown role of c-Myc in the formation of these structures. This deficiency is remarkable, as it points to c-Myc as an additional regulator of the TD immune response and CSR.

The large decrease in MZ B cells in the c-Myc-deficient mouse spleen is also noteworthy. MZ B cells are a non-recirculating 'innate-like' B cell population, as they provide a very effective, rapid TI response to blood-borne encapsulated bacteria due to their repertoire of Ig specificity and strategic position near the veins that enter the spleen. Their proximity to the MZ metallophilic macrophages that trap pathogens from the circulation optimize MZ B cell function as well. These MZ B cells bridge the temporal gap between the innate response, which is activated within hours, and the TD adaptive response, which requires approximately two weeks to peak. These cells originate from a transitional T2 population by several differentiation steps. The factors that influence the MZ vs FO phenotype decision at this branching point in differentiation are still unclear. Some authors propose that MZ arise from those B cells whose BCR signaling is weaker than those that give rise to FO cells (BCR strength or bottleneck hypothesis) (Lopes-Carvalho *et al.*, 2004; Pillai *et al.*, 2005). There are several models in which this B cell population is compromised; Btk$^{-/-}$ mice show an increased MZ B cell compartment at the expense of FO cells (Khan *et al.*, 1995), and Lyn$^{-/-}$ (a negative regulator of BCR signaling) mice have a deficiency in MZ B cells (Seo *et al.*, 2001). OBF.1 mutant mice also show defective MZ B cell development (Samardzic *et al.*, 2002). Nonetheless, we observe no differences in the expression of any of these molecules. Remarkably, the Notch2 KO mouse shows exactly the same alteration in the MZ/FO ratio as that described here. Theses authors also reported a decreased T1 population; this correlates with the c-Myc and Notch2 relationship, in which c-Myc is one of the target genes in the Notch pathway. Saito *et al.* (Saito *et al.*, 2003) suggest that Notch regulates CD21 expression (Makar *et al.* 2001) or that this gene is necessary to push the balance towards the MZ phenotype during differentiation. They propose that Notch ligands are expressed in the spleen by dendritic cells in the MZ, which trigger Notch signaling in MZ B cell precursors.

We here report abrogation of CSR *in vitro*. In contrast, we observed isotypes

other than IgM in the sera of mutant mice. We obtained a lower tendency in the IgG1, IgE and IgG2b isotypes in serum, although not statistically significant. This apparent paradox might be explained by the incomplete deletion of *c-myc* in *cmyc*$^{fl/fl}$ mice; transplant of c-Myc deficient mature B cells into Rag-deficient recipients might be very useful to clarify this question.

Alternatively, activation by TD antigens generates memory B cells that provide a rapid, robust and lasting immune response after re-encounter with the antigen. Memory B cells can be identified in mice as a antigen-specific cells that express CD38 on surface (Ridderstad *et al.*, 1998). We demonstrated that c-Myc controls generation and/or maintenance of memory B cells. Our results suggest that short-lived plasma cell, long-lived plasma cell and memory B cells differentiation pathways share some steps in the early stages of the process, as c-Myc abrogation resulted in the lack of these three populations.

To elucidate the details of the mechanism by which c-Myc controls all these processes, we considered rescue experiments using viral vectors to restore wild type expression levels of the altered transcription factors. The lack of proliferation by our c-Myc deficient B lymphocytes did not permit the use of retroviral vectors, as their infective capacity is restricted to proliferating cells. We thus proposed infection with lentiviral vectors bearing potent universal promoters. We overexpressed Blimp-1 to attempt to restore the normal B220^{lo}Synd-1^{+} plasma cell population; the experiments showed no effect on the c-Myc KO B lymphocytes or on control cells. We confirmed that upregulation of the gene carried by the lentivirus was approximately 9- to 10-fold compared to endogenous expression, but this was insufficient to rescue plasma cell differentiation. Bcl-6 overexpression might be preventing correct Blimp-1 function in PC generation in spite of Blimp-1 overexpression, as previously described (Diehl *et al.*, 2008), so this could be the reason why we do not rescue PC differentiation by lentivirus-medianted Blimp-1 upregulation.

Another possibility is irf4 overexpression, which we will attempt in the near future. The change in the flow cytometry staining profile and the decrease in CSR and Ig secretion (not shown) following infection nonetheless suggest that lentiviral transduction alters activation and/or differentiation, and the *in vitro* phenotype might not be rescued by this means. Another possibility is to breed these mice with transgenic mice overexpressing Irf4 or Blimp-1 in the B cell lineage, although the only candidates available are the Blimp-1^{EFGP} transgenic, which results in lack of function (Kallies *et al.*, 2007), and the ICSAT transgenic mouse, in which Irf4 is overexpressed only in T lymphocytes (Saito *et al.*, 1999). The generation of such transgenic mice, overexpressing one of the two main transcription factors involved in PC differentiation specifically in

the B cell lineage, would be a useful tool for the study of this process in the future.

In summary, based on our *in vitro* data it is clear that c-Myc is essential for the transition pre-plasmablast to plasmablast stage, pointing to c-Myc as a crucial transcription factor in the context of this Blimp-1/Bcl-6/Irf4/Pax5 ASC network. Moreover the c-Myc is directly or indirectly regulating the expression levels of Irf4, Blimp-1 and/or Bcl-6, and thus controlling the process of terminal B cell differentiation, apart from ruling proliferation of B lymphocytes. It is showed in figure D1.

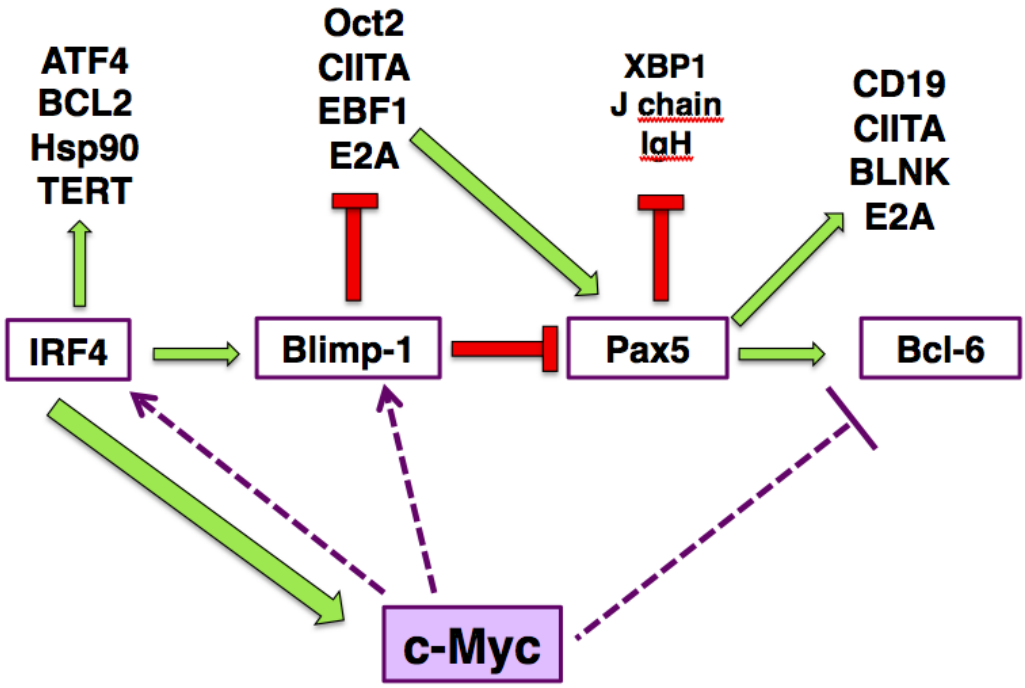


Figure D1. Model for c-Myc-dependent control of plasma cell differentiation process

immunization data demonstrated that c-Myc does not only regulate cell proliferation in terminal B lymphocyte differentiation. By a number of criteria, we have shown that c-Myc affects key functions of this process, including antibody secretion, CSR and SH, as well as immunological memory and alteration of populations that are 'innate-like' components of the immune response (MZ B cells).

Finally, we propose a model in which

6. Conclusions

1) c-Myc is necessary for the differentiation of naïve mature B cells into B220^{lo}Syndecan-1^{+} plasmablasts *in vitro*.

2) p27 deficiency is not sufficient to rescue defective proliferation in c-Myc knockout B cells.

3) c-Myc deficient B lymphocytes generate fewer antibody secreting cells than control cells *in vitro*.

4) The antibody secreting cells generated from *c-myc*$^{fl/fl}$ B cells hypersecrete IgM *in vitro* but do not secrete other immunoglobulin isotypes.

5) *c-myc* deletion causes deregulation in the expression of transcription factors involved in plasma cell differentiation: downregulation of *irf4* and *blimp-1* and upregulation of *bcl-6*.

6) *c-myc*$^{fl/fl}$ mice generate nor B220^{lo}Syndecan-1^{+} short-lived plasma cells in spleen neither long-lived PC in the BM, in response to T-dependent (TNP-KLH) or T-independent (LPS) antigens.

7) c-Myc-deficient B cells do not generate germinal centers following immunization with T-dependet antigens.

8) The marginal zone/follicular B cells cell ratio is decreased in c-Myc knockout mice.

9) c-Myc is necessary for the generation and/or maintenance of memory B lymphocytes after stimulation with T-dependent antigens.

7. Resumen

Introducción

El proto-oncogén *c-myc* fue identificado como el homólogo celular del oncogén del virus de la mielocitomatosis aviar *v-myc* (Vennstrom *et al*., 1982). La desregulación de *c-myc* está envuelta en70.000 casos anuales de muerte por cáncer en Estados Unidos (Dang, 1999; Nesbit *et al*., 1999). Por tanto, el esfuerzo para estudiar este proto-oncogén ha sido muy significativo en los últimos años, con miras terapéuticas.

c-Myc pertenece a la familia de factores de trascripción de hélice-bucle-hélice y cremallera de leucina (bHLH-LZ), junto con N-Myc, L-Myc, B-Myc y s-Myc (Henriksson *et al*., 1993).

El gen *c-myc* está localizado en el cromosoma 8 humano y contiene 3 exones. En el extremo carboxilo terminal c-Myc presenta el motivo bHLH-LZ para formar dímeros proteicos con Max, cuya unión es necesaria para el reconocimiento de las secuencias específicas de ADN, llamadas cajas-E (canónicas: 5'-CACGTG-3', y no canónicas: 5'-CANNTG-3'), que se hallan en los genes diana. En la región amino terminal contiene 2 cajas Myc altamente conservadas (MBI y MBII) para transactivar sus genes diana (Dang, 1999; Pelengaris *et al*., 2002). La región central alberga las cajas MBIII y IV y las secuencias de localización nuclear NLS1 y NLS2, junto con varios sitios de fosforilación (Dang *et al*., 1988; Henriksson *et al*., 1993).

La proteína nuclear c-Myc se expresa de forma ubícua durante la embriogénesis y en los tejidos adultos que poseen alta capacidad proliferativa. Sin embargo, es casi indetectable en las células quiescentes o totalmente diferenciadas. c-Myc se induce rápidamente ante la exposición a estímulos mitogénicos (Campisi *et al*., 1984; Kelly *et al*., 1983; Eilers, 1991), aunque su vida media es muy corta (20-30 minutos) (Hann *et al*., 1984).

La familia de proteínas Mad también juega un papel en la red de factores de transcripción Myc/Max/Mad, puesto que antagonizan el efecto de Myc gracias a su capacidad para heterodomerizar con Max e inhibir la expresión génica (Hurlin *et al*., 1995). El balance entre dímeros Myc/Max y Mad/Max determinará el estado de activación de sus genes diana comunes y el estado celular (Amati *et al*., 2001).

c-Myc está implicado en numerosas funciones biológicas, siendo las más destacables la proliferación, el crecimiento celular, la apoptosis y la diferenciación.

c-Myc promueve la progresión a lo largo del ciclo celular (Mateyak *et al*., 1997; Eilers *et al*., 1991). Esto se consigue mediante la activación de diferentes quinasas dependientes de ciclinas (CDK4, CDK2) (Steiner *et al*., 1995; Bouchard *et al*., 1999) y la represión de inhibidores de ciclo celular, como p15, p27 y p21 (Hermeking *et al*., 2000; Dang, 1999; Grandori *et al*., 2000). Además, c-Myc activa el metabolismo y la síntesis de proteínas, controla tanto el crecimiento celular como el tamaño corporal, como se demostró en estudios realizados en Drosophila melanogaster (Johnston *et al*., 1999), y también en linfocitos B y hepatocitos de ratón (de Alborán *et al*., 2001; Baena *et al*., 2005). En cuanto a la apoptosis inducida, se describió que Myc acelera la muerte celular en condiciones de deprivación de factores de crecimiento (Askew *et al*., 1991; Evan *et al*., 1992). Esta inducción se produce mediante distinas vías de señalización (Fas-Fas ligando, TNF y TRAIL), que culminan en la activación de la cascada de caspasas y la consiguiente degradación celular (Hueber *et al*., 1997; Klefstrom *et al*., 1994; Lutz *et al*., 1998).

Por otro lado, c-Myc ha sido identificado como un factor regulador de la diferenciación a distintos tipos celulares. Puesto que el objetivo de esta tesis es el proceso de diferenciación B, vamos a centrarnos en este campo. La expresión de c-Myc en el linaje B comienza en la fase pro-B tras la estimulación con IL-7 (Morrow *et al*., 1992). También se expresa en la población pre-B (Zimmerman *et al*., 1990). Esto animó a distintos grupos a estudiar el posible papel de c-Myc en la diferenciación de linfocitos B. Valiéndose de modelos de ganancia de función, como el modelo murino *Eμ-c-myc* (Iritani *et al*., 1999), se describió un papel para este gen en la transición pro/pre-B hacia células B inmaduras. También se ha estudiado el papel de c-Myc en la diferenciación de linfocitos B usando modelos de pérdida de función. Sin embargo, la letalidad embionaria en torno a día 10.5 de gestación causada por eliminación de *c-myc* en línea germinal (Davis *et al*., 1993) obligó a los investigadores a crear modelos condicionales de inactivación de c-Myc en tejidos adultos, creándose en 2001 el modelo *c-myc*^{flox} en el cual la tecnología Cre/LoxP permite la eliminación de *c-myc* en el ratón adulto (de Alborán *et al*., 2001). Estos ratones presentan una proliferación deficiente de linfocitos B en respuesta a anti-CD40 más IL-4, acumulándose en fase G0/G1, y mostrando un tamaño similar al de células no activadas. Además, poseen niveles anormalmente elevados de p27 y muestran resistencia a muerte inducida por la vía de Fas (de Alborán *et al*., 2001). Por otro lado, nuestro laboratorio ha descrito recientemente la regulación de la diferenciación temprana de linfocitos B por parte de c-Myc a través del factor de transcripción EBF1, gracias a la unión directa de c-Myc al promotor del gen *ebf1* (Vallespinós *et al*. 2011, *in press*).

Aparte del papel descrito para c-Myc en diferenciación temprana de linfocitos B,

este factor se expresa a distintos niveles a lo largo de todo el proceso de maduración, hasta que es inhibido cuando las células B maduras se diferencian a células plasmáticas (CP). El objetivo de esta tesis es estudiar la función de c-Myc en la diferenciación B terminal, puesto que creemos que puede estar jugando un papel importante, más allá del que juega en proliferación. No hay muchos estudios sobre este tema, pero se publicó un trabajo con la línea de linfoma B maduro BCL1 *in vitro*, en el cual se dice que la represión de c-*myc* es necesaria pero no suficiente para promover la diferenciación plasmacítica (Lin *et al*., 2000). Sin embargo, este trabajo deja una puerta abierta al requerimiento de c-Myc en las primeras fases de la diferenciación terminal de linfocitos B.

Vamos a repasar las distintas fases y algunos aspectos importantes del proceso de diferenciación de linfocitos B. La diferenciación B termpana es un proceso finamente regulado que ocurre en el hígado fetal o en la médula ósea adulta, desde progenitores linfoides hasta células B maduras. Está ligado al reordenamiento de los segmentos V(D)J de las cadenas pesada (IgH) y ligera (IgJ) de las inmunoglobulinas (Ig). El avance a lo largo de este proceso está acompañado de la expresión de varios marcadores de membrana que permiten la identificación de distinas subpoblaciones (Hardy *et al*., 2001). Una vez las células B han alcanzado la fase de células B maduras migran a los órganos linfoides periféricos, que incluyen los nódulos linfáticos, los parches de Peyer, las vegetaciones y amígdalas, el tejido linfoide asociado a las mucosas y la pulpa blanca del bazo. En estos órganos las células B naïve se activan mediante el reconocimiento de antígeno y se diferencian finalmente a CP y linfocitos B de memoria. Las CP con las mediadoras de la inmunidad humoral, puesto que secretan grandes cantidades de Ig, y las células B de memoria permiten una rápida y eficiente respuesta ante la re-exposición al antígeno, y pueden perdurar durante toda la vida del organismo.

Nuestro trabajo se centra en el bazo de ratón, que se divide en pulpa roja, encargada de eliminar eritrocitos viejos, y la blanca, donde hallamos las células del sistema inmune. La pulpa blanca engloba los folículos, las zonas T (zona periarteolar o PALS) y la zona marginal. Las células que entran al bazo provenientes de la médula presentan el fenotipo de membrana $IgM^{hi}IgD^{+}CD23^{int}CD21i^{nt\text{-}hi}$ y se llaman células B transicionales (T1 y T2). Éstas pueden poblar la zona marginal (MZ), entre las pulpas roja y blanca, u organizarse en folículos. Las células B de la zona marginal ($IgM^{hi}IgD^{lo}Cd21^{hi}CD21^{hi}CD23^{lo}$) se activan en respuesta a antígenos T-independientes (TI), generando plasmablastos (células con capacidad proliferativa y de secretar bajos niveles de anticuerpos, con características intermedias entre linfocito B y CP) y CP de vida corta. Sin embargo, las células B foliculares ($IgM^{lo}IgD^{hi}CD21^{lo}Cd23^{hi}$) pueden activarse mediante antígenos T-dependientes (TD), formando centros germinales (CG), que son

estructuras especializadas en las cuales ocurren ciclos consecutivos de división celular. En ellos también tienen lugar los procesos de hipermutación somática (CSR) y maduración de la afinidad o hipermutación somática (SH), que permiten generar anticuerpos de diferentes isotipos (distintas región constante y función efectora) y con afinidades mayores por el antígeno. En estas estructuras se generan CP de vida larga y células B de memoria (McHeyzer-Williams *et al*., 2001; Vinuesa *et al*., 2001; Shapiro-Shelef *et al*., 2005).

Este proceso de diferenciación B terminal está controlado por un programa transcripcional altamente regulado, en el cual la expresión de los genes necesarios para la diferenciación y la activación de linfocitos B y la de los que controlan la diferenciación de CP son mutuamente excluyentes. Blimp-1, codificada por el gen *prdm1*, era considerado el principal regulador de la diferenciación plasmacítica, ya que su expresión induce la diferenciación hacia células secretoras de anticuerpos (Turner *et al*., 1994; Shapiro-Shelef *et al*., 2003). Sin embargo, se publicó que en ausencia de Blimp-1 es posible generar una población de células que secretan bajos niveles Ig aunque son Syndecan1$^-$ (un proteoglicano que se usa como marcador de CP (Sanderson *et al*., 1989)), pero ya expresan genes específicos de secreción de Ig como *xbp1*, *flt3* e *igj*, gracias a la inhibición de *pax5* (Kallies *et al*., 2007). Blimp-1 reprime directamente *c-myc* (Lin *et al*., 1997), por lo que las CP dejan de proliferar. La disminución de los niveles de Pax5 permite la expresión de Xbp-1, que es otro factor de transcripción esencial para la diferenciación a CP, puesto que establece el fenotipo secretor de estas células. Gracias a sus funciones relacionadas con la respuesta a proteínas mal plegadas (UPR), media la expansión del retículo endoplasmático (ER) , aumento del tamaño celular, síntesis de Ig y aumento de la función mitocondrial (Reimold *et al*., 2001). Irf4 también participa en este proceso de diferenciación, realizando funciones no solapantes con Blimp-1, y su eliminación inhibe la generación de CP. Además, juega un papel en CSR a través de la activación directa de AID (Klein *et al*., 2996; Sciammas *et al*., 2006). Otro componente esencial de esta vía es Bcl-6, cuya expresión es máxima en los CG, debido a su función en el mantenimiento de estas estructuras (Cattoretti *et al*., 1995). Bcl-6 inhibe Blimp-1, por lo que debe ser reprimido para poder avanzar a lo largo del proceso de diferenciación hacia PC (Shaffer *et al*., 2000).

Objetivos

Todo esto, nos hizo plantearnos cuál es el papel de c-Myc en la diferenciación terminal de linfocitos B, y proponernos los siguientes objetivos:

1) Caracterizar el papel de c-Myc en la diferenciación terminal de linfocitos B mediante un modelo murino de pérdida de función: generación de células plasmáticas y secreción de inmunoglobulinas *in vitro*.

2) Caracterizar la respuesta inmune en ratones deficientes en c-Myc-deficient tras la inmunización con antígenos T-dependientes y T-independientes.

3) Analizar los niveles de expresión génica de los principales factores de transcripción implicados en la diferenciación terminal de linfocitos B, en ausencia de c-Myc.

4) Rescatar el fenotipo normal de diferenciación mediante ensayos de ganancia de función sobre-expresando potenciales genes diana de c-Myc.

Resultados

Modelo murino para el estudio de la diferenciación B: generación de los ratones *c-myc* $^{fl/fl}$;*cd19*$^{cre/+}$;*rosa26*$^{efgp/egfp}$

En esta tesis hemos utilizado el modelo condicional de inactivación de *c-myc* en ratón publicado anteriormente (de Alboran *et al*., 2001). En este modelo los exones codificantes 2 y 3 de *c-myc* están flanqueados por secuencias loxP, y la expresión específica en linaje B de la Cre recombinasa bajo el promotor de *cd19* media su eliminación. Cruzamos esta cepa *c-myc*$^{fl/f;l}$*cd19*$^{cre/+}$_con ratones que expresan la proteína verde fluorescente (EGFP) para poder identificar las células en las cuales la eliminación de *c-myc* se ha producido. Mediantecrucessucesivosobtuvimosratones homocigotos *c-myc*$^{fl/fl}$y controles *c-myc*$^{fl/+}$.

c-Myc es necesario para la diferenciación hacia células B220^{lo}Syndecan-1^{+} *in vitro*

Aislamos células B220^{+}GFP^{+} del bazo de ratones *c-myc*$^{fl/fl}$ o controles y estudiamos la generación de células plasmáticas *in vitro* mediante la activación con anti-CD40 más IL-4 o LPS. Observamos que las células B deficientes en c-Myc no generan células B220^{lo}Syndecan-1^{+} en respuesta a ambos antígenos. Cuando analizamos los niveles de expresión en membrana del marcador de activación CD69 no encontramos diferencias, lo que nos indica que este bloqueo no se debe a un defecto en la activación en respuesta a estímulo.

Las células B deficientes en c-Myc presentan una disminución dramática de la proliferación *in vitro*

Analizamos la capacidad de proliferar de nuestras células B deficientes en c-Myc. Por un lado, el tamaño de los blastos formado a día 4 de activación con anti-CD40 más IL-4 era mucho menor que los blastos de células control y, por otro lado, el número de células recuperada era también inferior. Además realizamos experimentos con yoduro de propidio mediante citometría de flujo, y observamos que la proliferación de linfocitos B deficientes en c-Myc está severamente impedida tanto en respuesta a anti-CD40 más IL-4 como a LPS, acorde con lo previamente publicado (de Alboran *et a*l., 2001).

La eliminación de p27 no rescata la proliferación en células B deficientes en c-Myc

Según lo publicado en (de Alboran *et a*l., 2001), sabemos que la molécula inhibidora de ciclo celular p27kip se haya sobre-expresada en los linfocitos B deficientes en c-Myc. Por tanto, tratamos de normalizar la proliferación en estas células mediante la eliminación de p27. Para ello cruzamos los ratones *c-myc*$^{fl/fl}$ y *c-myc*$^{fl/+}$;*cd19*$^{cre/+}$;*rosa26*$^{gfp/gfp}$ con ratones p27 "knockout" (p27KO) (Fero *et al*., 1996). Sin embargo, cuando analizamos el ciclo celular en estos ratones, no observamos diferencias

respecto a los KO simples *c-myc*$^{fl/fl}$.

Los linfocitos B deficientes en c-Myc son capaces de generar células secretoras de anticuerpos (ASC)

La ausencia de plasmablastos con el típico fenotipo de membrana B220$^{lo/-}$Synd-1^{+} no indica ausencia de células secretoras en nuestros cultivos c-Myc KO. Por tanto, analizamos la generación de Ig secretada en cultivo mediante ELISA, y la generación de células secretoras de anticuerpos mediante ensayo de ELISpot. Detectamos concentraciones similares de IgM, mediante ELISA, en los sobrenadantes de células KO y control en las activaciones con anti-CD40 más IL-4. Por otro lado, los ensayos de ELISpot indicaron que los linfocitos B deficientes en c-Myc son capaces de generar células secretoras de anticuerpos, aunque el número total generado *in vitro* era 6 veces menor que en cultivos control. Por tanto, dedujimos para que un número mucho menor de células secretoras diera lugar a un nivel de IgM soluble similar, estas células debían empezar a secretar antes que las células control, o bien secretar mayores cantidades de Ig por célula. Los estudios de cinética de secreción no mostraron ninguna diferencia; sin embargo, la medición del área de los "spots" de IgM secretada en ELISpot, junto con ensayos de inmunoprecipitacion de la IgM total en sobrenadantes y la tinción de IgM intracelular, demostraron que las células B deficientes en c-Myc son capaces de hiper-secretar IgM. En cambio, cuando analizamos otros isotipos como IgG1 e IgE, generados también en las activaciones con anti-CD40 más IL-4, no encontramos niveles detectables, indicando que c-Myc es necesario para realizar el proceso de CSR.

Expresión génica de células secretoras de anticuerpos deficientes en c-Myc

Analizamos los niveles de expresión de diferentes factores de transcripción implicados en la diferenciación de CP mediante qPCR. Extrajimos el RNA de células B220^{+}Syndecan-1^{-}GFP^{+} y/o B220^{lo}Syndecan-1^{+}GFP^{+} de ratones *c-myc*$^{fl/fl}$ y c-myc$^{fl/+}$ después de estimularlas *in vitro* con anti-CD40 más IL-4 durante 1 o 3 días. La población B220^{lo}Syndecan-1^{+}GFP^{+} no se genera todavía a día 1 ni en cultivos *myc*$^{fl/fl}$, ni en *c-myc*$^{fl/+}$; esta población fue purificada en las muestras de día 3 de cultivos control, ya que las células B deficientes en c-Myc no la generaron. Observamos varios cambios significativos en genes clave para este proceso: Irf4 y Blimp-1 se encuentran 4 y 3 veces disminuídos respectivamente, comparados con células control. Bcl-6 se haya sobre-expresado, y Xbp-1 no presenta cambios significativos. Estos cambios permiten explicar el bloqueo observado en la diferenciación de plasmablastos *in vitro*. Además, los niveles normales de Xbp-1 son compatibles con la capacidad de secretar Ig que presentan nuestras células. Por otro lado, aunque analizamos bastantes genes implicados en secreción y en la ruta de UPR, no observamos ningún cambio destacable que pudiera explicar la super-secreción de IgM.

Respuesta inmune impedida en ratones *c-myc*$^{fl/fl}$ y ausencia de centros germinales deficientes en c-Myc

Estudiamos la respuesta inmune en ratones deficientes en c-Myc y controles tras la inmunización con antígenos TD (TNP-KLH) y TI (LPS). En respuesta a ambos estímulos, observamos un gran defecto en la generación de células plasmáticas B220^{lo}Syndecan-1^{+}GFP^{+}, tanto las de vida corta que se generan en bazo, como de las de vida larga, que migran a la médula ósea.

Además, tras la inmunización con TNP-KLH observamos una falta de generación de células B de centro germinal (GL-7^{hi}) por citometría de flujo, y una ausencia de centros germinales generados a partir de linfocitos B deficientes en c-Myc mediante técnicas de inmunohistoquímica. Adicionalmente, nos llamó la atención la alteración de la proporción de células B de zona marginal respecto a las células B foliculares, disminuyendo drásctica mente en los ratontes homocigotos. Esto lo demostramos tanto por citometría de flujo mediante los marcadores CD23 y CD21, como por métodos de inmunohistoquímica.

Por último, el análisis de los niveles de distintos isotipos de Ig en suero tras la inmunización con TNP-KLH no reveló cambios significativos, pero sí sugieren una tendencia a la hipersecreción de IgM y disminución del resto de isotipos. La existencia de un porcentaje de células en las cuales no se elimina c-Myc dentro de los ratones homocigotos, debido a la incompleta acción de la recombinasa Cre, hace que nos sea imposible en este modelo determinar los niveles reales de Ig liberada al suero únicamente por las células c-Myc deficientes.

c-Myc es necesario para el mantenimiento de los linfocitos B de memoria

Estudiamos la generación de linfocitos B de memoria en ratones deficientes en c-Myc tras la inmunización con TNP-KLH. Los análisis de citometría de flujo tras re-estimulación con este antígeno mostraron una severa reducción de las células B220+IgG1^{+}CD38+TNP^{+}GFP^{+}, que son linfocitos B de memoria específicos de antígeno.

Infección lentiviral de linfocitos B deficientes en c-Myc para rescatar la diferenciación hacia células plasmáticas

Puesto que los niveles de Blimp-1 se hayan disminuidos significativamente en los linfocitos B deficientes en c-Myc, tratamos de restablecer los niveles de este factor de trancripción tan importante para la diferenciación de células plasmáticas. Para ello infectamos las células B con un vector lentiviral con el fin de sobre-expresar Blimp-1. No podemos utilizar vectores retrovirales en nuestro caso, puesto que infectan células en división. Utilizando el vector EF.CMV. Blimp-1-IRES-hCD2t o el vector control EF.CMV.IRES-hCD2t, infectamos células B220^{+}GFP^{+} de ratones *c-myc*$^{fl/fl}$ o control

y las activamos posteriormente con anti-CD40 más IL-4. Cuando analizamos por citometría de flujo la diferenciación hacia células $B220^{lo}Synd\text{-}1^{+}$, no observamos ningún efecto del vector que sobre-expresa Blimp-1 respecto del vacío. Sin embargo, la mera infección alteró bastante tanto la diferenciación de las células *in vitro* como el brillo de los marcadores analizados en citometría, e incluso la secreción de inmunoglobulinas. También nos planteamos infectar las células con un vector que sobre-expresase Irf4, pero el hecho de que la infección lentiviral altere tanto la diferenciación a células plasmáticas, nos hace dudar que mediante este procedimiento experimental se pueda rescatar el fenotipo normal de las células B deficientes en c-Myc. El uso de ratones transgénicos que sobre-expresen Blimp-1 o Irf4 serían de gran utilidad para este tipo de estudios.

Conclusiones

1) c-Myc es necesario para la diferenciación hacia plasmablastos B220^{lo}Syndecan-1^{+} *in vitro*.

2) La deficiencia en p27 no es suficiente para rescatar la proliferación en células decificientes en c-Myc.

3) Los linfocitos B deficientes en c-Myc generan menos células secretoras de anticuerpos que los linfocitos control.

4) Las células secretoras de Ig generadas a partir de células B *c-myc*$^{fl/fl}$ hiper-secretan IgM *in vitro*, pero no secretan otros isotipos de Inmunoglobulinas.

5) La eliminación de c-Myc causa la desregulación de la expresión de varios factores de transcripción implicados en diferenciación hacia células plasmáticas: disminución de la expresión *irf4* y *blimp-1*, sobre-expresión de *bcl-6*.

6) *c-myc*$^{fl/fl}$ no genera células plasmáticas de vida corta B220^{lo}Syndecan-1^{+} en el bazo ni de vida larga en la médula ósea, en respuesta a antígenos T-dependientes (TNP-KLH) o T-independientes (LPS).

7) Las células B deficientes en c-Myc no generan centros germinales tras inmunización T-depeniente.

8) El ratio entre linfocitos B de zona marginal y foliculares está severamente disminuído en ratones deficientes en c-Myc.

9) c-Myc es necesario para la generación y/o mantenimiento de linfocitos B de memoria tras la estimulación T-dependiente.

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c-Myc is essential for hematopoietic stem cell differentiation and regulates Lin$^-$Sca-1$^+$c-Kit$^-$ cell generation through p21

Esther Baena, Maitane Ortiz, Carlos Martínez-A, and Ignacio Moreno de Alborán

Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, Universidad Autónoma de Madrid, Madrid, Spain

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Objective. **The c-Myc protein is a member of the basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family, which is implicated in regulation of proliferation, differentiation, and apoptosis in multiple cell types. The aim of this study was to characterize the role of the proto-oncogene *c-myc* in hematopoietic stem cells (HSC) during postnatal development.**

Material and Methods. **We have generated a conditional mouse model that allows us to inactivate *c-myc* in bone marrow (BM) in an inducible fashion.**

Results. **We show that conditional inactivation of c-Myc in BM severely impairs HSC differentiation, leading to a striking decrease in the number of lymphoid and myeloid cells. *c-Myc* deletion in BM causes substantial accumulation of a Lin$^-$Sca-1$^+$c-Kit$^-$ cell population expressing high levels of the cell-cycle inhibitor p21, whose origin and function are otherwise poorly characterized. In vivo inactivation of p21 and c-Myc normalizes Lin$^-$Sca-1$^+$c-Kit$^-$ cell numbers and restores normal proliferation. The potential origin and function of these cells are discussed.**

Conclusions. **c-Myc plays a role in HSC maintenance and differentiation and might be regulating generation of Lin$^-$Sca-1$^+$c-Kit$^-$ through the cell-cycle regulator p21.**

Given the appropriate environment, hematopoietic stem cells (HSC) are able to perpetuate themselves by self-renewal and to differentiate into all blood cell types. Under normal physiological conditions, the number of HSC is kept constant. HSC must, therefore, maintain the balance between differentiation and self-renewal to provide an input of differentiated cells [1,2]. Numerous reports have focused on the understanding of the molecular mechanisms that govern HSC differentiation and self-renewal, with the aim to improve the potential clinical applications of these cells. HSC are included in a bone marrow (BM) population defined by surface expression of Sca-1 and c-Kit in the lineage-negative (Lin$^-$) fraction (Lin$^-$Sca-1$^+$c-Kit$^+$, LSK cells). LSK cells can be further characterized based on surface expression of different markers in cells with long-term multilineage reconstitution potential and self-renewal activity and short-term with limited self-renewal potential in irradiated mice [3].

Several molecular pathways have been implicated in the regulation of HSC self-renewal and differentiation. *Hoxb4*, a member of the homeobox gene transcription factor family, is expressed in HSC and promotes their in vivo and in vitro expansion [4,5]. Moreover, ectopic *hoxb4* expression confers multilineage engraftment potential to embryonic stem cells and yolk sac hematopoietic precursors. Wnt proteins have been shown to play an important role in vertebrate and invertebrate development. In HSC, activation of the Wnt signaling pathway and stabilization of β-catenin promote self-renewal in vitro and enhance the capacity of hematopoietic precursors to reconstitute lethally irradiated mice [6]. Another gene that has been shown to play a prominent role in HSC homeostasis is *Bmi-1*. This gene is a member of the polycomb family of genes involved in lymphocyte development, senescence, and neurological development. Inactivation of *bmi-1* gene in mice leads to progressive loss of all hematopoietic lineages due to a defect in HSC self-renewal capacity [7,8]. Other molecular pathways

Dr. Baena is currently at Children's Hospital, Division of Hematology/Oncology, Boston, MA.

Offprint requests to: Ignacio Moreno de Alborán, Ph.D. Universidad Autonoma de Madrid, Centro Nacional de Biotecnologia/CSIC, Department of Immunology and Oncology, Darwin 3, Madrid E-28049, Spain; E-mail: imoreno@cnb.uam.es

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B Lymphocyte Commitment Program Is Driven by the Proto-oncogene *c-myc*

Mireia Vallespinós[1*], David Fernández[1*], Lorena Rodríguez[1*], Josué Alvaro-Blanco[†], Esther Baena[*§], Maitane Ortiz[*], Daniela Dukovska[*], Dolores Martinez[‡], Ana Rojas[‡], Miguel R. Campanero[†] & Ignacio Moreno de Alborán[*]

[*]Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, Darwin 3, Cantoblanco, Madrid E-28049; [†]Instituto de Investigaciones Biomédicas/CSIC - UAM, Arturo Duperier 4, E-28029 Madrid; [‡]Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, E-28029 Madrid, Spain

[§]Present address: Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA

[1]These authors contributed equally to this work

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Corresponding author: Ignacio Moreno de Alborán

email: imoreno@cnb.csic.es

Fax: (+34) 91 372 0493

Phone: (+34) 91 585 4562