Mechanisms of B cell activation and lymphomagenesis

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Versicherung

Ich, Caroline Hojer, geboren in München, versichere hiermit ehrenwörtlich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Beihilfe angefertigt habe.

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List of abbreviations

α	anti
A	adenine
AID	activation induced cytidine deaminase
APC	allophycocyanin
APS	ammoniumpersulfate
BAFF	B cell activating factor of the TNF family
bp	base pair
B-CLL	B cell chronic lymphoblastic leukemia
BCR	B cell receptor
BrdU	Bromodesoxyuridine
BSA	bovine serum albumine
C	cytosin
CD	cluster of differentiation
CFSE	Carboxy-Fuorescin Diacetate Succinimidyl Ester
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
Cre	protein recombinase of the phage *P1* („Causes Recombination“)
CSR	class switch recombination
CTAR	C-terminal activating region
d	day
DMEM	Dulbecco’s modification of the „Eagle“ medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	desoxyribonucleotidetriphosphate
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EDTA	ethylene-diamine-tetra-acetate

EMSA	electromobility shift assay
Erk	extracellular signal-regulated kinase
ES cells	embryonic stem cells
et al.	"et alii"
FACS	fluorescence-activated cell scanning/ sorting
FCS	fetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
Fig.	Figure
Fo B cell	follicular B cell
G	guanin
g	gram
xg	x times gravity
GC	germinal center
GFP	green fluorescent protein
h	hour
HL	Hodgkin lymphoma
HRP	horse-raddish peroxidase
HRS cell	Hodgkin-Reed/Sternberg cell
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
Igα, β	immunoglobulin-associated signaling molecules α and β, respectively
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
IRES	internal ribosome entry site
ITAM	immunoreceptor tyrosine-based activation motif
JAK	janus kinase
Jnk	c-Jun N-terminal kinase
kb	kilo base
kDa	kilo dalton
ko	deletiertes Gen („knock-out")
l	liter
LMP	latent membrane protein
loxP	locus of crossover (x) of phage P1
M	molar
MACS	magnetic associated cell sorting
MAPK	mitogen activated protein kinase
MHC	major histocompability
min.	minute
mg	milligram
MZ B cell	marginal zone B cell
µCi	microcurie
µF	microfaraday
N	amino-terminus (protein)
NF-κB	nuclear factor κ B
NK cell	natural killer cell
NLS	nulear localization sequence
nm	nanometer
PAA	polyacrylamide
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	perdinin chlorophyll protein
RNA	ribonucleic acid
RNase	Ribonuclease
RBP-Jκ	recombination signal binding protein J kappa
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec.	second
SHM	somatic hypermutation
T B cell	transitional B cell
Tab.	Table
TAE	Tris-acetate-EDTA
TD	T cell dependent
TdT	terminal desoxynucleotidyl Transferase
TE	Tris-EDTA
TI	T cell independent
TNF-R	tumor necrosis factor receptor
TRAF	TNF receptor associated factor
tTA	tetracycline transactivator
U	units
UTR	untranslated region
UV	ultraviolet light
V	volt
v/v	volume per volume
w	weeks
w/o	without
wt	wildtype
w/v	weight per volume

1 Introduction

1.1 The immune system

Multicellular organisms evolved an immune system to protect themselves from pathogens and aberrant cells of the body. In vertebrates this system has grown more complex, in addition to the innate immune system, an adaptive immune system developed. This system specifically recognizes pathogens and keeps a memory for long periods of time. Cells of both the innate and the adaptive immune response act via a variety of receptors that for instance recognize antigen, serve as costimulatory signals or direct migration. In some cases, activation of these cells goes awry and leads to autoimmune diseases or tumorigenesis.

1.2 Humoral immunity and B lymphocytes

The term "humoral immunity" arose at a time where antibodies were thought to be the sole immune defense mechanism. In the late 19th century Ilya Mechnikov discovered phagocytes as cells of the immune system and the concept of "cellular immunity" was born. Today it is known that both arms, the humoral and the cellular, are important and interconnected in mounting an efficient immune response to a foreign antigen in vertebrates. The actual source of antibodies, namely B lymphocytes, was discovered not until some decades later, when Max Cooper published his results on the bursa of fabricus in chicken in 1966 (Cooper et al., 1966).

B lymphocytes are part of the adaptive immune response that -in contrast to the innate immune response- enables specific recognition and memory of pathogens. B lymphocytes like all cells of the mammalian immune system belong to the hematopoietic lineage. They derive from a pluripotent progenitor, the hematopoietic stem cell (HSC), in the bone marrow. This stem cell gives rise to two lines of differentiation, the myeloid and the lymphoid line. The myeloid compartment derives from the common myeloid progenitor (CMP) and the lymphoid compartment is generated by the common lymphoid progenitor (CLP), the latter differentiating into T, natural killer (NK), dendritic and B cells.

T cell progenitors migrate to the thymus to further mature whereas B cells reside in the bone marrow. During the maturation of B cells the gradual rearrangement of the Ig loci takes place. This genetic process allows an extreme and random diversity of Ig molecules that may then

recognize a large diversity of antigens. During development, the expression of a rearranged B cell receptor is important for the positive selection of B cells so that only successfully rearranged, non-self specific B cells can survive and migrate to the periphery. The first type of B cells to arise, the pro B cells, only expresses the signaling domains of the B cell receptor complex, namely Igα and Igβ as a heterodimer with calnexin. Induction of the expression of the *Rag-1* and *Rag-2* genes leads to the rearrangement of the Ig μ locus. The underlying genetic process is called somatic recombination and is characterized by the joining of D_H to J_H Ig heavy chain DNA segments in early and the joining of V_H to D_H segments in late pro B cells (Tonegawa, 1983). The enzyme terminal deoxynucleotidyl transferase (TdT) introduces N-nucleotides at the joining regions and thus further enhances Ig diversity (Gilfillan et al., 1993). The expression of a successfully rearranged IgH together with a surrogate light chain consisting of the VpreB and the λ5 chain leads to the positive selection of pre B cells (Melchers et al., 1993). A second recombination process occurs in the Ig light chain segments of either the λ or κ gene of the pre B cell genome that ultimately leads to the expression of the B cell receptor in immature B cells. A mechanism called allelic exclusion guarantees that only one of the two alleles of the Ig heavy and light λ or κ chain gene is successfully rearranged and expressed (Alt et al., 1984). This inhibits that the antibody molecules are formed by more than one IgH and one IgL and would thus have different specificities. To prevent autoimmunity, immature B cells that recognize self antigens are negatively selected or may undergo a process called receptor editing to change their specificity (Edry and Melamed, 2004). Only around 10% of these cells survive selection and migrate into the blood stream as transitional B cells (T1). After entering the spleen, T1 B cells experience another selection process and only about a third further differentiates to T2 and T3 to become mature B cells (Chung et al., 2003). Mature B cells receiving survival signals via BAFF receptor and tonic BCR signaling independently of antigenic contact can thus survive in the body for long periods of time (Forster et al., 1989; Kraus et al., 2004; Lam et al., 1997; Moore et al., 1999). Follicular (Fo) and marginal zone (MZ) B cells are the two main types of mature B cells found in the spleen, B1 cells predominantly reside in the peritoneal cavity. Follicular B cells circulate through the blood and lymph and can be found in spleen and lymph nodes, whereas MZ B cells only reside in the marginal zone of follicles in the spleen in rodents. Both MZ and B1 B cells are considered as the main source of IgM in response to T cell independent antigens and have the capacity of self-renewal (Martin and Kearney, 2000; Martin et al., 2001; Tarakhovsky, 1997).

Mature naïve B cells remain in a resting state until they encounter their cognate antigen. Depending on the nature of the antigen there are two general mechanisms of B cell activation. Crosslinking of the B cell receptor via T cell independent (TI) antigens (e.g. bacterial polysaccharides) leads to clustering of the receptor and thus signaling via the Igα and Igβ tail into the B cell is initiated. Such activated B cells may then proliferate to form extrafollicular foci in secondary lymphoid organs and differentiate into plasma cells that produce low affinity antibodies. This type of activation is commonly found in MZ B and B1 cells.
T cell dependent (TD) antigens (peptides) on the other hand bind specifically to the BCR and are then presented on MHC class II complexes after internalization. $CD4^+$ T cells that have been activated through binding of the antigen presented on a dendritic cell may then interact with the antigen-presenting B cells at the T-B-cell-zone border within follicles. In addition to the antigen-derived signal from the BCR T cells deliver costimulatory signals to B cells. One of these signals is mediated by the CD40 receptor that binds to the CD40 ligand expressed on activated $CD4^+$ T cells. In concert action, these signaling pathways stimulate B cells to either form extrafollicular foci or germinal centers (GCs).

Germinal centers are structures in follicles of secondary lymphoid organs that arise four to seven days after administration of antigen. They origin from only about one to ten activated B cells that proliferate with a doubling time of 6 to 8 hours. A network of follicular dendritic cells (FDC) and $CD4^+$ T cells can be found in between these B cell clones. Within these structures proliferating B cells are found in the dark zone (centroblasts), whereas the emerging B cell clones are selected in the light zone (centrocytes). Two important processes take place in germinal center B cells: somatic hypermutation (SHM) and class switch recombination (CSR). Both require the action of a specific enzyme, activation induced cytidine deaminase (AID) and of general DNA repair mechanisms. During somatic hypermutation, the variable (V) region of the Ig locus accumulates point mutations as well as small deletions or duplications with a mutation rate of about 10^{-3}/ nt. Due to this diversification process B cell clones that show a higher affinity to their cognate antigen are positively selected by FDCs. The second process, class switch recombination, leads to the expression of different antibody classes and thus influences the effector function of these molecules when they are secreted into the serum. Antibody classes are defined by the Fc (constant) region of the Ig heavy chain and can be altered genetically by class switch recombination, whereas the V region of the antibody molecule keeps the same antigen specificity. In response to different cytokines B cells can switch from IgM to IgG1, IgG2a,

IgG2b, IgG3, IgE or IgA. B cells that have undergone these processes and have been positively selected by FDCs and $CD4^+$ T cells in the germinal center may then differentiate into plasma or memory B cells. Plasma cells function as effector cells that migrate to the bone marrow where they produce and secrete antibodies that bind to the specific pathogen. This leads to neutralisation, opsonization and phagocytosis by macrophages or complement mediated lysis of the pathogen. Memory B cells on the other hand remain in a resting state in the body for long periods of time but can be activated promptly upon a second antigen challenge leading to a fast and specific secondary immune response.

The interaction between B and T cells during a TD immune response is mediated by a variety of cell surface molecules and promotes close contact of the cells, often called the "immunological synapse". This guarantees that e.g. released cytokines only bind to the activated and not to unspecific bystander cells. The pair of the CD40 receptor and CD40 ligand is part of the immunological synapse and plays a crucial costimulatory role in activating B cells during a TD dependent immune response.

1.3 The CD40 receptor

CD40 is a tumor necrosis factor (TNF) family receptor that is expressed on a variety of cells, among them epithelial and endothelial cells as well as antigen presenting cells of the immune system. *In vitro*, CD40 stimulated B cells start to proliferate, show prolonged survival, and display an activated phenotype as shown by the expression of activation and adhesion markers like CD23, CD95, CD80, CD86, MHC class II and ICAM and secretion of cytokines like IL6, IL10, TNFα and TNFβ (Dallman et al., 2003; Harnett, 2004). In conjunction with certain cytokines, CD40 stimulation of B cells leads to class switch recombination.
In vivo, CD40 plays an essential role during TD immune responses. Triggering of CD40 on B cells by CD40L expressed on activated $CD4^+$ T cells in combination with BCR signals leads to the activation and subsequent differentiation of B cells into either germinal center B cells or plasma cells derived from extrafollicular foci. Within the germinal center, CD40 signaling influences selection processes of B cells with highly affine antigen receptors that may then either differentiate into plasma or memory B cells. The importance of CD40 costimulation during TD immune responses became obvious when patients with the Hyper-IgM syndrome, an X-linked immunodeficiency syndrome resulting in reduced production of IgG, IgE and IgA antibodies as well as memory B cells upon challenge with T cell dependent antigens, were shown to carry mutations in the CD40L gene (Ferrari et al., 2001; Fuleihan et al., 1993;

Imai et al., 2003; Ramesh et al., 1994). This observation was further underlined by the analysis of CD40 as well as CD40L knock out mice that are not able to mount TD immune responses resulting in the lack of germinal centers, SHM and reduced CSR (Kawabe et al., 1994; Xu et al., 1994).

Once CD40 is activated via its ligand, the CD40L (CD154), it triggers the activation of distinct signaling pathways (Figure 1). The cytoplasmic signaling domain of CD40 lacks intrinsic catalytic activity but consists of two PxQxT motifs that bind to TRAF (TNF receptor associated factor) 1, 2, 3, 5 and 6 upon activation (Harnett, 2004). TRAFs associated with CD40 subsequently lead to the activation of the MAPKs (mitogen activated protein kinases) Erk (extracellular signal regulated kinase), Jnk (c-Jun N-terminal kinase) and p38 and the canonical and noncanonical NF-κB pathways. Erk, p38 and Jnk are activated by a cascade of MAP kinases and result in the activation of transcription factors, among them Elk-1, ATF1 and AP-1 (Fos/ Jun), respectively. The NF-κB pathway shows a complex way of regulation; activation is marked by degradation of an inhibitory protein and translocation of the NF-κB dimers to the nucleus where they act as transcription factors (Ghosh and Karin, 2002). In the case of the canonical NF-κB pathway, IκBα is the prominent inhibitor of p50/p65 and p50/c-Rel heterodimers that retains them in the cytoplasm. Upon activation of the canonical NF-κB pathway, IκBα is phosphorylated, ubiquitinated and subsequently degraded by the proteasome. This releases the heterodimer's nuclear translocation sequences and enables it to translocate to the nucleus. In the case of the noncanonical NF-κB pathway, the inhibitory function lies in the heterodimer of NF-κB itself. A protein called p100 is associated with RelB in the cytoplasm preventing the dimer's translocation to the nucleus. Activation leads to phosphorylation and ubiquitin-proteasomal processing of the p100 molecule that then remains as p52 associated with RelB. This active dimer of the noncanonical NF-κB pathway can then enter the nucleus and act as a transcription factor. Besides p52/RelB, p50/RelB heterodimers are regarded as part of the noncanonical NF-κB pathway, although p50 is thought to be constitutively processed from p105 by the proteasome (Derudder et al., 2003; Xiao et al., 2001). Further JAK-STAT (janus activated kinase - signal transducer and activator of transcription) as well as the PI3K (phosphoinositol 3 kinase) and PLCγ2 (Phospholipase Cγ2) pathways can be induced upon CD40 signaling (Hanissian and Geha, 1997; Harnett, 2004). Some studies support the idea that CD40 stimulation activates protein Tyr kinases, such as Lyn, Fyn and Syk (Faris et al., 1994; Ren et al., 1994). CD40 triggering thus leads to the

transcriptional activation of a variety of genes, including anti-apoptotic as well as cell cycle promoting genes.

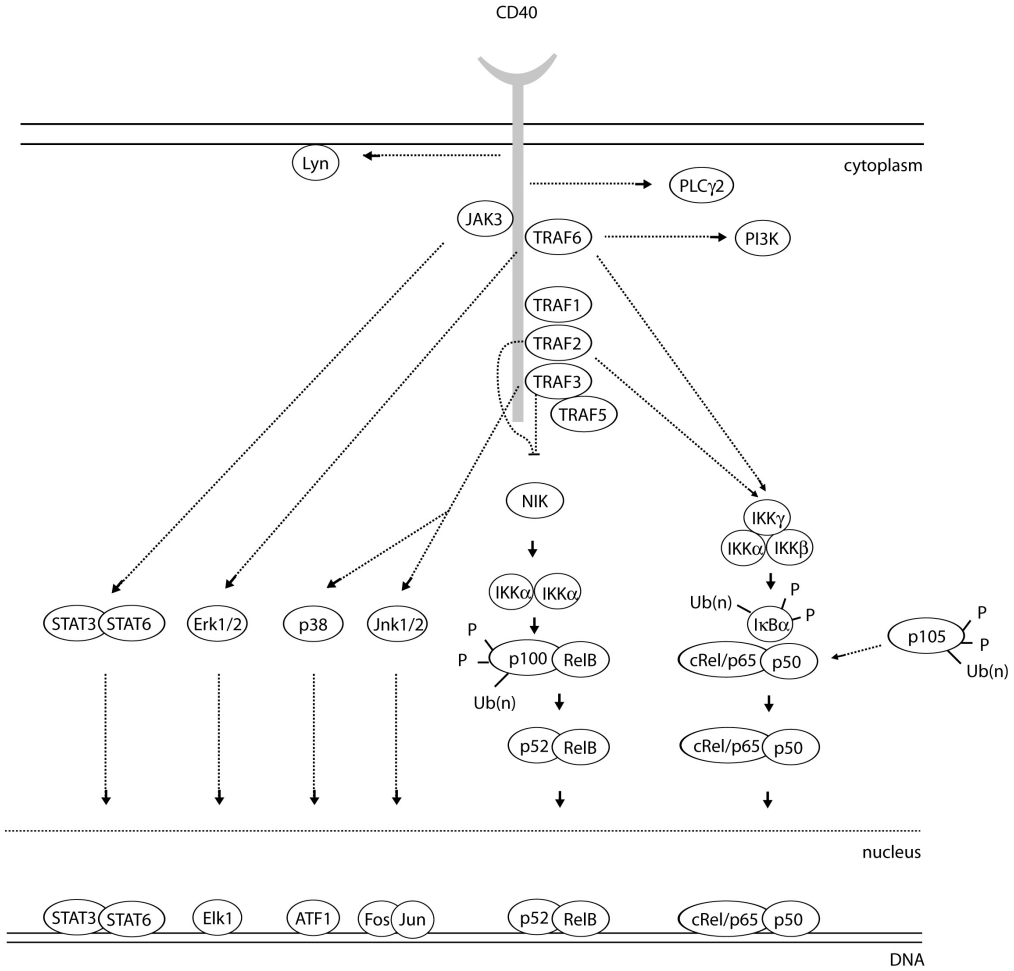


Figure 1 Simplified scheme of CD40 signaling (modified from Harnett, 2004; van Kooten and Banchereau, 2000)
Upon ligand-binding and trimerization, CD40 associates with TRAF1, 2, 3, 5, 6 and JAK3 leading to the activation of the MAPKs Erk, Jnk and p38, the canonical (p50/p65, p50/c-Rel) and the noncanonical (p52/RelB) NF-κB pathway, the PI3K and the PLCγ2 lipid-metabolizing enzymes as well as the JAK-STAT pathway. Erk, Jnk and p38 activate transcription factors that bind to DNA to activate gene transcription. The canonical and noncanonical NF-κB pathways are activated by specific kinases (NIK and IKKs) that phosphorylate inhibitors of NF-κB (p100, IκB) marking them for processing and degradation, respectively, by the ubiquitin-proteasomal pathway. P105 is processed constitutively to p50 by the proteasome. NF-κB heterodimers then translocate to the nucleus and act as transcription factors for specific genes. CD40 has also been shown to mediate phosphorylation of Lyn by unknown mechanisms. TRAF (TNF receptor associated factor), JAK (Janus kinase), NIK (nuclear κ B inducing kinase), IKK (IκB kinase), IκBα (Inhibitor of κ B α), Ub (Ubiquitin), P (phosphorylation), PI3K (Phosphoinositol 3 kinase), PLCγ2 (Phospholipase Cγ2), STAT (signal transducer and activator of transcription), Jnk (c-Jun N-terminal kinase), Erk (extracellular signal regulated kinase).

Interestingly, CD40 induces similar pathways as B cell receptor signaling. Indeed, CD40 signaling is known to protect immature B cells from BCR induced apoptosis, cooperating with it in different pathways in mature B cells and in generating a germinal center phenotype (Galibert et al., 1996; Mizuno and Rothstein, 2003, 2005).

1.4 The B cell receptor

The B cell receptor plays a role at many different stages of B cell development as described before. During development in the bone marrow its expression is critical for positive selection and survival of B cells. In contrast to the T cell receptor the BCR is even required for the survival of mature naïve B cells (Kraus et al., 2004; Labrecque et al., 2001; Lam et al., 1997). In the case of infection it leads to the activation and differentiation of specific B cells upon binding to their cognate antigen. The B cell receptor in mature B cells is a complex of molecules that resides in lipid rafts. It consists of the transmembrane Ig and the signaling molecules Igα and Igβ. The Ig molecule recognizes and binds antigen whereas Igα and β are required for downstream signaling (Figure 2). Antigen induced BCR signaling is very well studied, however the nature of ligand-independent, tonic BCR signals is not known. In the following, signal propagation after antigen binding to the receptor is described; ligand-independent signaling might be considered not as a distinct, non-overlapping process, but as 'the opposite end of a continuum' (Monroe, 2006).

Transmembrane receptors (e.g. the Ig complex) receive signals from the extracellular space that trigger receptor clustering and lead to the formation of lipid raft signalosomes. Proximal membrane-anchored (e.g. Lyn) and SH2 containing soluble proteins (e.g. Syk) nucleate signaling at the membrane. Adaptor proteins subsequently recruit proteins to the signaling complex that initiate intracellular signaling cascades to activate effector proteins.

Resting mature B cells exhibit an assembly of Ig, Igα and Igβ with the Src family tyrosine kinases Lyn, Fyn and Blk (Burkhardt et al., 1991). Upon activation of the B cell receptor the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Igα and β are phosphorylated by these Src family kinases (Kurosaki, 1999). Subsequently the SH2 domain containing kinase Syk is recruited and binds to the phosphorylated tyrosines of the ITAMs (Rowley et al., 1995). Syk itself was shown to phosphorylate the ITAMs in Igα and Igβ and to recruit and phosphorylate SH3 or PH domain containing proteins like SLP-65 that mediate signaling to effector proteins (Wienands et al., 1998). These effector proteins are lipid-metabolizing enzymes (PI3K, PLCγ2), GTP-binding proteins (Ras, Rac) and Ser-Thr-kinases (Akt, Erk, Jnk, p38, PKCs) as well as phosphatases (SHP-1, SHIP, PTEN) to tightly control and terminate signaling in a negative feedback loop (Fu et al., 1998; Kurosaki, 1999; Rolli et al., 2002).

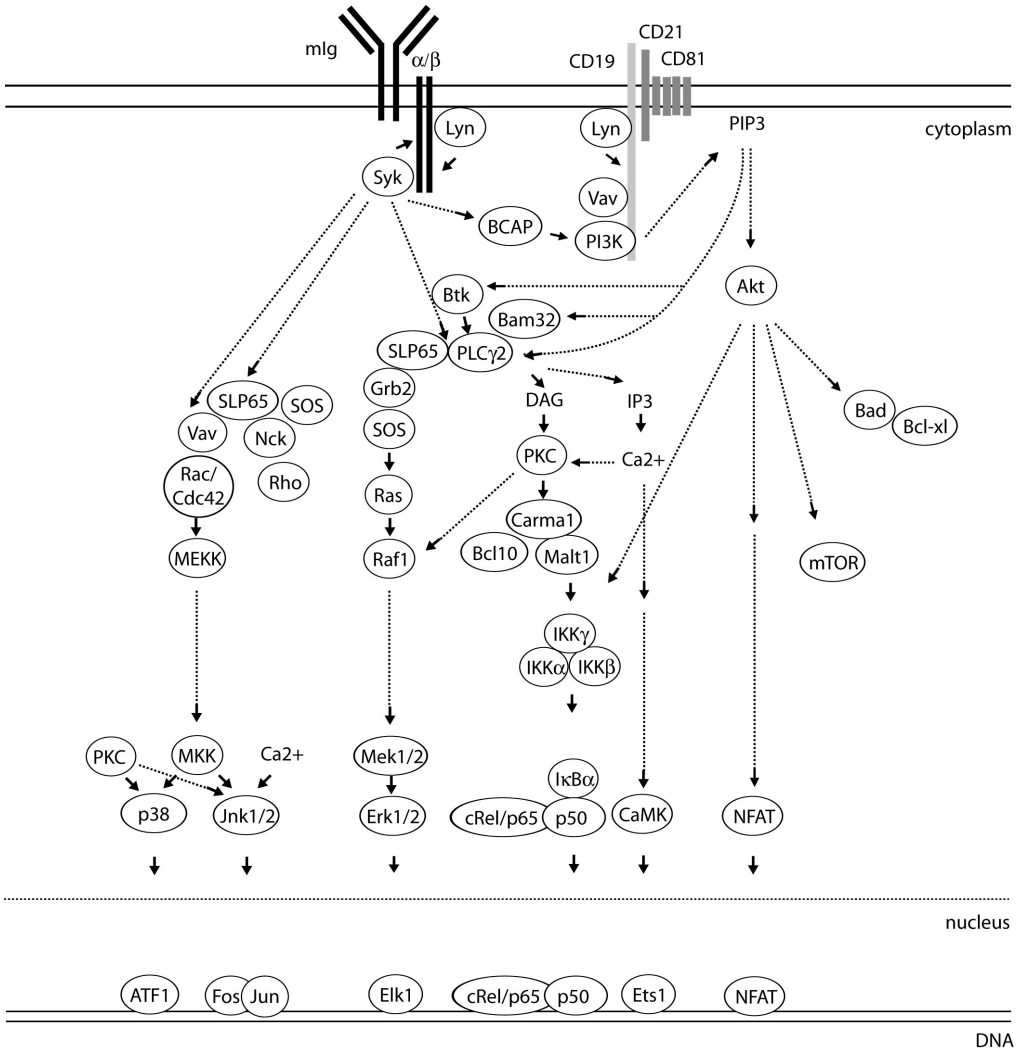


Figure 2 Simplified scheme of B cell receptor signaling (modified from Dal Porto et al., 2004)
The B cell receptor complex comprises of the Ig molecule and the signaling molecules Igα and Igβ. It resides in lipid rafts upon activation and associates with the coreceptor complex of CD19, CD21 and CD81. Proximate signaling events include the association of the Src-kinase Lyn with Igα and Igβ that mediates phosphorylation of the latter. During this early event CD19 has an important role in enhancing signaling via the recruitment of Lyn. The kinase Syk mediates signaling to downstream adaptors like SLP65, Vav and BCAP. These induce cascades that activate effector proteins like the MAPK Jnk, Erk and p38, the canonical NF-κB pathway and lipid-metabolizing enzymes like PLCγ2 and PI3K. Jnk and p38 are activated via a complex of SLP65, Vav and Rac that subsequently activates the MAPK cascade MEKK-MKK-Jnk/ p38. PKC and Ca^{2+} play an additional role in their activation. SLP65, SOS and Nck further promote actin cytoskeleton formation via Rho. The classical activation of Erk is mediated by the SLP65, Grb2, SOS complex that leads to the activation of the Ras-Raf-Mek-Erk cascade. PLCγ2 generates IP_3 and DAG that activate Ca^{2+}-release from the endoplasmatic reticulum and the activation of the kinase PKC, respectively. Ca^{2+} activates CaMK and PKC that is involved in the activation of the Ras-Raf-Mek-Erk cascade and NF-κB. More precisely, upon BCR signals, PKC activates the complex of Carma1, Bcl10 and Malt1 that activates the IKK complex. This results in degradation of the inhibitor IκBα so that NF-κB heterodimers of cRel/p50 and p65/p50 can translocate into the nucleus and act as transcription factors. PI3K activation is mainly mediated through CD19 that binds to PI3K, Lyn and Vav via phosphorylated Tyr residues in its cytoplasmic tail. PI3K is a lipid metabolizing kinase resulting in PIP_3 that activates Akt, PLCγ2, Btk and Bam32. Akt is a kinase that activates Bcl-x_L, mTOR and NFAT. Bam32 is an adaptor molecule that integrates PI3K and PLCγ2 signaling pathways. Btk is a kinase that activates PLCγ2. Ig (immunoglobulin), Btk (Bruton's tyrosine kinase), BCAP (B cell adaptor for PI3K), Bam32 (B lymphocyte adaptor molecule), CaMK (Calmodulin dependent protein kinase).

In mature B cells the BCR is functionally connected to a coreceptor complex consisting of CD19, CD21 and CD81, which is known to augment BCR signaling. CD19 had first been described as a coreceptor of the B cell receptor in 1987 (Pezzutto et al., 1987), while four years later it was shown to lower the threshold of BCR signaling in *in vitro* studies (Carter et al., 1991). With the help of knock out mice it became clear that it is critical in mounting an immune response to TD antigens and partly required for the generation and survival of resting B cells (Engel et al., 1995; Otero et al., 2003; Rickert et al., 1995). Thus Fo B cells have been shown to be reduced and B1 as well as MZ B cells are missing in CD19 deficient mice. CD19 is known to directly interact with PI3K, Lyn and Vav via phosphorylated Tyr residues in its cytoplasmic tail and to mediate signaling to PLCγ2, Ca^{2+} release, Akt as well as the MAPK Erk and Jnk (Buhl et al., 1997; Li and Carter, 1998; Otero et al., 2001; Wang et al., 2002). It has further been suggested to be involved in the transduction of other mitogenic signals like CD40 that was shown to trigger phosphorylation of Lyn as well as of CD19 (Uckun et al., 1993). The complement receptor CD21 binds to the complement cleavage fragment C3d but probably requires CD19 to signal into the cell. It is expressed solely on mature B lymphocytes. CD21 deficient mice display a similar phenotype to CD19 deficient mice; they lack B1a cells and are impaired in mounting a TD immune response (Croix et al., 1996; Molina et al., 1996). CD81, the third component of the BCR co-receptor, contains four membrane spanning regions and is known to be required for cell surface expression of CD19. Its inactivation in mice leads to an impairment in humoral immune responses (Maecker and Levy, 1997). It has been suggested to contain signaling activity via its cytoplasmic tail by activating integrins (Maecker et al., 1997).

A recent publication showed that both BCR and CD40 signals that are delivered to B cells upon activation during a TD immune response, collaborate with Notch receptor signals to enhance proliferation and to produce $IgG1^{+}$ cells *in vitro* and *in vivo* (Thomas et al., 2007). Furthermore the activation of the Notch pathway enhanced the activation of MAPKs upon stimulation of the BCR and CD40. These findings imply an interplay of BCR, CD40 and Notch signaling during immune responses. It is therefore tempting to speculate that deregulated signaling of these receptors cooperates in inducing B cell malignancies. In the case of B cell chronic lymphocytic leukemia (B-CLL) for example all three receptors have been suggested to be involved in the pathogenesis of the disease (Ghia et al., 2008; Hubmann et al., 2002).

1.5 The Notch receptor

Early studies on drosophila melanogaster by Thomas Hunt Morgan led to the discovery of Notch. He described a strain that showed notches in its wingblades while studying chromosomal inheritance (Thomas Hunt Morgan 1917). Decades later the four Notch genes coding for the Notch 1, 2, 3 and 4 receptors were identified in mammals. Notch plays a role in a broad spectrum of cell fate decisions, in differentiation processes as well as in proliferation and apoptosis. In mammals, Jagged 1 and 2 as well as Delta-like 1, 3 and 4 have been described to bind and activate the Notch receptor. Recently it became clear that different pairing of Notch receptors to ligands impacts the nature and outcome of the signal (Besseyrias et al., 2007; Santos et al., 2007).
In the hematopoietic system, the Notch receptors are expressed in a variety of compartments. Notch1 has been shown to play a role in stem cell self-renewal and in cell fate decision of the common lymphoid progenitor triggering T cell development (Han et al., 2002; Pui et al., 1999; Radtke et al., 1999; Stier et al., 2002). In B cell development, Notch2 is responsible for the development of MZ B cells and cooperates with CD40 and BCR/ LPS signaling in inducing proliferation, isotype switching and differentiation into plasma cells (Saito et al., 2003; Santos et al., 2007; Thomas et al., 2007).

The Notch receptor is a heterodimer consisting of an N-terminal extracellular and a C-terminal transmembrane and intracellular domain linked non-covalently to one another (Figure 3) (Blaumueller et al., 1997; Logeat et al., 1998). The N-terminal domain consists of EGF (epidermal growth factor) like repeats that mediate ligand binding while the C-terminal domain anchors the receptor to the membrane and mediates signaling into the cell. Upon binding of a ligand to the receptor, two proteolytic cleavages occur. ADAM proteases cleave part of the extracellular domain that will be internalized together with the ligand on the interacting cell. A γ-secretase cleaves part of the intracellular domain (Notch-IC) that will subsequently enter the nucleus via its NLS sequence and act as a transcription factor by recruiting a coactivator complex (Oswald et al., 2001).

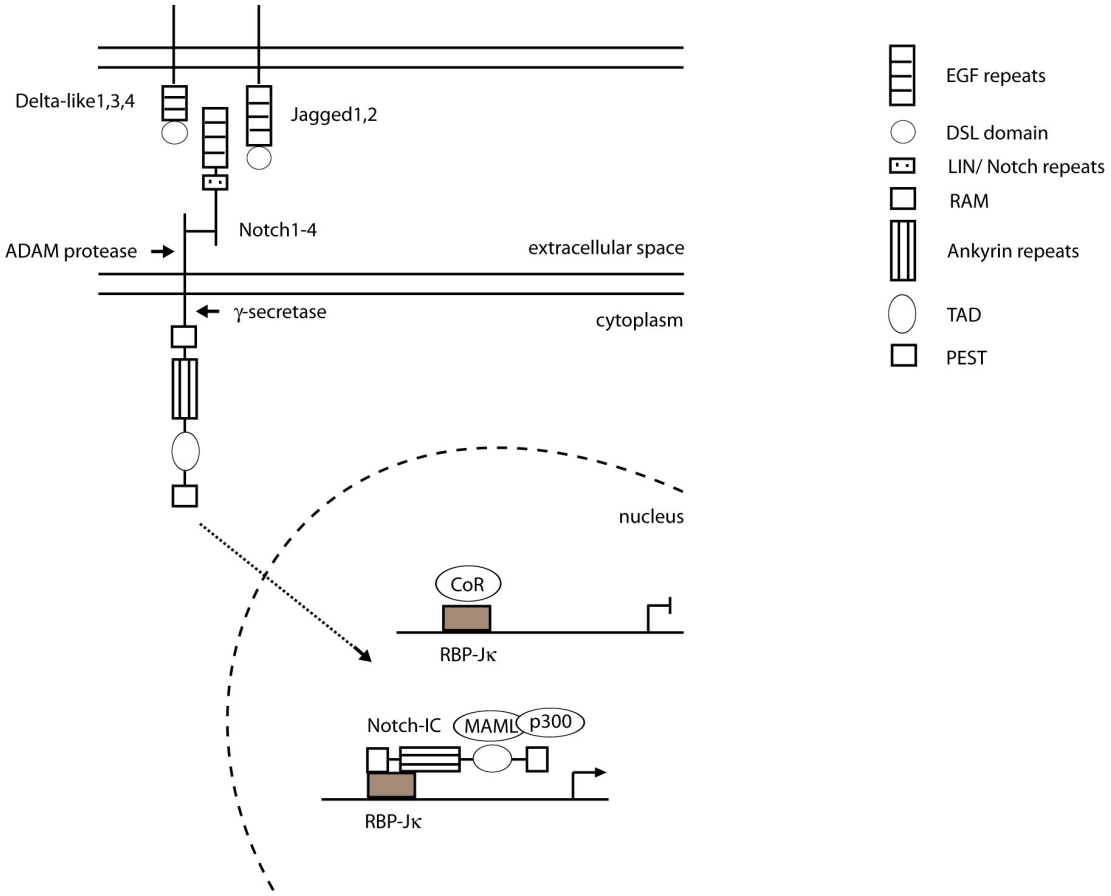


Figure 3 Notch receptor activation and signaling
Upon ligation of the Notch receptor, two proteases cleave the Notch molecule. The family of metallo-proteases ADAM cleaves the extracellular part whereas γ-secretase cleaves the intracellular part of Notch. The cleaved Notch-IC migrates to the nucleus where it binds RBP-Jκ, displaces the co-repressor complex and recruits a coactivator complex containing MAML and p300 and thus leads to gene transcription. CoR (corepressor), MAML (mastermind like), RBP-Jκ (recombination signal binding protein for immunoglobulin kappa J region), EGF (epidermal growth factor), DSL (delta serrate lag-2), TAD (transactivation domain), PEST (proline-glutamine-serine-threonine-rich).

RBP-Jκ mediates Notch-IC binding to the DNA since Notch-IC itself is not able to bind DNA directly. Notch-IC is known to activate gene transcription of the Hey and Hes family among others. It has been proposed that Notch-IC can act as a transcription factor without the help of RBP-Jκ, but the mechanism remains unresolved (Martinez Arias et al., 2002). Additionally, Notch-IC leads to the repression of gene transcription. In B cells, the Igμ locus is a classical example for such a negatively regulated gene. Once the intracellular part of Notch is cleaved off, it is rapidly degraded by the ubiquitin proteasomal pathway since it carries a PEST (proline-glutamine-serine-threonine-rich) domain marking it for ubiquititation. The overall mechanism of cleavage of an intracellular part of a receptor and rapid degradation of the product guarantees a rapid shut-down of the signal.

1.6 Signaling pathways in lymphomagenesis

The key feature of cells of the adaptive immune system lies in the ability to expand rapidly upon stimulation by a specific pathogen, even after long periods in a resting, non-dividing state. This and the ability of B cells to additionally mutate their DNA to further enhance specificity against the pathogen eventually leads to B cell lymphomagenesis. Although BCR, CD40 and Notch signaling physiologically play a central role in B cell activation and differentiation processes, deregulated or in the case of the BCR even tonic signals from these receptors are suspected to be involved in the development of B cell lymphomas. Interestingly, these three receptors are mimicked by an oncogenic virus, the Epstein-Barr virus (EBV). This virus predominantly infects human B cells and leads to their immortalization *in vitro*. During this process, EBV triggers the expression of a certain latency program, where LMP1 (mimicking a constitutively active CD40), LMP2A (mimicking tonic BCR signaling) and EBNA2 (mimicking a constitutively active Notch receptor) are produced among a few other viral gene products.

Aberrant Notch expression has been identified in various tumors, including breast, prostate and skin cancer as well as T cell acute lymphoblastic leukemia (T-ALL). The human Notch1 gene has been identified by the characterization of a chromosomal translocation t(7;9) being present in a few cases of T-ALL patients (Ellisen et al., 1991). In this translocation the active part of the Notch1 (Notch1-IC) gene was fused to the TCRβ locus. Later it was shown that the expression of constitutively active Notch1 and Notch3 in lymphoid progenitor cells leads to the development of T cell leukemias in mice (Bellavia et al., 2000; Pear et al., 1996). Till now it is not known whether deregulated Notch signaling in B cells leads to aberrant proliferation, however, members of the Notch receptor family are expressed on a variety of B cell malignancies. Notch1 and 2 are highly expressed on Hodgkin Reed Sternberg cells in Hodgkin's disease (HD) and Notch2 has been found to be overexpressed on neoplastic cells of B cell chronic lymphocytic leukemia (B-CLL) (Hubmann et al., 2002; Kapp et al., 1999).

Deregulated expression as seen in the case of Notch is not known for the BCR. Tonic, ligand-independent BCR signaling is of great importance for mature resting B cells and it has been speculated whether this might also account for B cell neoplasms since they mostly express the BCR and its coreceptor CD19. Syk for example was suggested as a target for the treatment of Non-Hodgkin lymphomas (Chen et al., 2008; Gururajan et al., 2006; Young et al., 2009). An exception is represented by Hodgkin lymphomas (HL) where most of the B cell receptor

associated proteins are diminished or absent on the malignant cells. It has been speculated whether high NF-κB activity –a hallmark of HL- substitutes for the survival signals provided by the BCR. However, it is not clear yet whether HL cells are completely independent of tonic BCR signals. All HL cells carrying crippled mutations in their Ig genes, completely inhibiting their expression, are EBV positive, suggesting that LMP2A, a viral mimic of the BCR, substitutes for the required survival signal (Kuppers et al., 2005). This is in contrast to EBV negative cases, where BCR components are only diminished, but no crippled mutations were found in the Ig loci. Thus it remains unclear whether malignant B cells require the survival signal from the BCR or whether the aberrant activation of other survival pathways renders them independent.

One such aberrantly activated pathway in Hodgkin lymphoma cells is represented by CD40. CD40 is highly expressed in HL and B-CLL and it has overall been shown to be present on several types of tumor cells (O'Grady et al., 1994; Westendorf et al., 1994). It had been suspected that CD40 might not only serve as a marker of these malignancies but could actually be involved in the formation or maintenance of the diseases (Pham et al., 2002). Pham *et al.* showed that Non-Hodgkin lymphoma (NHL) growth is mediated by autonomous CD40 ligation through the coexpression of CD40 and CD40L on NHL B cells. This ligation results in robust NF-κB activation. A similar scenario accounts for Hodgkin lymphoma where both CD40 and CD40L are expressed on HRS cells and NF-κB is highly active.

1.7 The LMP1/CD40 transgenic mouse strain

To study the malignant potential of CD40 signaling in B cells, a conditional mouse strain that allows cell type specific expression of a constitutively active CD40 molecule was generated in our lab by Cornelia Hömig-Hölzel (Homig-Holzel et al., 2008). The constitutive activity of CD40 was achieved by fusing the transmembrane domain of LMP1, to the signaling domain of CD40. LMP1 is expressed in Epstein-Barr virus immortalized B cells and is thought to be a functional homologue of CD40 with the exception that it is able to signal ligand independently through clustering of its transmembrane domain. Thus the fusion protein LMP1/CD40 is rendered ligand independent and signals constitutively via the CD40 tail. The LMP1/CD40 gene was targeted to the ubiquitously active *Rosa26* locus. To restrict LMP1/CD40 expression to B cells, the transgene was preceded by a loxP flanked STOP cassette that prevents its expression. Crossing mice that carry the LMP1/CD40^{flSTOP} in their

Rosa26 locus to mice that carry Cre under the control of a B cell specific promoter (e.g. CD19) results in the deletion of the STOP cassette and expression of the transgene in B cells (LMP1/CD40 mice).

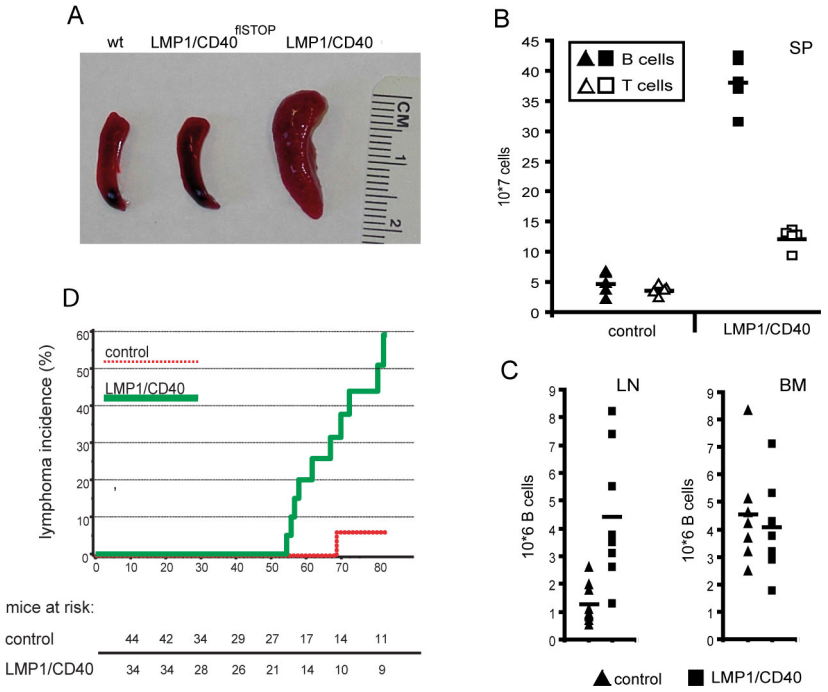


Figure 4 LMP1/CD40 mice show splenomegaly, an expansion of B cells and develop lymphomas
(A) LMP1/CD40 transgenic mice are characterized by a splenomegaly compared to control (wt and LMP1/CD40^{flSTOP}) mice. (B) (C) LMP1/CD40 transgenic mice have higher B and T cell numbers in the spleen (B) and in the lymph nodes (LN), but relatively normal B cell numbers in the bone marrow (BM) (C). (D) The Kaplan-Meier curve demonstrates the high lymphoma incidence in LMP1/CD40 transgenic mice (green line) plotted to the age of mice in weeks. In the control group (dotted red line) spontaneous lymphoma incidence was very low.

Transgenic mice expressing a constitutively active CD40 molecule (LMP1/CD40) in B cells show splenomegaly and enlarged lymphnodes as compared to control mice (wt and LMP1/CD40^{flSTOP}) (Figure 4A). Both B and T cell numbers in the spleen (SP) are found to be increased about eight and three times, respectively, in LMP1/CD40 expressing mice as compared to control mice (Figure 4B). Likewise, B cell numbers in the lymph nodes (LN) are increased about three times, whereas those in the bone marrow (BM) appear to be normal in LMP1/CD40-expressing mice (Figure 4C, right and left panel, respectively). The B cells display an activated phenotype but were not able to form germinal centers and to produce high-affinity antibodies (data not shown) upon immunization with TD antigens. After the age of one year more than 50% of LMP1/CD40 transgenic mice develop lymphomas as depicted in a Kaplan-Meier curve (Figure 4D).

2 Aim of the project

The unique feature of B cells to expand rapidly upon pathogen contact is of great importance for its function but carries substantial risk. Activation and expansion of resting cells is mediated by receptors like CD40 and Notch that bind to external stimuli and translate signals into the cell. If the induction of proliferation in these cells cannot be terminated due to aberrant activation, the expansion of B cells may ultimately result in lymphomagenesis.

The CD40 receptor is known to be a crucial element in the activation of B cells during a TD immune response. Its activation is normally strictly controlled, however, there is compelling evidence that deregulation of CD40 signaling is involved in tumorigenesis. We could show that constitutive activity of CD40 in murine B cells *in vivo* leads to B cell expansion and triggers lymphomagenesis. The constitutive activity of CD40 was achieved by fusing the transmembrane domain of LMP1, a viral constitutively active mimic of the CD40 receptor, to the signaling domain of CD40. The fusion protein LMP1/CD40 thus signals constitutively via the CD40 cytoplasmic tail. The first part of this thesis deals with the question which signaling pathways are induced upon constitutive CD40 signaling in the LMP1/CD40 transgenic mouse strain and how they contribute to lymphomagenesis.

In the second part, the role of tonic BCR signaling in LMP1/CD40 driven B cell expansion and lymphomagenesis will be investigated. Besides its function in antigen recognition, the B cell receptor is required for B cell development in the bone marrow, for selection processes of functional non-self specific B cells and for the maintenance of peripheral B cells. This maintenance survival signal is thought to be ligand-independent (‘tonic’), however, its nature it not known. Most B cell derived tumor cells still express the BCR and it is speculated whether its signaling is crucial for the initiation and maintenance of these tumors. To analyze how impairment of tonic BCR signaling influences aberrantly activated, premalignant B cells, two components of the BCR complex, Igβ and CD19, should be deleted in LMP1/CD40-expressing B cells *in vivo*.

CD40 and Notch are known to cooperate in activating B cells and in inducing their proliferation as well as their class switching. Our own results suggest that Notch has the potential to induce proliferation in B cells but strikingly not survival and even apoptosis in the absence of antiapoptotic stimuli *in vitro*. However, when aberrantly activated together with a survival promoting receptor like CD40, it might be able to promote lymphomagenesis. A very similar situation is seen in Hodgkin lymphomas, where CD40 and Notch are concomitantly expressed. To confirm this hypothesis *in vivo* a transgenic mouse strain should be established

allowing reversible, B cell specific expression of Notch2-IC. In this mouse strain, *Notch2-IC* is placed under the control of the TetO7 promoter. Crossing this strain to the R26STOPrtTA and a B cell specific Cre strain will result in inducible and reversible expression of a constitutively active Notch2 in B cells. This mouse strain will allow to study the effects of Notch2-IC in B cells *in vivo* and clarify how it contributes to lymphomagenesis.

3 Results

3.1 Constitutive CD40 signaling in B cells *in vivo*

Constitutive CD40 signaling in B cells *in vivo* leads to B cell expansion and ultimately triggers B cell lymphoma development. This observation awakened our interest in the question which signaling pathways are induced and might be responsible for these phenotypic effects. Since it is known from *in vitro* studies that α–CD40 stimulation of primary B cells leads to the activation of the MAPKs Jnk, Erk and p38 as well as the canonical and noncanonical NF-κB pathways, we investigated whether constitutive CD40 signaling via LMP1/CD40 leads to the constitutive activation of these signaling pathways *in vivo*.

3.1.1 Constitutive CD40 signaling *in vivo* selectively activates the noncanonical NF-κB pathway in B cells

The family of NF-κB transcription factors plays an important role in transmitting signals in the immune system. Deregulation of NF-κB signaling has been shown to be a common event in tumors and is often already found in premalignant, low grade growths of cells (Weinberg, 2007). The activation of the canonical and noncanonical NF-κB pathways can be analyzed by investigating the phosphorylation status of NF-κB inhibitory molecules like IκBα that are marked for degradation and by investigating the nuclear translocation of the NF-κB transcription factors.

A previous electromobility shift assay (EMSA) performed by Cornelia Hömig-Hölzel and Andreas Gewies revealed that -compared to control B cells- the nuclear fraction of LMP1/CD40-expressing B cells contains similar basal amounts of p50/p65 and p50/c-Rel complexes, but higher levels of RelB containing complexes. This result suggested that LMP1/CD40 signaling activates the noncanonical but not the canonical NF-κB pathway in B cells *in vivo*.

To confirm these findings, we analyzed the activity of the canonical and noncanonical NF-κB pathway in more detail. First we performed Western blots of whole cell extracts and probed them with α-p100/p52, α-IκBα and α-pIκBα antibodies (Figure 5). Three independent

experiments were blotted on one gel to exhibit natural deviations of total and phosphorylated protein levels (Figure 5A).

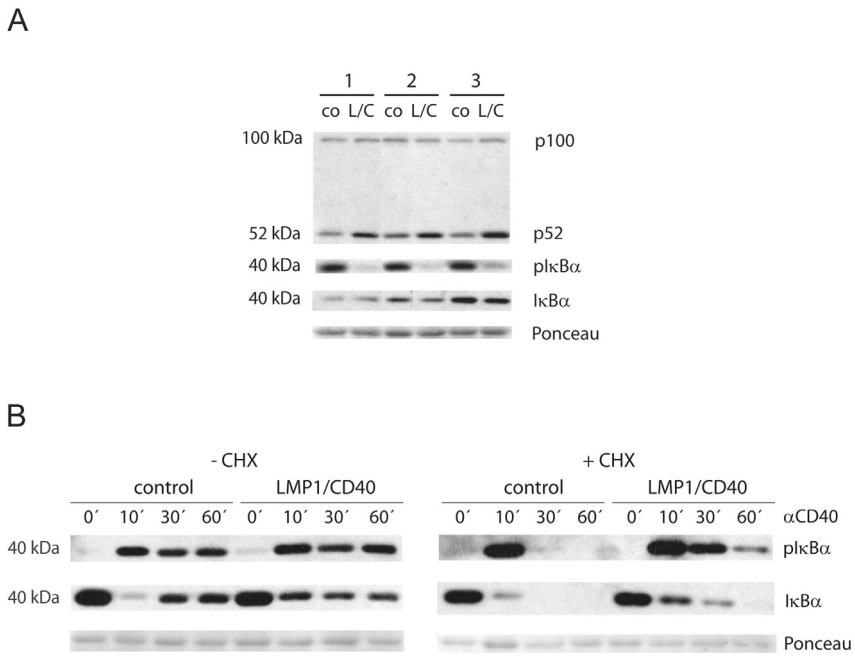


Figure 5 LMP1/CD40-expressing B cells do not activate the canonical NF-κB pathway
(A) Whole-cell extracts of splenic B cells from a set of three independent experiments were examined by Western blot analysis. Protein extracts were derived from unstimulated control and LMP1/CD40-expressing B cells and probed for α-p100/p52, α-pIκBα (Ser32/36) and α-IκBα. Protein loading was controlled by Ponceau-S staining. The independent experiments were performed five times. (B) LMP1/CD40 and control B cells were stimulated with α-CD40 at the indicated time points with (right panel) and without (left panel) prior addition of CHX for 30 minutes (‘) and protein extracts were subjected to Western blot analysis for α-pIκBα and α-IκBα. Equal protein loading was controlled by Ponceau-S staining. The experiment was performed three times.

P52 levels are higher in LMP1/CD40-expressing B cells as compared to control B cells whereas p100 levels are similar, suggesting that the noncanonical NF-κB pathway is activated in these cells and leads to a constant processing of p100 to p52. LMP1/CD40-expressing B cells (L/C) show lower levels of phosphorylated IκBα and similar levels of total IκBα in comparison to control B cells (co). This result implies that the canonical NF-κB pathway is not activated in LMP1/CD40-expressing B cells. Next we investigated whether the canonical NF-κB pathway can still be activated by an external CD40 stimulus or whether it is actively repressed by constitutive CD40 signaling. We stimulated control and LMP1/CD40-expressing B cells *in vitro* with an agonistic α-CD40 antibody for the indicated time points and analyzed the levels of phosphorylated and total IκBα of whole cell extracts on a Western blot (Figure 5B). IκBα is phosphorylated, but compared to control cells IκBα degradation is delayed in LMP1/CD40-expressing B cells when stimulated by an external CD40 stimulus. Thus,

LMP1/CD40 expression dampens the external activation of the canonical NF-κB pathway in B cells. To exclude that IκBα phosphorylation can be only weakly detected in the Western blot in LMP1/CD40-expressing B cells at time point 0 due to high turnover rates, cycloheximide (CHX) that prevents protein synthesis, was added to primary B cells isolated from LMP1/CD40 and control mice 30 minutes before whole cell extracts were prepared (Figure 5B, right panel). LMP1/CD40-expressing B cells do not show lower levels of IκBα after treatment with CHX as compared to control cells and LMP1/CD40 cells in the absence of CHX (-CHX, time point 0) leading to the conclusion that IκBα is not constantly re-synthesized and degraded (Figure 5B). Stimulation of LMP1/CD40-expressing B cells by an agonistic α-CD40 antibody in the presence of cycloheximide confirmed that IκBα is indeed more slowly degraded upon external stimulation (+CHX, time points 10' 30' 60') compared to control cells (Figure 5B). These findings suggest that constitutive CD40 signaling in B cells *in vivo* does not activate the canonical NF-κB pathway and might even dampen its activation, whereas the noncanonical NF-κB pathway seems to be constitutively activated.

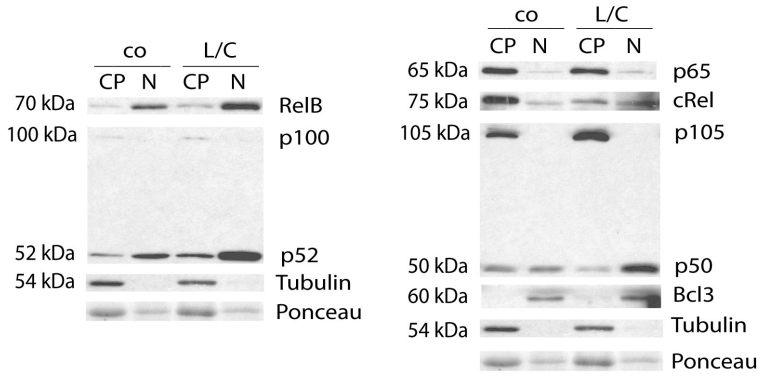


Figure 6 LMP1/CD40 expression induces the activity of the noncanonical NF-κB pathway
Cytoplasmic (CP) and nuclear (N) levels of the NF-κB components RelB, p100/p52, p65, c-Rel, p105/p50 and Bcl-3 of LMP1/CD40 and control B cells were analyzed by Western blotting. Purity of cytoplasmic and nuclear extracts was verified by α-tubulin and α-Bcl3 probing, which are preferentially located in the cytoplasm and nucleus in murine B cells, respectively. Equal protein loading was controlled by Ponceau-S staining. The experiment was performed three times.

The above findings were supported by fractionation experiments where cytoplasmic and nuclear fractions of LMP1/CD40 and control B cells were isolated and subjected to Western blot analysis (Figure 6). This experiment revealed a preferential nuclear appearance of p52 and RelB in LMP1/CD40-expressing B cells in comparison to control B cells, whereas the canonical NF-κB components p65 and c-Rel in the nucleus of LMP1/CD40-expressing B cells are similar to that of control B cells, thus again strengthening the finding that the noncanonical but not the canonical pathway is activated by LMP1/CD40. Of note, c-Rel

levels are reduced in the cytoplasm of LMP1/CD40-expressing B cells as compared to control B cells (Figure 6). Additionally we found elevated p50 levels in the nucleus and higher p105 levels in the cytoplasm of LMP1/CD40-expressing in comparison to control B cells. It is known that p50 as well as p52 can form homodimers that -due to the lack of a transactivation domain- act as transcriptional repressors on NF-κB target genes. However, an unusual member of the IκB familiy, Bcl3, has been shown to be able to bind to these homodimers and to render the complex into a transactivator of NF-κB target genes (Fujita et al., 1993). B cell malignancies like B-CLL and a subgroup of anaplastic large B cell lymphoma have indeed been shown to express high levels of Bcl3 and p50 (McKeithan et al., 1990; Nishikori et al., 2003). Thus we investigated whether the high nuclear level of p50 in LMP1/CD40-expressing B cells correlates with high nuclear levels of Bcl3, hinting that the canonical NF-κB pathway might be activated via p50/p50/Bcl3 in these cells. However, Bcl3 levels are similar in the nuclei of LMP1/CD40 and control cells, thus we concluded that this mode of action does not apply for LMP1/CD40-expressing B cells (Figure 6). These nuclear translocation experiments support the finding that constitutive CD40 signaling in B cells leads to the preferential activation of the noncanonical NF-κB pathway, presumably consisting of RelB/p52 and RelB/p50 heterodimers.

3.1.2 Constitutive CD40 signaling *in vivo* activates the MAPK Jnk and Erk

CD40 signaling leads to the activation of the MAPK Jnk, Erk and p38, which is triggered by phosphorylation at a tyrosine and threonine residue. To determine the activation status of MAPKs in LMP1/CD40-expressing B cells in comparison to unstimulated control B cells, B cells were isolated from the spleen and the phosphorylation status of the MAPKs was analyzed by Western blotting of whole cell extracts. To exhibit the natural variation of phosphorylated and total protein amounts in LMP1/CD40 and control B cells (isolated from CD19-Cre$^{+/-}$ mice), protein extracts from three independent experiments were blotted on one gel and probed for (p)Jnk and (p)Erk (Figure 7A, left panel). The results of at least five independent experiments were additionally evaluated by calculating the mean values of fold induction of the signals from the total and phosphorylated forms of Jnk, Erk and p38 of LMP1/CD40 compared to control B cells (Figure 7A, right panel). Figure 7A shows that total protein levels of the MAPKs are similar in LMP1/CD40 and control B cells whereas LMP1/CD40-expressing B cells show a significantly higher basal phosphorylation and thus constitutive activation of the MAPK Jnk and Erk but not of p38 compared to control B cells

(Figure 7A). Observing that constitutive CD40 signaling *in vivo* in B cells activates Jnk and Erk, we wondered whether these MAPKs can be further activated in LMP1/CD40-expressing B cells by an external CD40 stimulus. We were especially interested in addressing this question since upon TD immunization LMP1/CD40 B cells were not able to mount normal immune responses (Homig-Holzel et al., 2008). The reason for this might be that constitutive CD40 signaling in B cells blocks their further activation to form germinal centers. Stimulation of LMP1/CD40-expressing B cells by an agonistic α-CD40 antibody at the indicated time points *in vitro* revealed a further enhancement in the phosphorylation of MAPKs, although to a lower extent (especially for Erk) as compared to control B cells, suggesting that the constitutive activity of CD40 leads to a low basal activation of Jnk and Erk but dampens their further activation by external CD40 stimulation (Figure 7B).

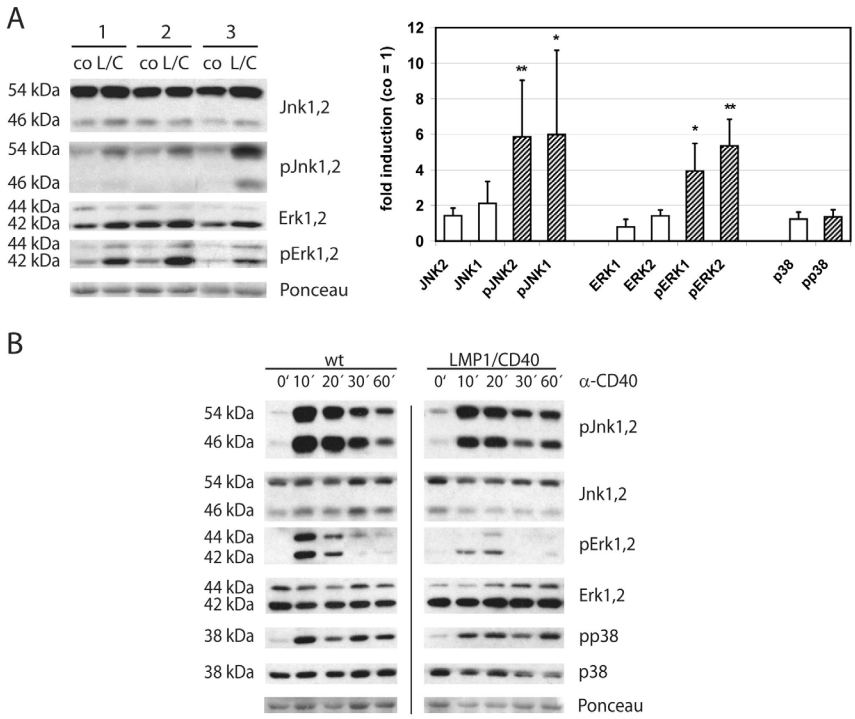


Figure 7 The MAPKs Erk and Jnk are activated in LMP1/CD40-expressing B cells
(A) Whole-cell extracts from a set of three independent experiments were examined by Western blotting. Protein extracts were derived from unstimulated control (CD19-Cre$^{+/-}$) and LMP1/CD40-expressing B cells and probed for α-pJnk1/2 (Thr 183/ Tyr 185), α-Jnk1/2, α-pErk1/2 (Thr 202/ Tyr 204) and α-Erk1/2. Equal protein loading was controlled by Ponceau-S staining. The graph on the left shows mean values of the fold induction of phosphorylated and basal protein levels of Jnk1/2, Erk1/2 and p38 (Thr 180/ Tyr 182) in LMP1/CD40 compared to control B cells. Mean values were calculated from at least five independent experiments, standard deviation (SD) is shown by error bars, * $P<0.05$, ** $P<0.001$, calculated by the two-tailed student's t test. (B) LMP1/CD40 and control B cells were stimulated with α-CD40 for the indicated time points (minutes) and protein extracts were subjected to Western blot analysis using α-pJnk1/2, α-Jnk1/2, α-pErk1/2, α-Erk1/2, α-p-p38, and α-p38. Equal protein loading was controlled by Ponceau-S staining. The experiment was performed three times.

3.1.3 Constitutive activation of Erk is essential for the improved survival of LMP1/CD40-expressing B cells

The observation that constitutive CD40 signaling in B cells *in vivo* activates both the MAPKs Jnk and Erk raised the question whether one or the other pathway is responsible for the improved survival of LMP1/CD40-expressing B cells (Homig-Holzel et al., 2008). Since we did not have the tools to genetically delete one or the other pathway *in vivo*, we decided to make use of small chemical inhibitors in an *in vitro* experiment. Figure 8A shows the survival advantage of LMP1/CD40-expressing B cells over five days *in vitro* as compared to control B cells.

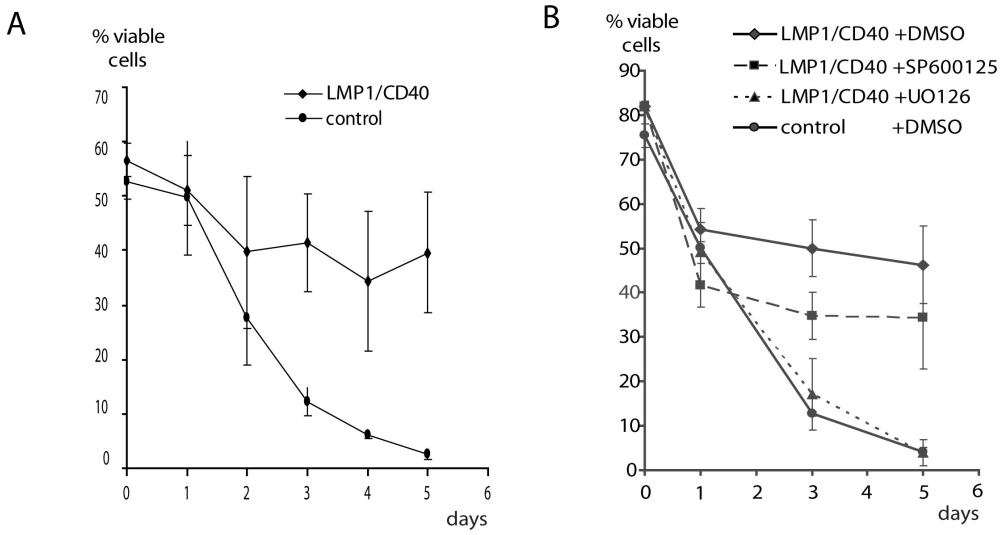


Figure 8 Survival of LMP1/CD40-expressing B cells *in vitro* depends on Erk activity
(A) Splenic B cells from LMP1/CD40 and control mice were cultured for up to 5 days and analyzed for TOPRO-3 negative cells by flow cytometry each day (triangle and circle, respectively). Percentages of living cells (TOPRO-3 negative) are plotted against days in culture. The bars show mean percentages of living cells of three independent experiments. Error bars show the SD. (B) Splenic B cells from LMP1/CD40 were cultured for up to five days with the Mek1/2 inhibitor UO126 (dotted line, triangles) and the Jnk-inhibitor SP600125 (dashed line, squares). As control, B cells from LMP1/CD40 and control mice were cultured in the presence of DMSO (continuous lines, rectangle and circle, respectively). Percentages of living cells (TOPRO-3 negative) were determined by flow cytometry at day 0, 1, 3 and 5. The bars show mean percentages of living cells of three independent experiments. Error bars show the SD.

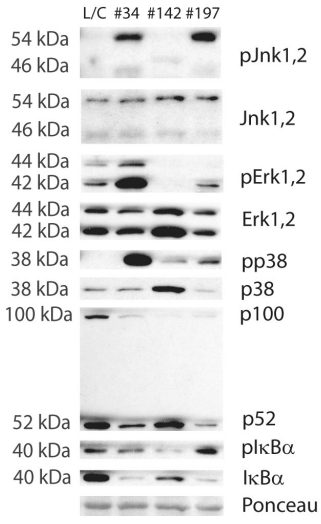
To study whether this enhanced survival is dependent on the activity of Jnk and Erk we cultured LMP1/CD40-expressing and control B cells over a time period of five days *in vitro* with and without small chemical inhibitors for the Jnk (SP600125) and the Erk (UO126) pathway. Survival was measured at day 0, 1, 3 and 5 by subjecting cells to flow cytometry analysis and evaluating the percentage of TOPRO-3 negative cells (Figure 8B). The survival assay revealed that LMP1/CD40 mediated survival of B cells *in vitro* is only marginally

dependent on Jnk activity but strongly dependent on the constitutive Erk phosphorylation. Though addition of SP600125, inhibiting Jnk activity, dampens survival of LMP1/CD40-expressing B cells at day 1 of culture, but does not have further effects on survival from day 3 to 5, where LMP1/CD40-expressing B cells show the same survival kinetics as untreated LMP1/CD40-expressing B cells (+DMSO as the solvent of the inhibitors). In contrast, treatment with UO126, that inhibits the kinase activity of the MAPKK Mek1/2 and therefore the activation of Erk, abrogated enhanced survival of LMP1/CD40 B cells completely; the percentage of surviving cells was similar to that of untreated control B cells (Figure 8B).

3.1.4 Malignant B cells exhibit a diverse pattern of activated signaling pathways

From the age of 12 months on, LMP1/CD40 transgenic mice develop B cell lymphomas with high incidence. In order to investigate if the same signaling pathways are active in premalignant and malignant cells, we performed Western blot analysis of whole cell extracts from splenic tumor samples (Figure 9A).

A



B

	#34	#142	#197	#376	#364	#546	#674
pJnk	++	-	++	-	-	-	-
pErk	+++	-	+	+	++	++	+++
pp38	++	(+)	(+)	++	++	++	++
p52	+	++	+	(+)	+++	+	++
pIκBα	(+)	(+)	++	-	+++	+	++
IκBα	+	++	+	-	+++	+	+

Figure 9 Lymphoma cells from LMP1/CD40 mice show a diverse pattern of activated signaling pathways

(A) Western Blot analysis for the activation of MAPKs and NF-κB in splenic tumor samples (#34, #142, #197) compared to pre-malignant LMP1/CD40-expressing splenic cells (L/C). Whole cell extracts were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Ponceau S staining is shown as a loading control. (B) Table showing the different signaling patterns of seven tumor samples (#) from malignant LMP1/CD40-expressing mice. The signs show the activation status of the proteins in malignant LMP1/CD40-expressing B cells: – not detectable, (+) marginally detectable, + detectable, ++ well detectable, +++ highly detectable.

Since we could not purify tumor B cells from other splenic cells by MACS sorting due to death of the cells during the process, we cultured the cells for two days to obtain a purer tumor cell pool. These were compared to premalignant LMP1/CD40 splenic cells that were treated equally. Within the tumor samples investigated, we could not find a homogenous pattern of activated signaling pathways as we had seen for premalignant cells. Phosphorylation of the MAPK Erk and p38 was found in most of the tumor samples (#34, #197, #376, #364, #546, #674 and #34, #376, #364, #546, #674, respectively) whereas Jnk was only rarely activated (#34, #197). Both the canonical and the noncanonical NF-κB pathway were activated in various samples, but not in all (#197, #364, #674 and #34, #142, #197, #364, #546, #674, respectively) (Figure 9A, B). The detectability of signaling components in tumor samples, estimating the activity, is shown in Figure 9B. To conclude, tumor cells do not activate the same pattern of signaling pathways as premalignant B cells from LMP1/CD40 mice do, suggesting that second events take place that render the cells partly independent of these pathways.

3.2 The role of B cell receptor signaling in LMP1/CD40-activated B cells

Resting peripheral B cells strictly depend on the expression of a B cell receptor as had been shown by Lam *et al*. and Kraus *et al*. (Kraus et al., 2004; Lam et al., 1997). The nature of this so-called tonic BCR signal leading to the survival of B cells is not known. Yet, it is not clear whether the initiation and maintenance of B cell lymphomas, the malignant counterpart, depends on a functional BCR. Most B cell malignancies express the BCR and interestingly, also its coreceptor CD19. Hodgkin lymphoma cells show a similar pattern of activated signaling pathways as LMP1/CD40-expressing B cells and highly express CD40, but are exceptional in that they do not or only weakly express BCR components. Might aberrant CD40 activity thus be complementing the loss of tonic BCR signaling through the chronic activation of NF-κB and MAPKs? To address this issue we examined whether LMP1/CD40 driven B cell expansion is dependent on B cell receptor signals. We did so by deleting two different components of the complex: the signaling molecule Igβ and the coreceptor CD19. The first approach aims to completely abolish tonic survival signals from the BCR by deleting one of the Ig signaling molecules, Igβ. In the second approach we wanted to investigate how deletion of a BCR coreceptor, CD19, affects LMP1/CD40-expressing B cells.

3.2.1 The role of Igß in LMP1/CD40-expressing B cells

It is known from Igα knock-out studies that resting peripheral B cells depend on a functional BCR *in vivo* (Kraus et al., 2004). A former member of our institute, Nathalie Uyttersprot, generated a conditional Igβ-deficient mouse strain. Ablation of Igβ in mature B cells led to a strong reduction of Fo B cells and the disappearance of MZ B cells suggesting that both Igα and Igβ are necessary for tonic BCR signaling and survival of resting B cells (N. Uyttersprot, personal communication; (Kraus et al., 2004). Igα deficient B cells have a half life of 3-4 days and the little B cells observed in the spleen probably still express and signal via Igα due to protein stability despite deletion of the gene locus (Kraus et al., 2004). Since we had the Igβ deficient strain at hand, we used it to answer the question whether constitutive CD40 signaling in B cells overcomes the need for a functional BCR. LMP1/CD40 transgenic mice were crossed to Igβ deficient and CD21-Cre mice to achieve mature B cell specific expression of LMP1/CD40 and deletion of Igβ, thus of tonic BCR signaling.

3.2.1.1 The Igβ deficient mouse strain

Igβ deficient mice were designed as described in Figure 10. Since the loss of Igβ from early B cell stages on results in a complete block of B cell development, N. Uyttersprot generated a conditional knock out of the Igβ C-terminus (the ITAM containing signaling domain) based on the Cre/loxP system. The *Igβ* gene contains six exons of which exon 1 encodes the leader sequence, exon 2 and 3 compose the extracellular domain, exon 4 the transmembrane and exon 5 and 6 the C-terminal domain with the ITAM signaling motif. A termination sequence is located downstream of the *Igβ* gene in the UTR that contains MAZ sites (Myc-associated zinc finger protein). Exon 5 and 6 of the *Igβ* gene were flanked by loxP sites and a cassette consisting of a truncated exon 5, an IRES-GFP flanked by frt sites, as well as the 3' UTR including the termination sequences (MAZ sites) was placed downstream of the Igβ gene. This strategy allows tracking of deleted cells by GFP expression after Cre-mediated recombination in a very elegant way. Excision of the loxP flanked gene segment leads to splicing from exon 4 to the truncated exon 5, removing the signaling domain. Since the transcription termination sequence follows the IRES-GFP, not only a truncated Igβ but also GFP will be translated from the resulting mRNA.

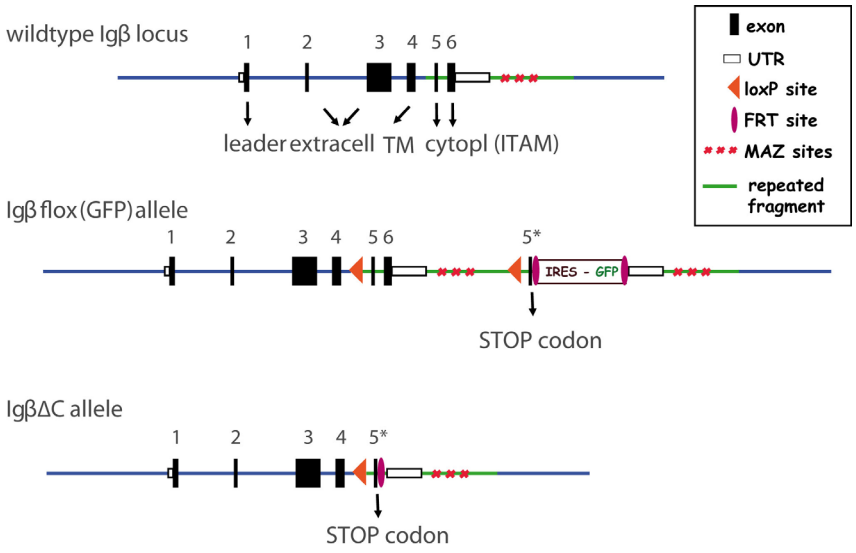


Figure 10 Targeting strategy for the generation of an Igβ deficient mouse strain (adapted from N. Uyttersprot)
The wildtype Igβ locus consists of six different exons, wherein exon 5 and 6 code for the cytoplasmic ITAM-containing signaling domain. These are followed by an UTR (untranslated region) and MAZ (Myc-associated zinc finger protein) sites. The Igβfl(GFP) allele contains two loxP sites that flank exon 5 and 6. Downstream of the second loxP site are a shortened exon 5 with a stop codon and a frt flanked IRES-GFP. Deletion of Exon 5 and 6 by Cre recombinase and deletion of the IRES-GFP results in the IgβΔc allele.

To obtain mice that delete Igβ only in mature B cells, $Igβ^{fl(GFP)/Δc}$//CD21-Cre mice were generated through mouse crossings. In the CD21-Cre mouse strain Cre is expressed under the control of the CD21 promoter, which is only active from the T2 B cell stage on, resulting in the deletion of floxed alleles mainly in mature B cells. Since CD21-Cre does not only delete in mature B cells but also in germ cells, $Igβ^{fl(GFP)/Δc}$//CD21-Cre mice had to be generated by crossing $Igβ^{Δc/Δc}$//CD21-Cre, carrying a deletion of Igβ in all cells of the body, with $Igβ^{fl(GFP)/fl(GFP)}$ mice, resulting in $Igβ^{fl(GFP)/Δc}$//CD21-Cre mice with a Mendelian ratio. In these mice one Igβ allele is deleted in all cell types whereas the second allele ($Igβ^{fl(GFP)}$) will be deleted dependent on Cre, resulting in homozygous inactivation of Igβ and in concomitant GFP expression only in mature B cells. By this method, N. Uyttersprot showed that deletion efficiency in $Igβ^{fl(GFP)/Δc}$//CD21 mice, tracked via GFP expression, is quite low (20-30%) in mature B cells, suggesting that deleted cells are negatively selected and non-deleted cells have a selective advantage and fill up the remaining B cell pool.

3.2.1.2 LMP1/CD40//Igβ$^{fl/\Delta}$ mice and corresponding controls

By crossings, the Igβ$^{fl(GFP)/\Delta c}$//CD21-Cre alleles were combined with the LMP1/CD40 allele to obtain LMP1/CD40//Igβ$^{fl(GFP)/\Delta c}$//CD21-Cre (referred to as LMP1/CD40//Igβ$^{fl/\Delta}$) mice. These were compared to Igβ$^{fl(GFP)/wt}$//CD21-Cre (referred to as Igβ$^{fl/wt}$), Igβ$^{fl(GFP)/\Delta c}$//CD21-Cre (referred to as Igβ$^{fl/\Delta}$) and LMP1/CD40//Igβ$^{fl(GFP)/wt}$//CD21-Cre (referred to as LMP1/CD40//Igβ$^{fl/wt}$) mice. Heterozygous loss of Igβ does not affect B cell development and activation, thus all mice were kept on a heterozygous background to be able to analyze and compare deletion efficiency via GFP expression.

3.2.1.3 Lymphoid compartment characterization of LMP1/CD40//Igβ$^{fl/\Delta}$ mice

To analyze how the loss of Igβ signaling affects LMP1/CD40-expressing B cells, secondary lymphoid organs were obtained from 8 to 12 week old transgenic mice. B cells in the periphery are present in abundant numbers in spleen, lymph nodes and the peritoneal cavity. Fo B cells circulate throughout the body whereas MZ and B1 B cells reside in the spleen and peritoneal cavity, respectively. Since splenomegaly is a hallmark of LMP1/CD40-expressing B cells, we measured the weight of the spleen as a first indication. The splenic weight of LMP1/CD40//Igβ$^{fl/\Delta}$ mice is about half times lower than that of LMP1/CD40//Igβ$^{fl/wt}$ mice; the same can be observed for the splenic weight of Igβ$^{fl/\Delta}$ compared to Igβ$^{fl/wt}$ mice (Figure 11). This observation suggests that B cell numbers are reduced in LMP1/CD40//Igβ$^{fl/\Delta}$ as compared to LMP1/CD40//Igβ$^{fl/wt}$ mice.

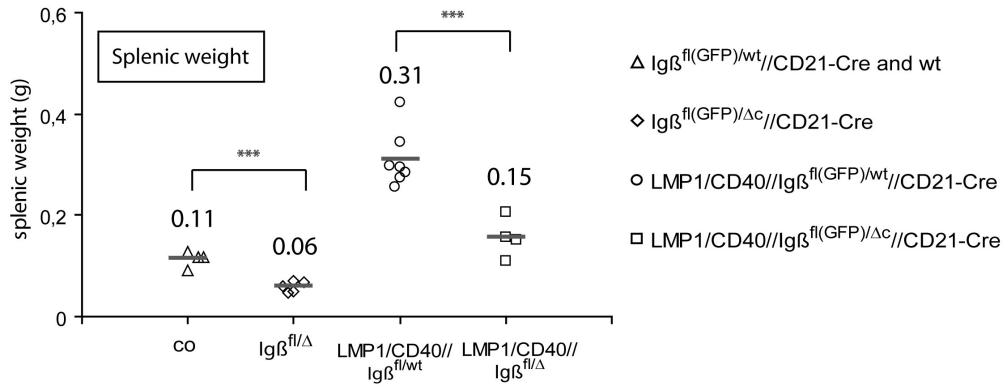


Figure 11 Splenic weight is reduced in LMP1/CD40//Igβ$^{fl/\Delta}$ mice
Mean values of the weight of spleens from the indicated genotypes are shown in a graph. Each symbol represents a single mouse. Mean percentages are indicated by horizontal bars. *** P<0.001, calculated by the two-tailed student's t test.

It is known that the loss of Igβ in peripheral B cells leads to a dramatic reduction of total B cells numbers in peripheral organs. To analyze B cell numbers, we calculated the total number of B cells (TOPRO-3^{-} B220^{+}) in the spleen (SP), inguinal lymph nodes (iLN) and peritoneal cavity (PC) (Figure 12).

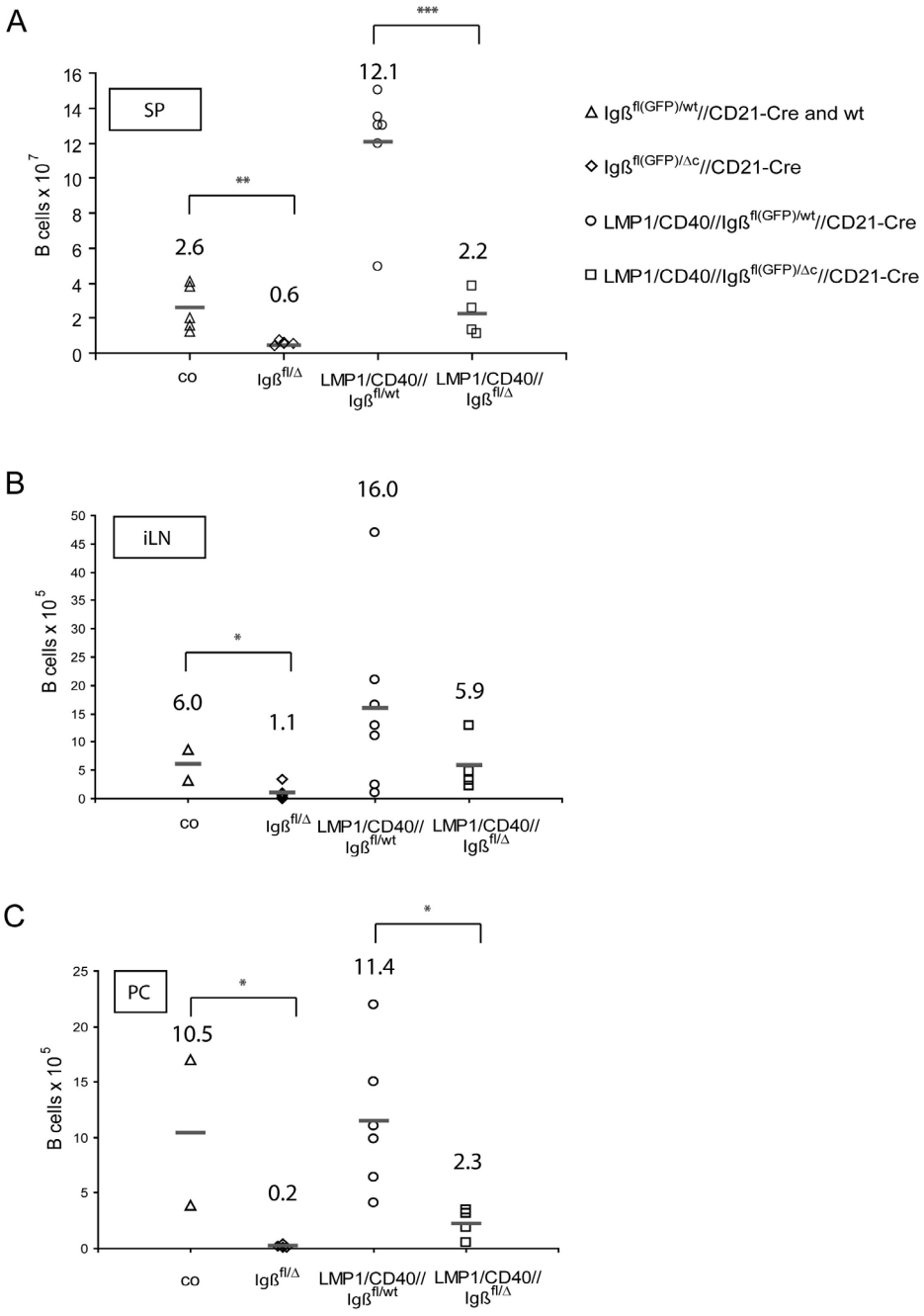


Figure 12 B cell numbers in the peripheral lymphoid organs of control, Igβ$^{fl/\Delta}$, LMP1/CD40//Igβ$^{fl/wt}$ and LMP1/CD40//Igβ$^{fl/\Delta}$ mice

Absolute numbers of B220^{+} B cells in (A) spleen (SP), (B) inguinal lymph nodes (iLN) and (C) peritoneal cavity (PC) of Igβ$^{fl/wt}$, Igβ$^{fl/\Delta}$, LMP1/CD40//Igβ$^{fl/wt}$ and LMP1/CD40//Igβ$^{fl/\Delta}$ mice. Each symbol represents a single mouse. The horizontal bars indicate mean values. * P<0.05, ** P<0.01, *** P<0.001, calculated by the two-tailed student's t test.

This analysis revealed a significant reduction of B cells in all secondary lymphoid organs tested of LMP1/CD40//Igβ$^{fl/\Delta}$ as compared to LMP1/CD40//Igβ$^{fl/wt}$ mice (Figure 12). However, when comparing B cell numbers of LMP1/CD40//Igβ$^{fl/\Delta}$ to Igβ$^{fl/\Delta}$ mice that show a dramatic loss of B cells in all investigated peripheral lymphoid organs as compared to control mice, LMP1/CD40//Igβ$^{fl/\Delta}$ mice have more remaining B cells in the spleen, the inguinal lymph nodes and the peritoneal cavity (three, six and ten times, respectively). Thus, LMP1/CD40-expressing B cells are not independent but less dependent on BCR signaling through Igβ than wildtype B cells.

Deletion of a gene locus via the Cre-loxP system does normally not occur in all targeted cells, a few cells circumvent this process due to various mechanisms. If deletion of a locus is disadvantageous, deleted cells might be negatively selected and non-deleted cells fill up the cell pool. This phenomenon is called "counterselection" and results in a low deletion efficiency. It is known from studies by N. Uyttersprot that Igβ deficient cells are counterselected and show a deletion efficiency of only 20-30% in the spleen. Hence, we were interested whether LMP1/CD40-expressing B cells that are deficient for Igβ are counterselected as well or whether they do not have a disadvantage compared to non-deleting cells. GFP expression analysis of B cells from SP, iLN and PC revealed that deletion efficiency of the Igβ locus (GFP$^+$ among B220$^+$ B cells) is almost as high in LMP1/CD40//Igβ$^{fl/\Delta}$ (75, 84 and 66% for SP, iLN and PC, respectively) as in control (88, 86 and 87%, respectively) and in LMP1/CD40//Igβ$^{fl/wt}$ B cells (90, 90 and 92%, respectively), whereas the efficiency in Igβ$^{fl/\Delta}$ B cells is very low (26, 30 and 9%, respectively) (Table 1). This shows that LMP1/CD40//Igβ$^{fl/\Delta}$ B cells are almost not negatively selected and thus do not have the same survival disadvantage as Igβ B cells, again strengthening the notion that constitutive CD40 signaling renders B cell less dependent on tonic BCR signaling.

Table 1

Deletion efficiency in B cells from SP, iLN and PC

	Mice (n)	SP	iLN	PC
Igß$^{fl/wt}$	2	88±11	86±12	87±14
Igß$^{fl/\Delta}$	5	26±7***	30±12**	9±4***
LMP1/CD40//Igß$^{fl/wt}$	6	90±5	90±2	92±4
LMP1/CD40//Igß$^{fl/\Delta}$	3-4	75±5	84±4	66±11

Percentages within the B lymphocyte gate are shown, values represent the mean ± SD.
** P<0.01, *** P<0.001 in comparison to Igß$^{fl/wt}$

Further, we aimed to analyze how Igβ deficiency in LMP1/CD40-expressing mice influences different B cell subsets. Since we deleted Igβ and expressed LMP1/CD40 through CD21-controlled Cre, we investigated mature B cell subsets. To analyze the level of Ig expression on the surface of these cells and characterize IgM^+IgD^- and IgM^+IgD^+ subsets, we stained splenic B cells from $Ig\beta^{fl/wt}$, $Ig\beta^{fl/\Delta}$, LMP1/CD40//$Ig\beta^{fl/wt}$ and LMP1/CD40//$Ig\beta^{fl/\Delta}$ mice with α-IgM and α-IgD antibodies and subjected them to FACS analysis. In Figure 13A, the expression profile of IgM and IgD is shown in dot plots; the upper row shows $B220^+$ gated and the lower row $B220^+$ and GFP^+ gated cells. The first observation to be made is that LMP1/CD40//$Ig\beta^{fl/\Delta}$ B cells show a dramatic reduction of surface IgM and IgD levels, that becomes even more clear when gating on deleted cells (GFP^+) only (Figure 13A, lower row). In contrast, the few deleted, surviving $Ig\beta^{fl/\Delta}$ B cells retain IgD surface expression, albeit slightly reduced in comparison to control B cells. This indicates that LMP1/CD40//$Ig\beta^{fl/\Delta}$ B cells lose Ig expression from the surface to a certain extent and that they are less sensitive to this loss than $Ig\beta^{fl/\Delta}$ B cells. Similar results were obtained for B cells from the iLNs (data not shown). Moreover, IgM^+IgD^- B cells (transitional and MZ B cells) almost disappear when gating B cells from $Ig\beta^{\Delta/\Delta}$ and LMP1/CD40//$Ig\beta^{fl/\Delta}$ mice on GFP. Thus the IgM^+IgD^- B cells found in $Ig\beta^{fl/\Delta}$ and LMP1/CD40//$Ig\beta^{fl/\Delta}$ mice are undeleted B cells, that constitute either of $CD21^-$ and thus non-deleting transitional B cells deriving from the bone marrow or of undeleted MZ B cells.

To reveal whether the preferential loss of Igβ deleted IgM^+IgD^- B cells can indeed be attributed to MZ B cells, we stained B cells from the spleen for CD21 and CD23 expression (Figure 13B). MZ B cells are $CD21^{high}CD23^-$ whereas Fo B cells are $CD21^{low}CD23^+$. The upper panel of Figure 13B shows dot plots gated on B cells only, the lower panel on GFP^+ B cells. Considering both total and deleted (GFP^+) B cells, $Ig\beta^{fl/wt}$ mice show the normal distribution of about 4 to 5 % MZ and 75% Fo B cells whereas LMP1/CD40-expressing B cells show an increase up to 22 % of MZ B cells as described before (Homig-Holzel et al., 2008). A strong reduction of MZ B cells can be observed in both LMP1/CD40//$Ig\beta^{fl/\Delta}$ and $Ig\beta^{fl/\Delta}$ mice (2%), indicating that MZ B cells are more sensitive to the loss of tonic BCR signaling. A relative reduction of Fo B cells can also be seen in $Ig\beta^{fl/\Delta}$ mice but not in LMP1/CD40//$Ig\beta^{fl/\Delta}$ mice when calculating the percentages within all B cells. The percentages of MZ B cells of $Ig\beta^{fl/\Delta}$ and LMP1/CD40//$Ig\beta^{fl/\Delta}$ mice do not differ between total and deleted B cells (2%), whereas the percentages of Fo B cells do (44 to 75% and 68 to 88%, respectively).

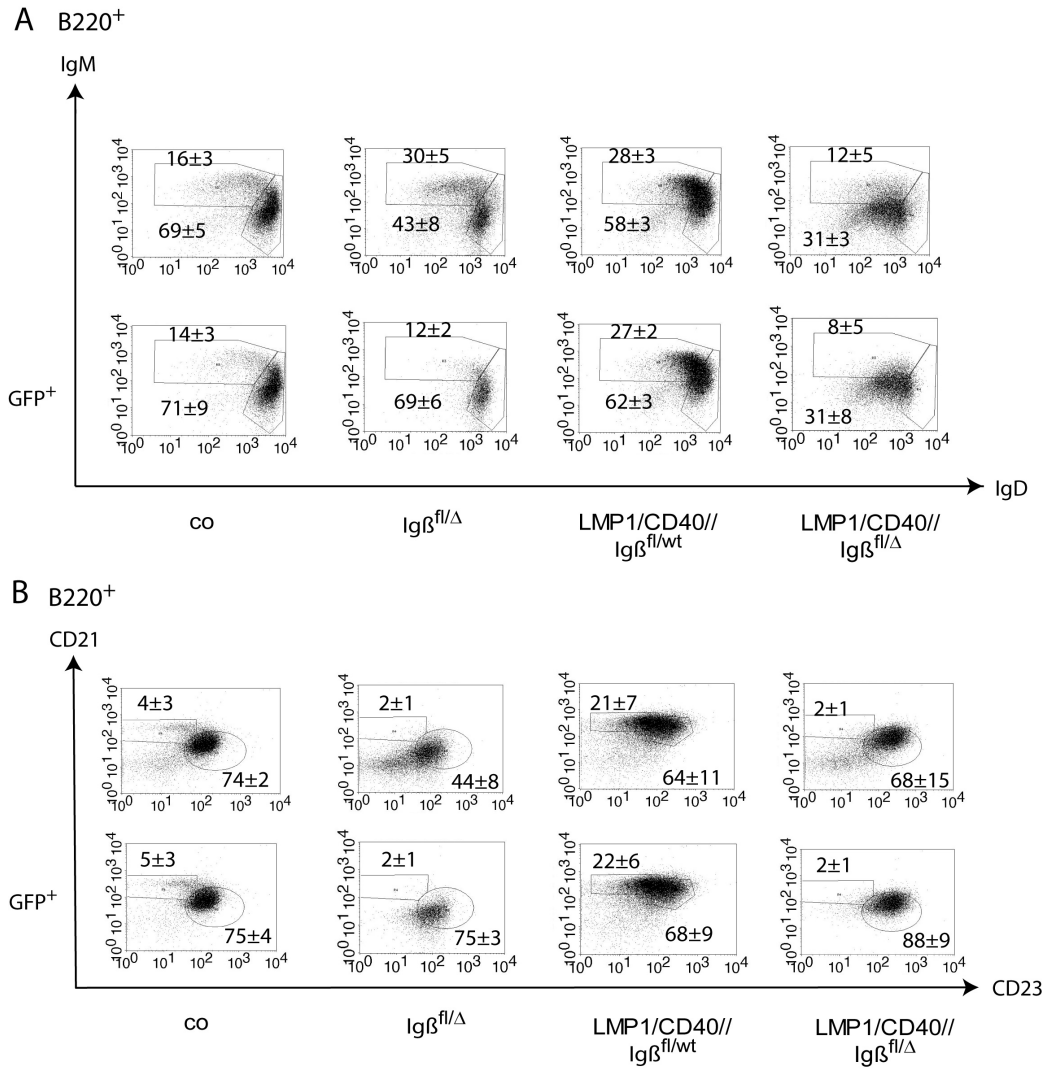


Figure 13 Characterization of the lymphoid compartment
Lymphocytes from the spleen (SP) were gated on B220 and analyzed for the expression of IgM, IgD (A) and CD21 and CD23 (B) by flow cytometry. The respective upper panel is only gated on living, $B220^+$ lymphocytes whereas the respective lower panel is additionally gated on GFP, thus depicting Igβ deleted cells. Numbers are mean values and SD of percentages of gated populations from at least three independent experiments.

This can be attributed to relatively higher percentages of undeleted $CD21^-CD23^-$ B cells as compared to control and LMP1/CD40//$Igβ^{fl/wt}$ mice that disappear when gating on GFP^+ B cells. The splenic $CD21^-CD23^-$ B cell subset represents transitional B cells entering the spleen from the bone marrow. These B cells do not delete Igβ since they do not express CD21. Thus the appearance of undeleted (GFP^-) IgM^+IgD^- B cells can be attributed to relatively higher numbers of transitional B cells ($B220^+CD21^-CD23^-$) in $Igβ^{fl/Δ}$ and LMP1/CD40//$Igβ^{fl/Δ}$ mice that result from relatively lower numbers of mature B cells.

From the relative percentages of total B cells, we calculated total cell numbers of MZ and Fo B cells as shown in Table 2. LMP1/CD40//Igβ$^{fl/wt}$ mice show an expansion of Fo and MZ B cells as compared to control mice (8.2x10^7 and 2.8x10^7 as compared to 2.3x10^7 and 1.5x10^6, respectively). This expansion cannot be seen in LMP1/CD40//Igβ$^{fl/\Delta}$ mice; however, they reveal higher numbers of Fo and MZ B cells than Igβ$^{fl/\Delta}$ mice (1.7x10^7 and 5x10^5 as compared to 3.7x10^6 and 1x10^5, respectively). To conclude, LMP1/CD40-expressing B cells depend less on Igβ signaling than wildtype B cells, however, MZ B cells are more dependent than Fo B cells.

Table 2

Absolute cell numbers in control, Igß$^{fl/\Delta}$, LMP1/CD40//Igß$^{fl/wt}$, and LMP1/CD40//Igß$^{fl/\Delta}$ mice

	Mice (n)	Fo B cells	MZ B cells
SP [x 10^6]			
co	4	22.5±10.9	1.5±1.5
Igß$^{fl/\Delta}$	5	3.7±2.4**	0.1±0
LMP1/CD40//Igß$^{fl/wt}$	7	81.7±20***	27.8±12.9**
LMP1/CD40//Igß$^{fl/\Delta}$	4	17.1±9	0.5±0.5

Values represent the mean ± SD.
** P<0.01, *** P≤0.001, in comparison to control cells

3.2.2 The role of CD19 in LMP1/CD40-expressing B cells

The depletion of Igβ in LMP1/CD40-expressing B cells leads to an abrogation of CD40 mediated expansion of B cells, but rescues Igβ$^{fl/\Delta}$ B cells from loss in the periphery to a certain degree showing that LMP1/CD40-expressing B cell are less dependent on BCR signals than wildtype B cells. CD19, described as a coreceptor of the BCR, is expressed from pro B cells to B cell blasts but is lost during differentiation to plasma cells. Deletion of CD19 results in strong reduction of MZ and B1 B cells and reduction of Fo B cells of about 50%, revealing the involvement of CD19 in mature B cell development and/ or maintenance (Engel et al., 1995; Rickert et al., 1995). CD19 deficient mice are not able to mount TD immune responses suggesting a role for CD19 during B cell activation. Strikingly, CD19 is highly expressed on nearly all B cell malignancies, however, an involvement of CD19 during aberrant B cell activation has not been described (Uckun et al., 1988). Thus we further asked whether CD19 provides survival signals that are required for aberrant, chronic B cell activation and premalignant expansion. To test this assumption, we deleted CD19 in

LMP1/CD40 transgenic mice and analyzed whether aberrant CD40 signaling is able to mediate B cell activation and expansion when CD19 is absent.

CD19 deficiency was achieved by generating homozygous CD19-Cre mice. Cre is placed into and disrupts the CD19 locus and thereby results in a knock out of endogenous CD19. These mice were crossed to LMP1/CD40 transgenic mice to obtain LMP1/CD40-expressing, CD19 deficient mice (LMP1/CD40//CD19$^{-/-}$). They were compared to LMP1/CD40//CD19$^{+/-}$, CD19$^{+/-}$ and CD19$^{-/-}$ mice. CD19 heterozygosity does not show any significant phenotype and serves as a control to reflect the heterozygous situation in LMP1/CD40//CD19$^{+/-}$ mice.

3.2.2.1 B cell numbers in peripheral lymphoid organs

First of all, we investigated how CD19 deficiency in LMP1/CD40-expressing B cells affects B cell numbers in the peripheral organs. 8 to 14 week old mice with the described genotypes (3.2.2) were analyzed. Splenomegaly as a hallmark of LMP1/CD40//CD19$^{+/-}$ mice was not observed in age-matched LMP1/CD40//CD19$^{-/-}$ mice, their spleen showed a similar weight and size as the CD19$^{+/-}$ control (Figure 14A, B).

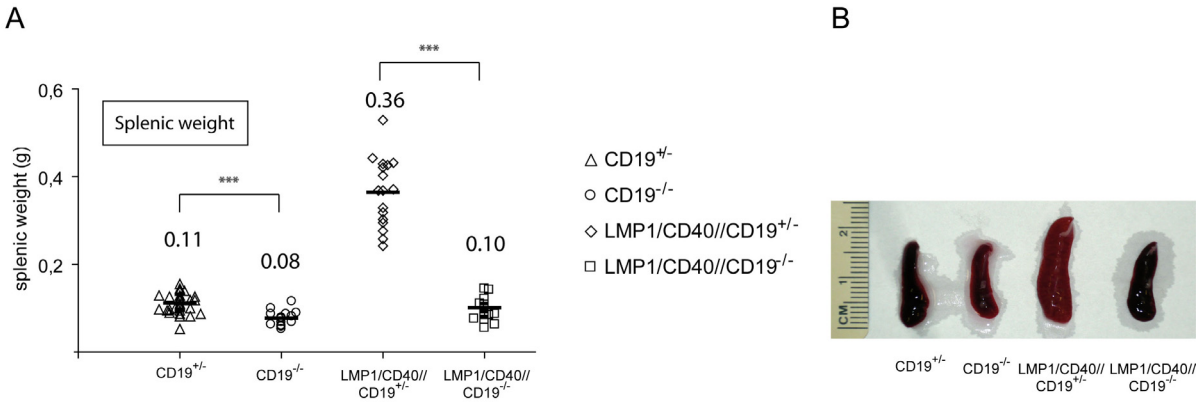


Figure 14 Splenomegaly cannot be observed in LMP1/CD40//CD19$^{-/-}$ mice
(A) Splenic weight of CD19$^{+/-}$, CD19$^{-/-}$, LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ mice was measured and is depicted as a graph. Each symbol represents a single mouse. Mean values of the weight of spleens from each genotype are shown as horizontal bars. *** P<0.001, calculated by the two-tailed student's t test. (B) Spleens dissected from the indicated mice were compared. The ruler indicates the size of the organs.

To determine the total numbers of B cells in SP, iLN and PC, single cell suspensions were prepared and the total amount of cells was counted. Cells were stained with TOPRO-3 and α-B220 to exclude dead cells and stain B cells, respectively. Obtained percentages of living B cells were multiplied with total cell numbers (Figure 15).

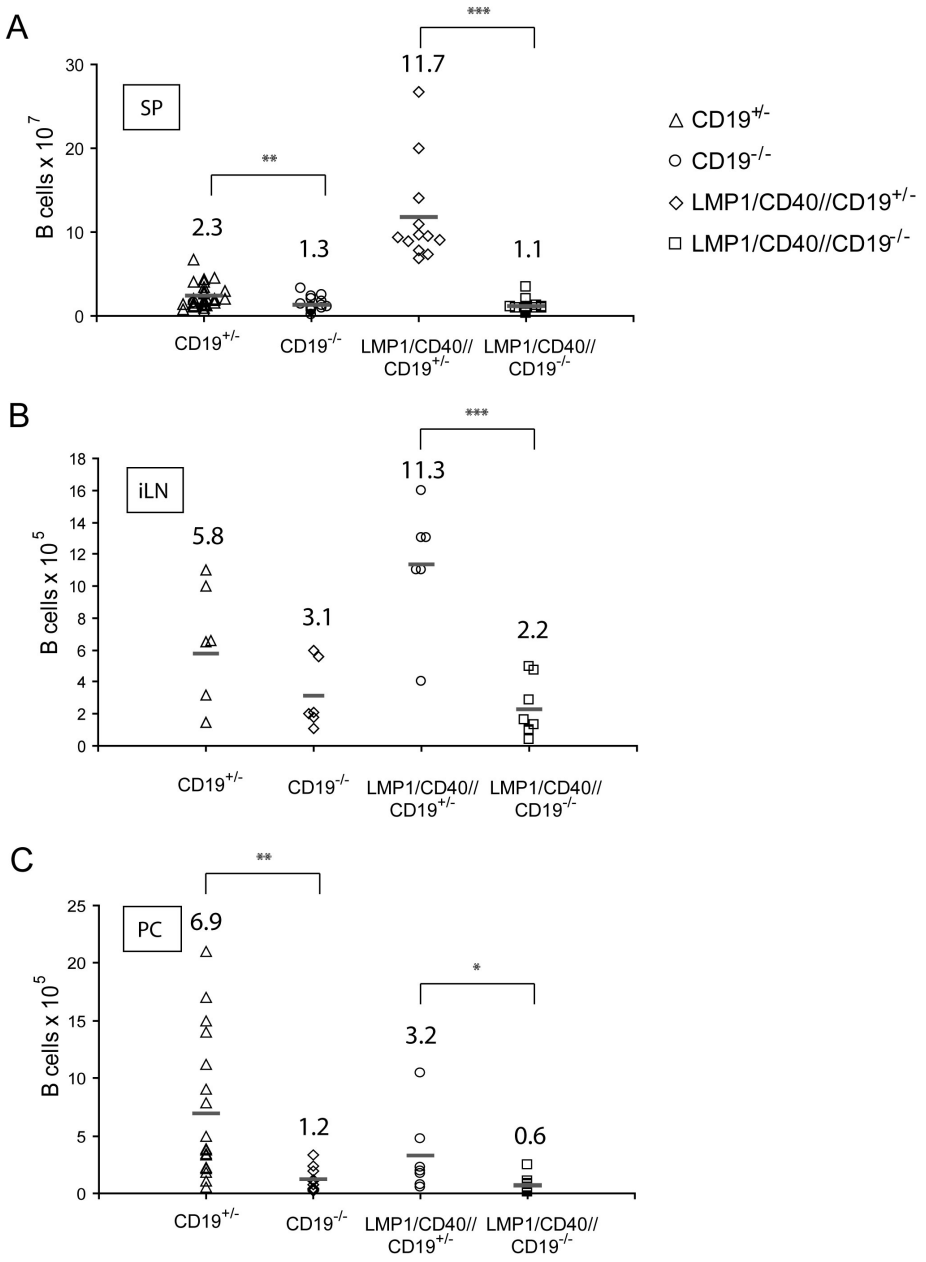


Figure 15 B cell numbers in peripheral lymphoid organs are reduced in LMP1/CD40//CD19$^{-/-}$ mice

Absolute numbers of B220^{+} B cells in (A) spleen (SP), (B) inguinal lymph nodes (iLN) and (C) peritoneal cavity (PC) of CD19$^{+/-}$, CD19$^{-/-}$, LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ mice. Each symbol represents a single mouse. The horizontal bars indicate mean values. * P<0.05, ** P<0.01, *** P<0.001, as calculated by the two-tailed student's t test.

The calculation of B cell numbers in the spleen, lymph nodes and peritoneal cavity revealed that LMP1/CD40//CD19$^{-/-}$ mice exhibit significantly lower numbers of B cells in the periphery than LMP1/CD40//CD19$^{+/-}$ mice (Figure 15). Their numbers in the SP and iLN are comparable to those of CD19$^{-/-}$ mice and are thus about 50% of control CD19$^{+/-}$ mice (Figure 15A, B). In the peritoneal cavity LMP1/CD40-expressing B cells were even almost completely lost in the absence of CD19 and the presence of LMP1/CD40 (Figure 15C). These

results show that loss of CD19 in LMP1/CD40-expressing B cells abolishes their expansion *in vivo* and does not lead to a rescue of $CD19^{-/-}$ B cells as seen for $Ig\beta^{fl/\Delta}$ B cells, suggesting that LMP1/CD40 mediated effects are strictly dependent on CD19.

3.2.2.2 B cell development in the bone marrow

Unlike CD21 whose promoter was used to delete Igβ in mature B cells via Cre expression, CD19 is expressed from the late pro B cell stage on. Thus we analyzed whether its deletion and the expression of LMP1/CD40 disturbs B cell development in the bone marrow explaining the reduction of peripheral B cells. Figure 16 shows a graph of total B cell numbers in the bone marrow (BM) that were calculated by counting absolute numbers of cells and multiplying them with percentages of living $B220^+$ cells obtained by flow cytometry. B cell numbers were reduced in both LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$ as compared to control mice.

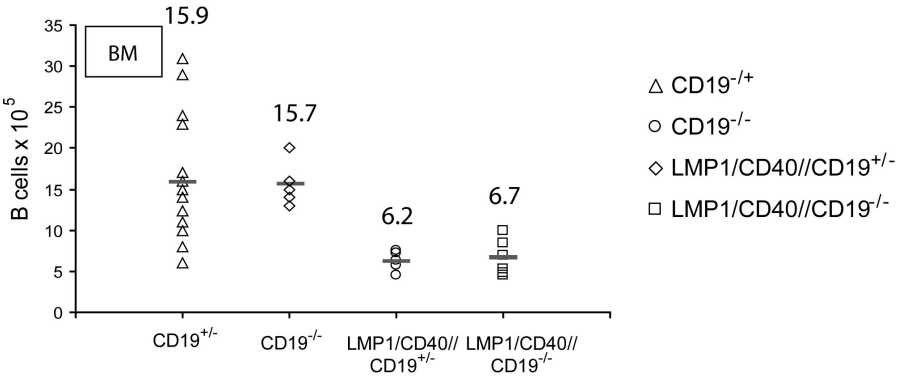


Figure 16 B cell development in the BM in LMP1/CD40//$CD19^{-/-}$ mice
Absolute numbers of $TOPRO3^-$ $B220^+$ B cells in the BM of mice with the indicated genotypes. Each symbol represents a single mouse. Mean values are indicated as horizontal bars.

We further analyzed whether a certain subset of B cells in the BM was responsible for this reduction. For this purpose we stained single cell suspensions from the BM with α-B220, α-CD43, α-IgM and α-IgD antibodies and subjected the cells to flow cytometry. When differentiating the B cell subsets found in the BM, pro ($IgM^-B220^{low}CD43^{high}$) B cell percentages were higher in LMP1/CD40//$CD19^{-/-}$ mice whereas pre ($IgM^-B220^{low}CD43^{neg}$) B cell percentages were slightly reduced in LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$ mice as compared to $CD19^{+/-}$ and $CD19^{-/-}$ mice (Table 3 and Figure S1, upper panel). When differentiating immature ($B220^{low}IgM^+IgD^-$) from mature, recirculating ($B220^{high}IgM^+IgD^+$) B cells, the latter were strongly reduced in LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$

mice (Table 3 and Figure S1, lower panel). The calculation of total B cell numbers showed that not only mature B cells are reduced in LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ as compared to control mice, but also immature B cells (Table 3). The pro and pre B cell stage however was not significantly affected. Hence, LMP1/CD40 expression in BM B cells as such has a slight effect on B cell development, which can be seen in both CD19 pro- and deficient mice. Additionally, the recirculation of mature B cells to the BM seems to be impaired by LMP1/CD40 expression.

Table 3

B cell populations in the BM of CD19$^{+/-}$, CD19$^{-/-}$, LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ mice

%		Mice (n)				
	Gated on: % of:		IgM$^-$ B220^{low}CD43$^+$ Pro B	IgM$^-$ B220^{low}CD43$^-$ Pre B	B220$^+$ IgM$^+$IgD$^-$ Immature B	B220$^+$ IgM$^+$IgD$^+$ Mature B
	CD19$^{+/-}$	5	8±2	40±12	20±4	17±8
	CD19$^{-/-}$	5	8±3	37±9	19±5	21±8
	LMP1/CD40//CD19$^{+/-}$	4	8±3	24±12	20±3	6±3
	LMP1/CD40//CD19$^{-/-}$	6	16±3**	27±6	17±3	2±1**
[x 10^5]						
	CD19$^{+/-}$	5	1.5±0.8	6.1±0.8	2.6±1.2	3.1±1.9
	CD19$^{-/-}$	5	1.8±0.6	8.5±2	2.5±0.5	2.8±0.7
	LMP1/CD40//CD19$^{+/-}$	4	2±1.3	4.7±1.4	1.5±0.6	0.7±0.6
	LMP1/CD40//CD19$^{-/-}$	6	1.9±1.0	3.6±2.2	1.1±0.4*	0.2±0.2*

Percentages within the lymphocyte gate are shown. Values represent the mean ± SD.
* P<0.05, ** P<0.01, in comparison to CD19$^{+/-}$

3.2.2.3 Analysis of the B cell compartments in LMP1/CD40//CD19$^{-/-}$ mice

Since the deletion of CD19 in LMP1/CD40-expressing B cells led to a complete abolishment of B cell expansion in spleen and lymph nodes, we were interested how the different B cell subsets are affected. B cell subsets can be characterized by staining of different surface molecules and analysis via flow cytometry. Since CD19 is deleted in all B cell subsets, we analyzed transitional and MZ B cells in the spleen, Fo B (B2) cells in the spleen and lymph nodes and B1 B cells in the peritoneal cavity.

3.2.2.3.1 LMP1/CD40 transgenic mice show reduced numbers of transitional B cells

Bone marrow derived, transitional B cells enter the spleen from the blood stream and differentiate through T1, T2 and T3 stages to become mature B cells. Transitional B cells can be stained by α-AA4.1 and α-B220 antibodies and their different stages can be separated by

the expression of IgM and CD23. The numbers of transitional B cells in LMP1/CD40//CD19$^{+/-}$, LMP1/CD40//CD19$^{-/-}$ and CD19$^{-/-}$ mice are slightly reduced as compared to control CD19$^{+/-}$ mice (Table 4). These three genotypes show a relative reduction of T1 B cells (B220^{+}AA4.1^{+}IgM^{high}CD23^{-}), but normal percentages of T2 (B220^{+}AA4.1^{+}IgM^{high}CD23^{+}) and T3 (B220^{+}AA4.1^{+}IgM^{low}CD23^{+}) B cells as compared to CD19$^{+/-}$ B cells (Table 4 and Figure S2). The calculation of total numbers of T1, T2 and T3 transitional B cells pointed to a reduction of predominantly T1 B cells in LMP1//CD40//CD19$^{-/-}$, LMP1/CD40//CD19$^{+/-}$ and CD19$^{-/-}$ mice (Table 4). Thus, LMP1/CD40//CD19$^{-/-}$ mice harbor less transitional B cells in the spleen.

3.2.2.3.2 CD19 deficiency in LMP1/CD40 transgenic mice leads to a reduction of mature peripheral B cells

Furthermore we analyzed how the two main subsets of mature B cells in the SP -MZ and Fo B cells- contribute to the reduced B cell numbers present in LMP1/CD40//CD19 mice. These two cell types can be differentiated via the expression of CD21 and CD23 (Figure 17).

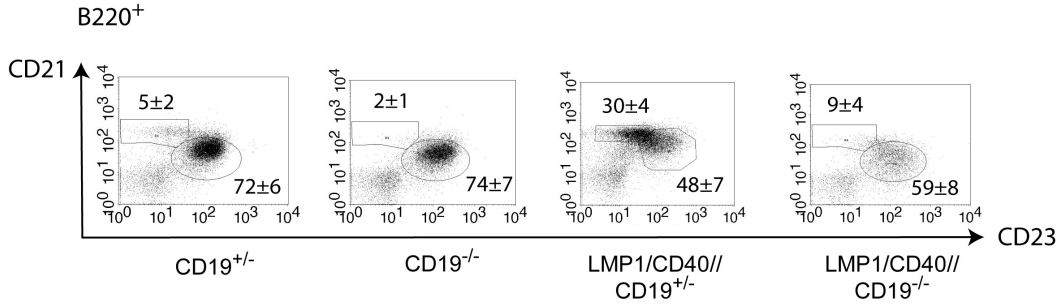


Figure 17 Distribution of MZ and Fo B cells in LMP1/CD40//CD19$^{-/-}$ mice
TOPRO3^{-} lymphocytes from SP were gated on B220^{+} cells and analyzed for the expression of CD21 and CD23 by flow cytometry. MZ (CD21^{high}CD23^{low}) and Fo (CD21^{int}CD23^{+}) B cells. Numbers are mean values and SD of percentages of gated populations from seven independent experiments.

The calculation of total cell numbers revealed that Fo (CD21^{int}CD23^{+}) B cells of LMP1/CD40//CD19$^{-/-}$ mice are not expanded as seen in LMP1/CD40//CD19$^{+/-}$ mice (8.5x10^{6} versus 5.3x10^{7}); they are even reduced as compared to CD19$^{+/-}$ mice (1.6x10^{7}) similar to CD19$^{-/-}$ mice (1.2x10^{7}) (Table 4). The MZ (CD21^{high}CD23^{low}) B cell compartment of LMP1/CD40//CD19$^{-/-}$ mice seems to be about the same size as that of CD19$^{+/-}$ mice (1.3x10^{6} versus 1.2x10^{6}), thus the expansion observed in LMP1/CD40//CD19$^{+/-}$ mice cannot be seen (3.7x10^{7} MZ B cells) (Table 4).

Table 4

B cell populations in SP of $CD19^{+/-}$, $CD19^{-/-}$, LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$ mice

%	Mice (n)						
Gated on: % of:		$B220^+$ $AA4.1^+$ T B	$B220^+AA4.1^+$ $IgM^{high}CD23^-$ T1 B	$B220^+AA4.1^+$ $IgM^{high}CD23^+$ T2 B	$B220^+AA4.1^+$ $IgM^{low}CD23^+$ T3 B	$B220^+$ $CD21^+CD23^-$ MZ B	$B220^+$ $CD21^{low}CD23^+$ Fo B
$CD19^{+/-}$	12-16	15±5	33±9	28±8	18±6	5±2	72±6
$CD19^{-/-}$	15-17	12±4	23±7**	36±10*	17±5	2±1**	75±6
LMP1/CD40//$CD19^{+/-}$	5-7	2±1**	20±12*	34±10	18±7	31±3***	48±7***
LMP1/CD40//$CD19^{-/-}$	13-14	9±3*	19±15**	35±10	16±7	9±4**	59±8***
[x 10^6]							
$CD19^{+/-}$	12-16	3±2.4	0.9±0.5	0.6±0.2	0.4±0.2	1.2±0.8	16.1±8.2
$CD19^{-/-}$	15-17	2.4±1.6	0.6±0.4	0.7±0.4	0.4±0.2	0.4±0.2***	11.7±6.0
LMP1/CD40//$CD19^{+/-}$	5	1.6±1.1	0.6±0.3	0.7±0.6	0.4±0.2	37±8.7***	53.2±8.2***
LMP1/CD40//$CD19^{-/-}$	12-14	0.9±0.5*	0.1±0.1***	0.4±0.3	0.2±0.2	1.3±0.9	8.5±5.4*

Percentages within the lymphocyte gate are shown. Values represent the mean ± SD.
* P<0.05, ** P<0.01, *** P<0.001, in comparison to $CD19^{+/-}$

But, compared to CD19$^{-/-}$ mice, which lack nearly all MZ B cells (4x10^5), the MZ B cell compartment might be rescued to some extent by LMP1/CD40 expression. However, immunohistochemistry of splenic sections showed that the appearance of MZ B cells in LMP1/CD40//CD19$^{-/-}$ mice in the flow cytometry assay might be due to a marker shift rather than to B cells residing in the marginal zone of the spleen (data not shown). These results show that both MZ and Fo B cells are reduced in LMP1/CD40//CD19$^{-/-}$ as compared to LMP1/CD40//CD19$^{+/-}$ mice.

3.2.2.3.3 Mature LMP1/CD40//CD19$^{-/-}$ B cells display an activated phenotype

Another hallmark of constitutive CD40 signaling in mature B cells *in vivo* is the expression of several activation markers on the cell surface. Splenic B cells were stained for CD95, ICAM-1 and MHC class II and analyzed by FACS (Figure 18).

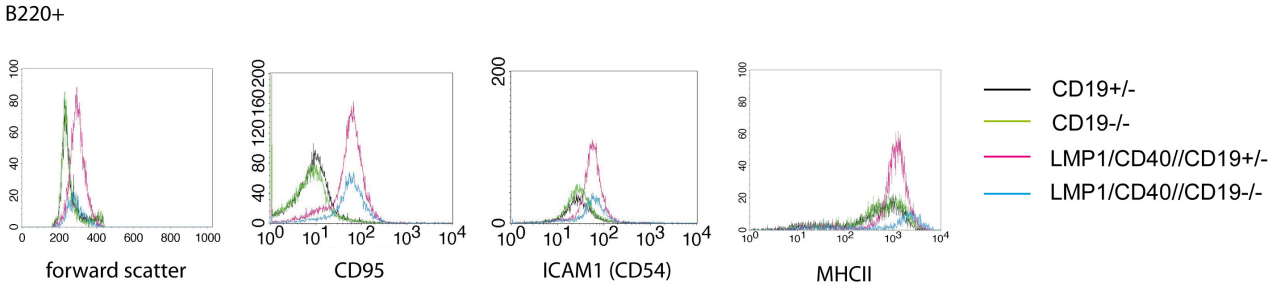


Figure 18 LMP1/CD40//CD19$^{-/-}$ B cells display an activated phenotype
The analysis of cell size and surface expression of CD95, ICAM-1 and MHCII on splenic TOPRO3$^-$ B cells was performed by flow cytometry. Histograms show an overlay of cell size (1st histogram) and overlays of surface expression levels of the indicated molecules on B cells (2nd to 4th histogram) from the indicated genotypes in a representative experiment of at least three. CD19$^{+/-}$ (black line), CD19$^{-/-}$ (green line), LMP1/CD40//CD19$^{+/-}$ (red line), LMP1/CD40//CD19$^{-/-}$ (blue line).

LMP1/CD40//CD19$^{-/-}$ B cells show high expression of CD95, ICAM-1 and MHC class II as LMP1/CD40//CD19$^{+/-}$ B cells do (Figure 18). Interestingly, analysis of the forward scatter revealed that the size of LMP1/CD40//CD19$^{-/-}$ B cells is comparable to control cells and is thus significantly smaller than that of LMP1/CD40//CD19$^{+/-}$ B cells. Similar results were obtained for B cells from the iLNs. Hence, LMP1/CD40 expression still maintains an activated B cell phenotype when CD19 is lost from the cells but does not lead to an increase in cell size.

3.2.2.3.4 B cells are lost in the peritoneal cavity of LMP1/CD40//CD19$^{-/-}$ mice

B1 cells in the peritoneal cavity (and in few numbers in the SP) are IgM only cells that have self-renewing capacity. They can be divided into B1a and B1b cells upon their expression of CD5. B1 cells have been shown to require strong BCR signals (maybe by self-antigens) to be generated and are dependent on CD19 expression. B1 cells are also diminished upon LMP1/CD40 expression. Since we saw that B cells are almost completely lost in the PC of LMP1/CD40//CD19$^{-/-}$ mice, we did not calculate absolute numbers of the B cells subsets, the relative percentages can be seen in Table 5. To analyze whether both B1 and B2 cells of the PC are reduced, we stained PC derived cells with α-B220, α-IgM and α-CD5 antibodies and subjected the cells to flow cytometry analysis. Interestingly, LMP1/CD40//CD19$^{-/-}$ mice did not only lose B1 cells (CD5$^{+/-}$IgM^{+}B220^{low}), therein preferentially B1a B cells (CD5^{+}IgM^{+}B220^{low}), in the peritoneal cavity but also B2 cells (CD5^{-}IgM^{+}B220^{high}) as shown by staining of IgM and B220 (Table 5 and Figure S3, upper panel). These results show that CD40 activated B cells of the peritoneal cavity are particularly dependent on CD19 signaling.

Table 5

B cell populations in the PC of CD19$^{+/-}$, CD19$^{-/-}$, LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ mice

%	Mice (n)				
% of:		B220^{+} B	B220^{low}CD5^{+} B1a	B220^{low} CD5^{-} B1b	B220^{int}CD5^{-} B2
CD19$^{+/-}$	4-11	44±17	37±11	14±5	43±18
CD19$^{-/-}$	4-9	33±16	5±3***	16±9	57±21
LMP1/CD40//CD19$^{+/-}$	3-5	42±14	21±5**	12±5	51±15
LMP1/CD40//CD19$^{-/-}$	5-9	16±13***	7±3***	16±8	48±17

Percentages within the IgM^{+} lymphocyte gate are shown. Values represent the mean ± SD.
** P<0.01, *** P<0.001, in comparison CD19$^{+/-}$

3.2.3 LMP1/CD40 signaling and function depend on CD19

The analysis of the role of Igβ and CD19 in premalignant B cells of the LMP1/CD40 transgenic mice reveals that peripheral, mature LMP1/CD40-expressing B cells are less dependent on Igβ-mediated tonic BCR signaling than control B cells, however they depend on CD19 to the same extent as control B cells. This is striking since we expected the loss of Igβ to have a more dramatic effect on LMP1/CD40 B cells than the loss of CD19. Our results imply that CD19 signaling has a distinct role from being a coreceptor of the B cell receptor during activation of B cells by (constitutive) CD40 signaling. Indeed, it has been speculated whether CD19 serves a coreceptor for CD40 as well. Out of these unexpected findings a new

question formed, namely, why is CD40 activation of B cells strictly dependent on CD19? To investigate this question in further depth, we analyzed how CD19 loss impairs LMP1/CD40 induced B cell survival, proliferation, lymphomagenesis and signaling.

3.2.3.1 LMP1/CD40 mediated survival and proliferation *in vitro* are dependent on CD19

Constitutive CD40 signaling via LMP1/CD40 was shown to increase survival and to induce spontaneous cell division of B cells *in vitro,* which might be the reason for the expansion of B cells observed *in vivo* (Homig-Holzel et al., 2008). Consequently, we asked whether CD19 deletion inhibits improved proliferation and enhanced survival of LMP1/CD40-expressing B cells.

For that purpose, LMP1/CD40//CD19$^{-/-}$ B cells were isolated from the spleen and cultured *in vitro* for five days without additional stimuli (Figure 19). Proliferation and survival of cells was determined by counting of living cells, CFSE (Carboxyfluorescein succinimidyl ester) and TOPRO-3 staining. In comparison to LMP1/CD40//CD19$^{+/-}$, B cell numbers of LMP1/CD40//CD19$^{-/-}$ decreased faster from the beginning of the culture (day 0, 5x10^5 cells) to day 1 but showed similar kinetics from day 2 on and were slightly higher than CD19$^{+/-}$ and CD19$^{-/-}$ cells from day 3 to 5 in culture, suggesting that a small percentage of cells shows improved survival or proliferation (Figure 19A). In parallel, proliferation was measured by labeling the cells with CFSE, a dye that binds to proteins on the inner membrane of cells and is thus transmitted equally to each daughter cell after cell division, resulting in a decline of fluorescence intensity per cell. At day 3 the proliferation rate of LMP1/CD40//CD19$^{-/-}$ B cells was comparable to that of control cells and averaged out at 11% whereas LMP1/CD40-expressing B cells showed around 24% proliferating cells (Figure 19B).

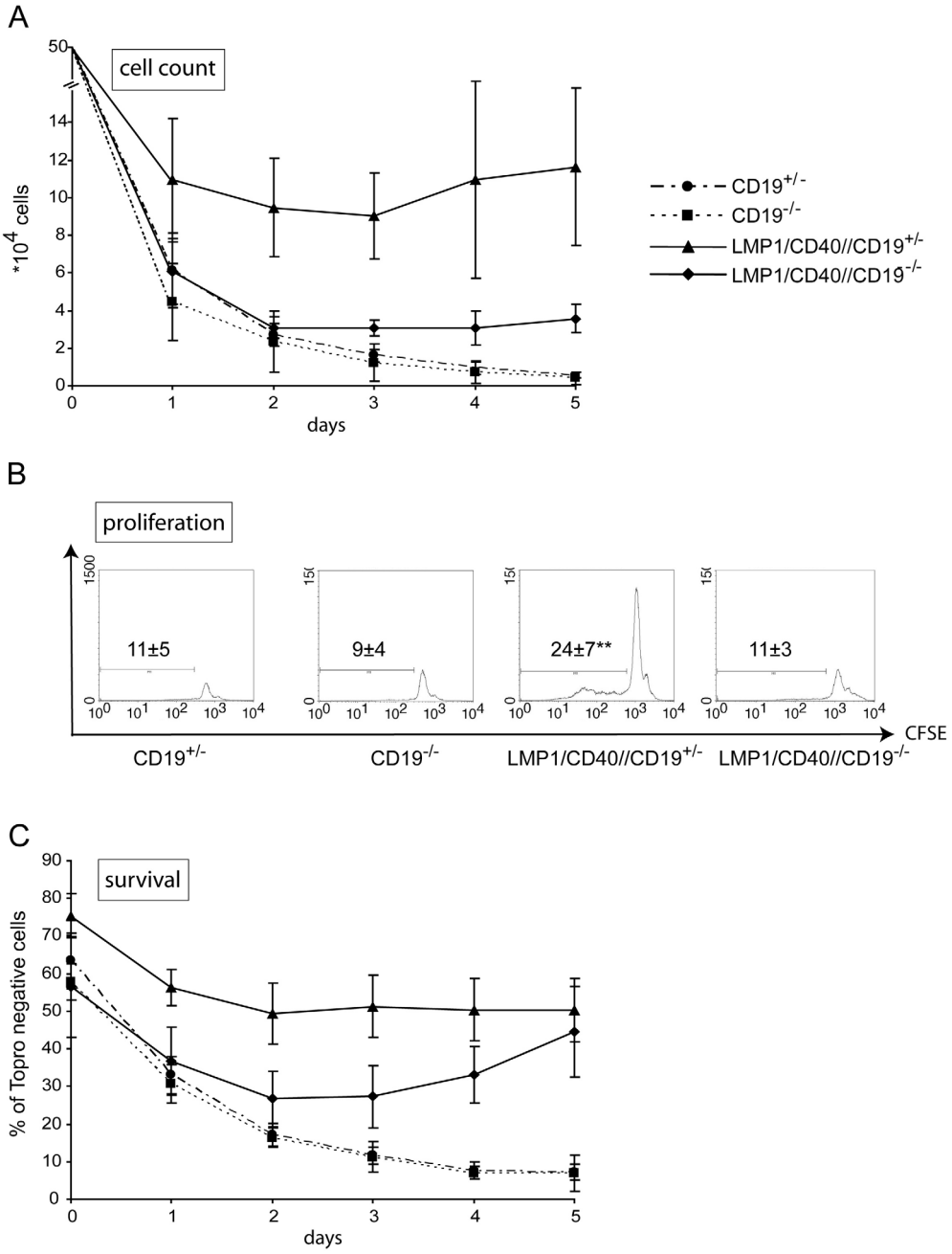


Figure 19 LMP1/CD40 mediated survival and proliferation *in vitro* are dependent on CD19
$5x10^5$ MACS-purified splenic B cells from the indicated genotypes were cultured for 5 days. (A) Numbers of viable cells were determined at day 1, 2, 3, 4 and 5 by flow cytometry based counting of living cells (TOPRO-3^-). The symbols show mean percentages of living cells of at least three independent experiments. Error bars indicate SD. (B) B cells were labeled with CFSE before culture. At day 3, the proliferation rate of TOPRO-3 negative cells was determined by FACS analysis and is shown in a histogram. Numbers indicate mean values and SD from at least three independent experiments. ** $P<0.01$ (C) Percentages of living cells were determined daily by flow cytometry. The symbols show mean percentages of living cells of at least three independent experiments. Error bars indicate SD.

The survival of LMP1/CD40//$CD19^{-/-}$ B cells was measured by TOPRO-3 staining of cultured cells at the indicated time points over five days (Figure 19C). In the first days of culture the

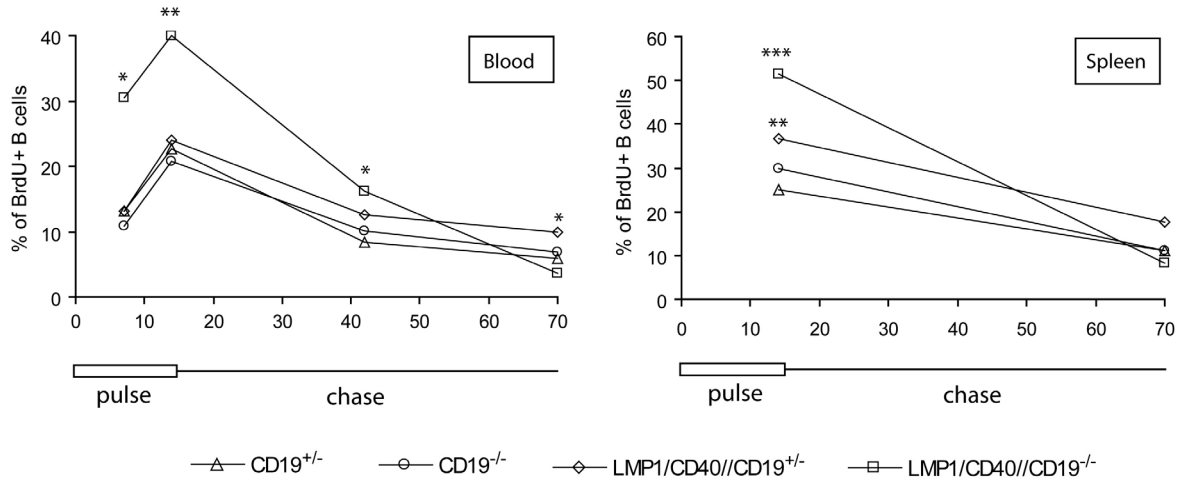
pool of LMP1/CD40//CD19$^{-/-}$ cells showed low percentages of living cells as observed for unstimulated CD19$^{+/-}$ and CD19$^{-/-}$ cells. Interestingly, after three to five days the percentages of living cells increased again. These results show that LMP1/CD40//CD19$^{-/-}$ B cells do not proliferate and show impaired, but not abolished survival as compared to LMP1/CD40//CD19$^{+/-}$ cells *in vitro*.

3.2.3.2 LMP1/CD40//CD19$^{-/-}$ B cells have a reduced life span *in vivo*

To analyze whether the reduced numbers of B cells in the peripheral lymphoid organs from LMP1/CD40//CD19$^{-/-}$ as compared to LMP1/CD40//CD19$^{+/-}$ mice result from reduced generation, longevity or the lack of proliferation *in vivo*, we performed BrdU pulse chase assays. BrdU (Bromodeoxyuridine) is a thymidine analog that is incorporated into cells during DNA synthesis. It thus labels cells that have replicated their DNA in the S-phase of the cell cycle (pulse). These labeled cells can be traced over periods of time without further labeling in order to determine their life span (chase). Thus during the pulse, the percentage of B cells that entered the S-phase of the cell cycle can be measured whereas during the chase the survival of B cells can be determined. Peripheral, resting B cells do not proliferate so that BrdU positive B cells in the periphery arise from transitional B cells emerging from the bone marrow where they underwent several cycles of cell division. However due to LMP1/CD40 expression, proliferation in peripheral B cells might take place in transgenic mice. To perform this assay *in vivo*, mice were fed with BrdU in their drinking water over 14 days (pulse) and further inspected over a period of 56 days until day 70 (chase). At time points 7 and 14 of the pulse and 42 and 70 during the chase mice were bleeded and lymphocytes were isolated from the blood. At day 14 and 70 splenic B cells were analyzed in parallel to blood-derived B cells. At day 7 and 14 of the pulse period, CD19$^{+/-}$, CD19$^{-/-}$ and LMP1/CD40//CD19$^{+/-}$ mice showed relatively similar percentages of BrdU^{+} B cells in the blood, whereas LMP1/CD40//CD19$^{-/-}$ mice showed an increase of about three fold in blood and spleen (Figure 20B). In contrast to the cells derived from the blood, splenic LMP1/CD40//CD19$^{+/-}$ mice exhibited a slight increase in BrdU^{+} B cells as compared to control cells. The increase of BrdU^{+} B cells during the pulse may have two different reasons: if B cells are rapidly lost in the periphery due to cell death, their pool will be constantly refilled by cell influx from the bone marrow. Since cells transitioning from the bone marrow underwent several cycles of cell division during their maturation, they are labeled with BrdU. Thus this event does not reflect cell division in the periphery but increased turnover rates of B cells. On the other hand the increase can reflect

cells that proliferate in the periphery due to transgene mediated effects on the cells and thus take up BrdU into their DNA during S-phase of the cell cycle.

A



B

d	$CD19^{+/-}$	$CD19^{-/-}$	$L/C//CD19^{+/-}$	$L/C//CD19^{-/-}$
7	13±4	11±4	13±9	31±15*
14	23±8	21±4	24±11	40±13**
42	8±2	10±1	13±3	16±4*
70	6±0	7±1	10±2*	4±0**

d	$CD19^{+/-}$	$CD19^{-/-}$	$L/C//CD19^{+/-}$	$L/C//CD19^{-/-}$
14	25±5	30±6	37±6**	51±10***
70	11±3	11±3	18±3	8±3

* P<0.05, **P<0.01, *** P<0.001

Figure 20 LMP1/CD40//$CD19^{-/-}$ B cells have a reduced life span *in vivo*
Mice were fed with 0.8 mg/ml BrdU in their drinking water for 14 days during the pulse period and tracked until day 70 during the chase period. Blood samples were taken at day 7, 14, 42 and 70 (left panel), splenic samples at day 14 and 70 (right panel). $BrdU^+$ $B220^+$ B cells were determined by flow cytometry analysis. (A) The graphs show the mean results (symbols) of three different BrdU pulse chase experiments to determine the survival of B cells *in vivo*. The four different genotypes are indicated below the graphs. y-axis= % of $BrdU^+$ B cells, x-axis=time as days (B) $BrdU^+$ B cells from the indicated four genotypes at the indicated timepoints are shown as mean values with SD. Left panel shows results from blood derived B cells, right panel those from spleen. d=day (time point of taken sample), L/C=LMP1/CD40.

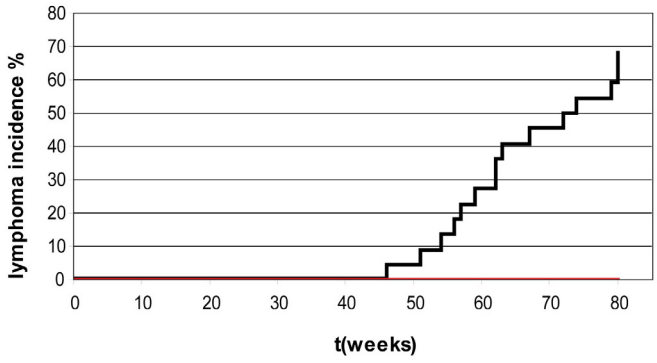
Since we could not observe an increased percentage of cells in the S-phase within all LMP1/CD40//$CD19^{-/-}$ B cells as measured by DNA content (data not shown), we investigated whether LMP1/CD40//$CD19^{-/-}$ are lost rapidly in the periphery as compared to control cells by evaluating the chase period of the BrdU experiment arguing for a high turnover. Tracing of BrdU labeled B cells over time (day 14, 42 and 70) revealed that LMP1/CD40//$CD19^{-/-}$ B cells are indeed rapidly lost in blood and spleen (Figure 20A, B). They drop from a mean value of 40% $BrdU^+$ B cells to 4% in the blood and from 51% to 8% in the spleen, whereas

$CD19^{+/-}$ control cells drop from 23% to 6% in the blood and 25 to 11% in the spleen from day 14 to 70 (Figure 20B). This is visualized in the graphs of Figure 20A showing an increased decline of the curve of LMP1/CD40//$CD19^{-/-}$ as compared to $CD19^{+/-}$ $BrdU^{+}$ B cells from day 14 to 70 in blood and spleen (about three times higher in the SP, as calculated with descending slope rates). Thus LMP1/CD40//$CD19^{-/-}$ B cells have a reduced life span in the periphery and enhanced BrdU incorporation is most likely due to increased influx of B cells from the bone marrow and not to proliferation in the periphery. In the blood, LMP1/CD40//$CD19^{+/-}$ B cells show slightly better survival than $CD19^{+/-}$ B cells (visualized by a less descending curve), however in the spleen, LMP1/CD40//$CD19^{+/-}$, $CD19^{+/-}$ and $CD19^{-/-}$ mice showed a similar decline in BrdU labeled B cells, suggesting a similar life span. The differences in spleen and peripheral blood in LMP1/CD40//$CD19^{+/-}$mice have to be investigated in further depth.

3.2.3.3 LMP1/CD40//$CD19^{-/-}$ mice do not develop lymphomas

LMP1/CD40//$CD19^{+/-}$ mice develop lymphomas with high incidence from the age of one year on. Hence, constitutive CD40 signaling *in vivo* promotes lymphomagenesis, possibly through the above described signaling pathways (3.1). After observing that CD19 loss in LMP1/CD40- expressing B cells leads to reduced proliferation and survival *in vitro* and rapid loss of B cells from the spleen and blood *in vivo*, we were interested whether LMP1/CD40//$CD19^{-/-}$ mice still develop lymphoma. Over a period of 19 months, 22 mice from each genotype, LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$, were monitored for tumor development by abdominal palpation. Mice with suspected disease were sacrificed and lymphoid organs were analyzed. A high percentage of LMP1/CD40//$CD19^{+/-}$ mice showed obvious signs of disease from the age of 12 months on whereas none of the LMP1/CD40//$CD19^{-/-}$ mice did (displayed as a graph of lymphoma incidence in Figure 21). The diseased LMP1/CD40//$CD19^{+/-}$ mice mostly displayed dramatic splenomegaly, enlarged lymph nodes and sometimes metastases in other non-lymphoid organs as had been described before (data not shown, (Homig-Holzel et al., 2008). To confirm lymphoma development, Southern blot analysis of the IgH locus of splenic DNA samples was performed. Using a J_H probe (spanning the J_H3-4 region of the IgH locus) no specific bands can be detected in polyclonal preparations due to the different rearrangments of the IgH locus of B cells. In contrast in mono- or oligoclonal populations, as found in lymphomas, one or a few specific bands can be detected due to the outgrowth of one or a few populations. This Southern blot analysis revealed that in addition to the germline fragment, that derives from the non-B cells

in all splenic samples, further distinct fragments could only be detected in tumor derived LMP1/CD40//CD19$^{+/-}$ (Figure S4 left side, + marked) but in none of the LMP1/CD40//CD19$^{-/-}$ samples. These data demonstrate that CD19-deficiency abolishes lymphomagenesis in LMP1/CD40 mice.



Mice #									
– LMP1/CD40//CD19$^{+/-}$	22	22	22	21	21	20	16	13	6
– LMP1/CD40//CD19$^{-/-}$	22	22	22	18	14	8	5	4	4

Figure 21 Lymphoma development occurs only in CD19 proficient LMP1/CD40 transgenic mice
The graph shows the lymphoma incidence in mice aged 0 to 80 weeks. Compared are LMP1/CD40//CD19$^{+/-}$ (black line) and LMP1/CD40//CD19$^{+/-}$ (red line) mice. The table below lists mice at risk from both genotypes. The reduction of mice was caused by analysis and drop-outs by lymphoma-unrelated death.

3.2.3.4 Analysis of the signaling pathways in LMP1/CD40//CD19$^{-/-}$ mice

Since we observed that LMP1/CD40-expressing B cells are particularly dependent on CD19, we were interested whether CD19 is required for the induction of certain signaling pathways activated by constitutive CD40 signaling. As shown in the first chapter of this work, LMP1/CD40 signaling in B cells induces activation of the noncanonical NF-κB pathway and the MAPK Erk and Jnk. We were interested whether the loss of the LMP1/CD40 phenotype by ablation of CD19 is reflected in the capacity to activate these signaling pathways. 8 to 16 week old mice with the described genotypes (3.2.2) were analyzed.

3.2.3.4.1 LMP1/CD40//CD19$^{-/-}$ B cells activate the noncanonical NF-κB pathway

The noncanonical NF-κB pathway is mostly associated with survival. In order to investigate whether impaired survival of LMP1/CD40//CD19$^{-/-}$ B cells is due to an inability to activate the noncanonical NF-κB pathway, we examined the status of both NF-κB pathways. Western blotting of whole cell extracts from the indicated genotypes revealed that LMP1/CD40//CD19$^{-/-}$ B cells show increased p52 levels as seen in LMP1/CD40//CD19$^{+/-}$ but

not in CD19$^{+/-}$ or CD19$^{-/-}$ B cells (Figure 22A). Strikingly, both LMP1/CD40//CD19$^{-/-}$ and CD19$^{-/-}$ B cells showed a noticeable reduction of p100 levels, independent of their p52 levels. LMP1/CD40-expressing B cells further exhibit a reduction of pIκBα irrespective of their CD19 status. These results suggest that LMP1/CD40 signaling is still able to activate the noncanonical NF-κB pathway when CD19 is absent. Fractionation experiments supported this observation (Figure 22B). Both LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ B cells showed higher levels of p52, p50 and RelB in their nuclei fractions. Of note, RelB was sometimes slightly degraded in fractionation experiments, which might be due to its relatively high instability (Fusco et al., 2008). In contrast to the results described in 3.1.1, we could only inconsistently observe an increase in p105 and a decrease in c-Rel levels. The obtained results suggest that CD19 deficiency does not impair the activation of the noncanonical NF-κB pathway.

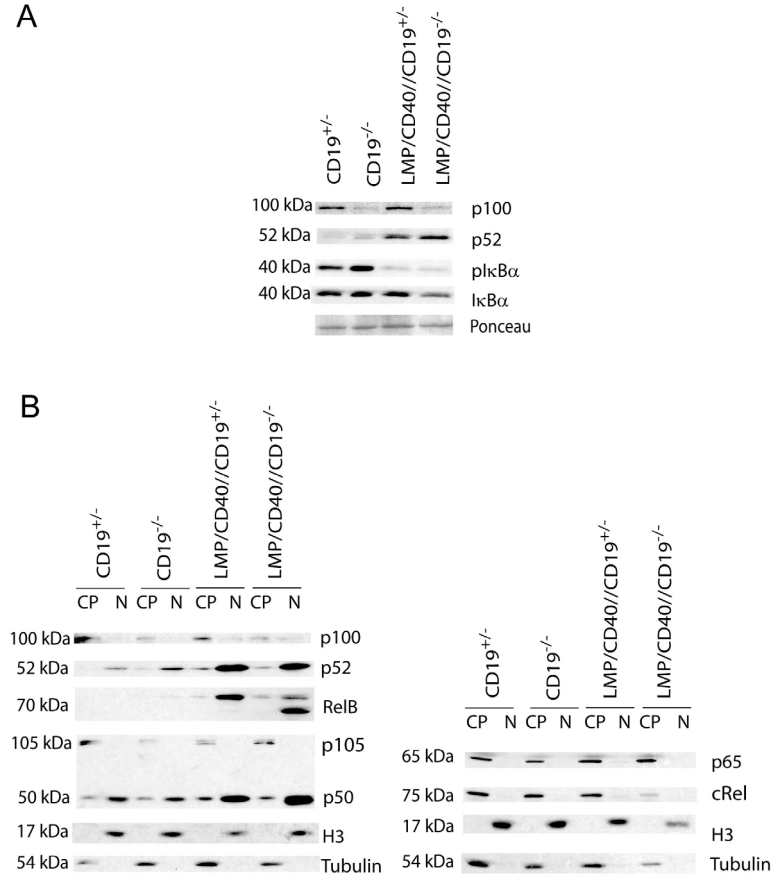


Figure 22 LMP1/CD40//CD19$^{-/-}$ B cells activate the noncanonical NF-κB pathway
(A) Whole-cell extracts were examined by Western blotting. Protein extracts were derived from unstimulated B cells from the indicated genotypes and probed with α-p100/p52, α-pIκBα and α-IκBα antibodies. Equal protein loading was controlled by Ponceau-S staining. The results are representative for four independent experiments (B) Cytoplasmic (CP) and nuclear (N) levels of the NF-κB components RelB, p100/p52, p65, c-Rel, p105/p50 of B cells from the indicated genotypes were analyzed by Western blotting. Purity and equal loading of cytoplasmic and nuclear extracts was verified by α-tubulin and α-H3 probing, respectively. The experiment was performed three times.

3.2.3.4.2 LMP1/CD40 mediated activation of Erk is dependent on CD19 expression

Secondly, we investigated the activation status of the MAPK Erk and Jnk as has been described before (3.1.2). MAPK are important players in the immune system and are known to promote survival and proliferation (Dong et al., 2002). Protein samples from all four genotypes ($CD19^{+/-}$, $CD19^{-/-}$, LMP1/CD40//$CD19^{+/-}$ and LMP1/CD40//$CD19^{-/-}$) were loaded on one gel and analyzed for the phosphorylation status and total protein amounts of Erk and Jnk. This analysis revealed that Erk phosphorylation is impaired in LMP1/CD40//$CD19^{-/-}$ as compared to LMP1/CD40//$CD19^{+/-}$ B cells, whereas the levels of pJnk were quite similar (Figure 23). In contrast, total protein amounts of both Jnk and Erk are still as high in LMP1/CD40//$CD19^{-/-}$ as in LMP1/CD40//$CD19^{+/-}$ B cells. No significant difference was observed between $CD19^{+/-}$ and $CD19^{-/-}$ protein samples. We draw the conclusion that Erk activation by constitutive CD40 signaling requires CD19.

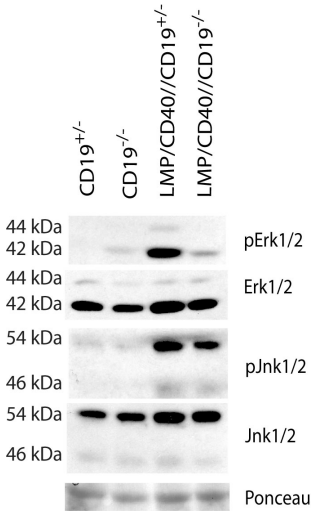


Figure 23 LMP1/CD40 mediated activation of Erk requires CD19
Whole cell extracts from unstimulated B cells from the indicated genotypes were examined by Western blot analysis using antibodies against pErk1/2, Erk1/2, pJnk1/2 and Jnk1/2. Equal loading was controlled by Ponceau S staining. The blot is representative for five independent experiments.

3.2.3.4.3 LMP1/CD40 signaling in B cells involves PI3K

The mutation of tyrosine residues Y513 and Y482 in the signaling domain of CD19 ablates all known functions of CD19 *in vivo*, uncovering the importance of these two tyrosine residues (Wang et al., 2002). Y513 and Y482 were shown to bind PI3K after phosphorylation, thus CD19 function *in vivo* was mainly attributed to its ability to activate PI3K. Indeed, PI3K activation upon BCR activation seems to be dependent on CD19 (Buhl et al., 1997).

To investigate if CD19 signal transduction in LMP1/CD40//$CD19^{+/-}$ B cells involves PI3K, we made use of small chemical inhibitors. We treated LMP1/CD40//$CD19^{+/-}$ B cells with the PI3K inhibitor LY294002 or with medium plus DMSO *in vitro* (Figure 24A). The cells were

tracked over five days in culture and the percentage of living cells was determined by TOPRO-3 staining. As expected, untreated LMP1/CD40-expressing B cells (LMP1/CD40 DMSO) showed enhanced survival compared to control B cells ($CD19^{+/-}$ DMSO) (Figure 8) (Homig-Holzel et al., 2008). However inhibition of PI3K in these cells (LMP1/CD40 LY) led to a decrease in the overall survival, indicating that LMP1/CD40 expressing B cells are dependent on PI3K in their survival *in vitro* (Figure 24A).

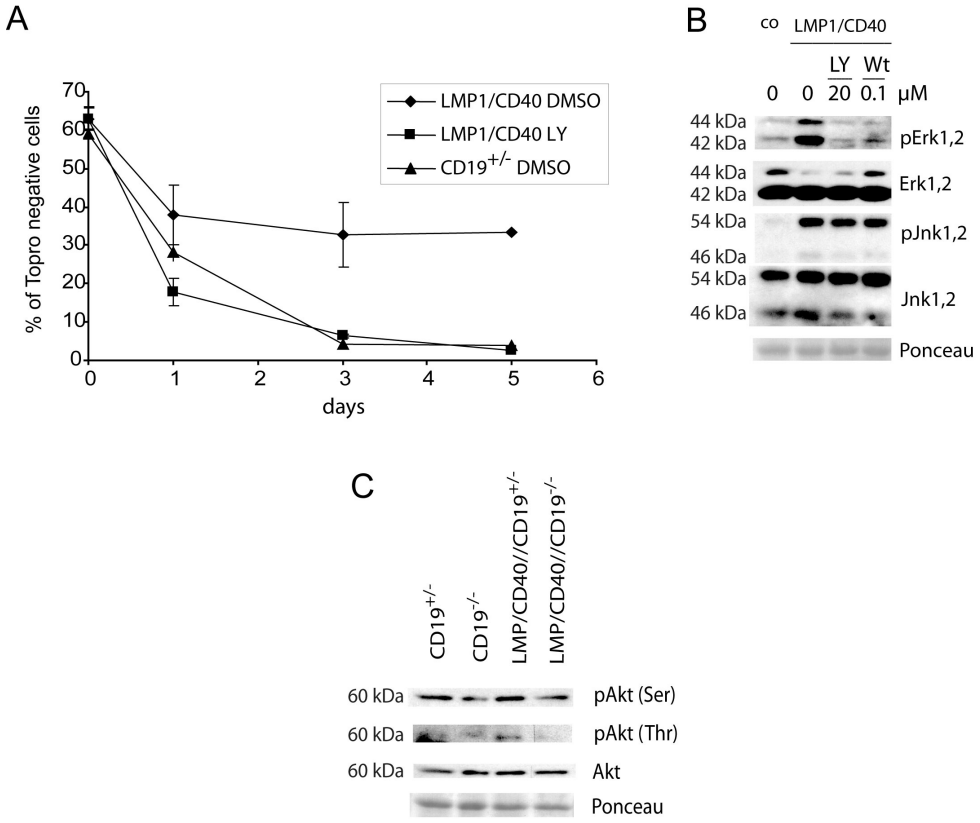


Figure 24 LMP1/CD40 mediated survival and Erk phosphorylation are dependent on PI3K
(A) Splenic B cells from LMP1/CD40 and control mice were cultured for up to five days with the PI3K inhibitor LY294002 (20µM) (squares). As control, B cells from LMP1/CD40 and control mice were cultured in the presence of DMSO, which was used to solve the PI3K inhibitor (rectangles and triangles, respectively). Percentages of living cells (Topro negative) were determined by flow cytometry at day 0, 1, 3 and 5. The bars show mean percentages of living cells of three independent experiments. Error bars show the SD. (B) Protein extracts derived from LMP1/CD40 B cells that were treated for one hour without (0) or with the PI3K inhibitors LY294002 (LY 20µM) and Wortmannin (Wt 0.1µM) were investigated by immunoblot and probed with the indicated antibodies. Untreated $CD19^{+/-}$ B cells (0) served as a control (co). Equal protein loading was controlled by Ponceau-S staining. The results are representative for three independent experiments. (C) Akt phosphorylation was examined on a Western blot from whole cell extracts of the indicated genotypes probed for pAkt(Ser473), pAkt(Thr308) and total Akt. Ponceau S staining served as a loading control. The results are representative of four independent experiments.

To analyze whether inhibition of PI3K results in impaired phosphorylation of Erk, LMP1/CD40//$CD19^{+/-}$ B cells were treated with the PI3K inhibitors LY294002 and

Wortmannin and assayed for Erk phosphorylation by Western blotting. To control whether PI3K would specifically affect Erk and not Jnk in LMP1/CD40 expressing B cells as CD19 does, we investigated (p)Jnk as well. Treatment of LMP1/CD40//CD19$^{+/-}$ B cells with the PI3K inhibitors LY294002 as well as with Wortmannin abolished Erk phosphorylation in LMP1/CD40 expressing B cells completely but left Jnk phosphorylation unharmed (Figure 24B). The PI3K inhibitors did not have any effect on total protein levels of Erk and Jnk. This indicates that Erk phosphorylation in LMP1/CD40 expressing cells is dependent on PI3K. Since PI3K is known to signal to Akt predominantly, we reassessed whether LMP1/CD40//CD19$^{+/-}$ B cells would also activate Akt, which was not investigated in previous experiments (3.1). This kinase shows phosphorylation at a serine and threonine residue upon activation which can be analyzed by Western blotting. Neither residue was found to be constitutively phosphorylated by LMP1/CD40 signaling as compared to CD19$^{+/-}$ control B cells (Figure 24C). Nevertheless, CD19 deficiency in B cells leads to an impairment of Akt activation irrespective of their LMP1/CD40 expression. To conclude, LMP1/CD40 mediated survival *in vitro* and activation of Erk are dependent on PI3K. However, the classical PI3K target Akt is not constitutively activated upon LMP1/CD40 signaling and thus cannot be attributed to enhance survival in LMP1/CD40-expressing B cells.

3.2.3.4.4 The protein tyrosine kinase Lyn as a candidate linking LMP1/CD40 to CD19

Lyn is a protein tyrosine kinase (PTK) that is involved in proximal signaling events at the BCR. Upon activation it becomes phosphorylated and associates with CD19. In its function as a kinase it phosphorylates CD19 as well as Igα and Igβ. Interestingly, Lyn has been shown to become phosphorylated upon CD40 stimulation of cells as well (Ren et al., 1994). We hypothesized that Lyn might be the kinase to link constitutive CD40 signaling to CD19 signaling.

In Western blot experiments we investigated the status of Lyn phosphorylation in whole cell extracts of unstimulated cells by probing the membrane with α-pLyn and α-Lyn antibodies. The analysis revealed that Lyn is constitutively phosphorylated in LMP1/CD40//CD19$^{+/-}$ but not in CD19$^{+/-}$, CD19$^{-/-}$ or LMP1/CD40//CD19$^{-/-}$ B cells (Figure 25). The overall level of Lyn was also increased in LMP1/CD40//CD19$^{+/-}$ but not in LMP1/CD40//CD19$^{-/-}$ B cells. Thus, LMP1/CD40 signaling leads to the constitutive upregulation and phosphorylation of Lyn.

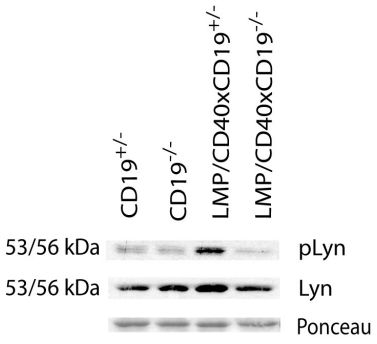


Figure 25 LMP1/CD40 expression leads to phosphorylation of the PTK Lyn
Whole-cell extracts were examined by Western blot. Protein extracts were derived from unstimulated B cells from the indicated genotypes and probed for α-pLyn (Tyr507) and α-Lyn. Equal protein loading was controlled by Ponceau-S staining. The results are representative for three independent experiments.

3.3 Generation of a tetracycline regulatable Notch2-IC transgenic mouse strain

Deregulated Notch1-IC expression is known to promote T cell lymphomagenesis, however, the role of Notch signaling during B cell lymphomagenesis is not known (Ellisen et al., 1991; Pear et al., 1996). In the absence of pro-survival pathways Notch leads to apoptosis in B cells (Kohlhof et al., 2009). However, in cooperation with CD40, Notch has been shown to enhance proliferation of B cells *in vitro* (Thomas et al., 2007). Additionally, Notch2 might be partly responsible for the phenotype of certain B cell malignancies, e.g. of Hodgkin lymphoma cells. These cells almost completely lose the expression of B cell specific genes but show high expression of receptors like CD40, CD30 and Notch1 and 2. Indeed, members of our lab showed that Notch-IC expression in a B cell line leads to the downregulation of B cell specific genes (H. Kohlhof, F. Hampel, personal communication).
With the generation of a Tet regulatable Notch2-IC-expressing strain we aim to investigate whether constitutively active Notch2 is able to induce B cell lymphomas *in vivo* and if so whether its expression is continuously required for maintenance of the tumor. To approach the question whether constitutive activity of Notch2 and CD40 cooperates in driving lymphomagenesis *in vivo*, Notch2-IC transgenic mice will be crossed to LMP1/CD40 transgenic mice.

3.3.1 The Tet system

In vitro and in several *in vivo* settings, the Tet system is a well established bacteria-derived tetracycline dependent expression system (Gossen and Bujard, 2002). It allows conditional expression of a transgene in a temporal, quantitative and reversible manner. In combination with the cre-loxP system it additionally enables to express a transgene in a tissue-specific manner. Two Tet systems are commonly used: the Tet-off and the Tet-on system. The Tet-off

system is directed by a tetracycline-transactivator (tTA) that is only active on the TetO7 promoter when tetracycline (or derivates like doxycycline) is absent. In the Tet-on system on the other hand, a mutant form of tTA, rtTA (reverse tTA), is only active when tetracycline is present. So either the deprivation or the addition of tetracycline leads to gene expression from the TetO7 promoter and can be reversed by addition or removal of it, respectively. So far, the Tet-system has not been shown to work in B cells *in vivo* with the exception of the cMYC mouse strain. Studies on cMYC transgenic mice revealed that cMYC driven tumorigenesis is reversible upon cMYC deprivation (Felsher and Bishop, 1999; Marinkovic et al., 2004). Using the Tet-off system, the human *cMYC* was placed under a bidirectional TetO7 promoter and was controlled by Eμ-tTA (tetracycline transactivator under the control of the enhancer of Igμ and a minimal promoter). Expression of cMYC was shown to induce preferentially T cell and to a lesser extent B cell lymphomas that regressed when tetracycline (Tet) was fed to the mice and thus tTA induced cMYC expression was lost. These studies elegantly showed that even after the formation and progression of cMYC-induced tumors, the protein was required for their maintenance.

3.3.2 Strategy

The following strategy was designed for the Tet-regulatable Notch2-IC transgenic mouse strain. Three different transgenic mouse strains should be crossed to obtain tetracycline inducible, tissue-specific expression of Notch2-IC in B cells (Figure 26). These strains are the TetO7-Notch2-IC transgenic mouse strain, the CD19-, CD21-, mb-1- or γ1-Cre transgenic strains to allow B cell specific expression of the rtTA and the R26STOPrtTA strain received by Wei Hsu (Yu et al., 2005). In the latter strain the Tet-regulatable transactivator rtTA2^{S}-M2 preceded by a loxP-flanked STOP cassette is placed into the *Rosa26* locus. rtTA2^{S}-M2 is a variant of rtTA that is more sensitive to doxycycline and thus minimizes toxic effects by the latter. Crossing of the R26STOPrtTA strain and a B cell specific Cre-strain to a mouse strain that is transgenic for *Notch2-IC* placed under a Tet-regulatable promoter would consequently allow Tet-regulatable and thus inducible and reversible Notch2-IC expression in B cells (Figure 26). The Notch2-IC transgenic mouse strain was generated within the scope of this thesis.

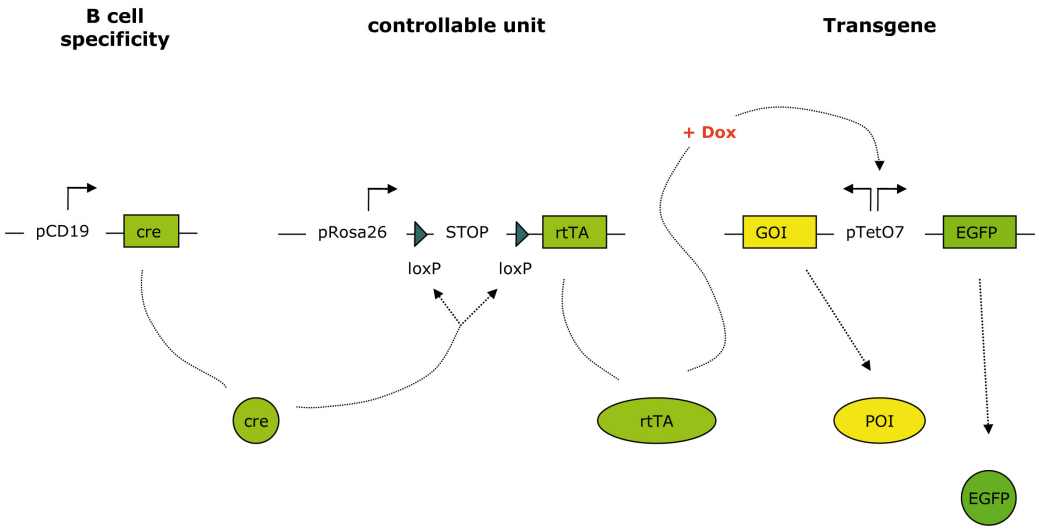


Figure 26 Reversible Notch2-IC expression in B cells *in vivo*
B cell specificity is achieved by B cell restricted Cre-expression (e.g. from the CD19 promoter) excising the STOP-cassette, which is located upstream of the rtTA, tissue specifically (Yu, 2005). The gene of interest (GOI) is placed under a bidirectional TetO7 promoter that is dependent on rtTA activation. Addition of doxycycline (Dox) leads to binding of rtTA to the TetO7 promoter and activates GOI expression. Concomitant EGFP expression allows tracking of cells that activate the TetO7 promoter. rtTA (reverse tetracycline dependent transactivator), STOP (cassette containing stop sequences for transcription and translation), GOI (gene of interest), POI (protein of interest).

In detail, murine *Notch2-IC* cDNA was placed on one side of a bidirectional TetO7 promoter (TetO7BI) and *EGFP* on the other side (Figure 27 (3)). Via concomitnant EGFP expression, Notch2-IC-expressing B cells can be traced by green fluorescence. The *Notch2-IC* cDNA was derived from a pTracer_CMV_Notch2cDNA vector (kindly provided by F. Radtke) by PCR. An endogenous ATG-Kozak sequence, located at the 5´end of *Notch2-IC*, was used as translational start site. Since it is known that the addition of a structure that contains splice sites leads to a higher transcription rate of otherwise unspliced cDNA, we cloned the *Rosa26* Exon1 and Intron1 upstream of the *Notch2-IC* cDNA. Since Exon1 of the *Rosa26* locus does not contain an ATG, translation will start at the Kozac-ATG of *Notch2-IC*. PolyA signal sequences were cloned downstream of *Notch2-IC* and *EGFP* to ensure termination of transcription. To achieve different expression levels of Notch2-IC, the enhancer of the Ig light chain κ locus (Eκ), flanked by frt sites (to be able to remove it via Flp-recombinase) was cloned downstream of *Notch2-IC*. With this system we expect the following expression levels: (i) without enhancer/ without Tet: no expression of Notch2-IC, (ii) without enhancer/ with Tet: weak expression, (iii) without enhancer/ with Tet: intermediate expression, (iv) with enhancer/ with Tet: high expression.

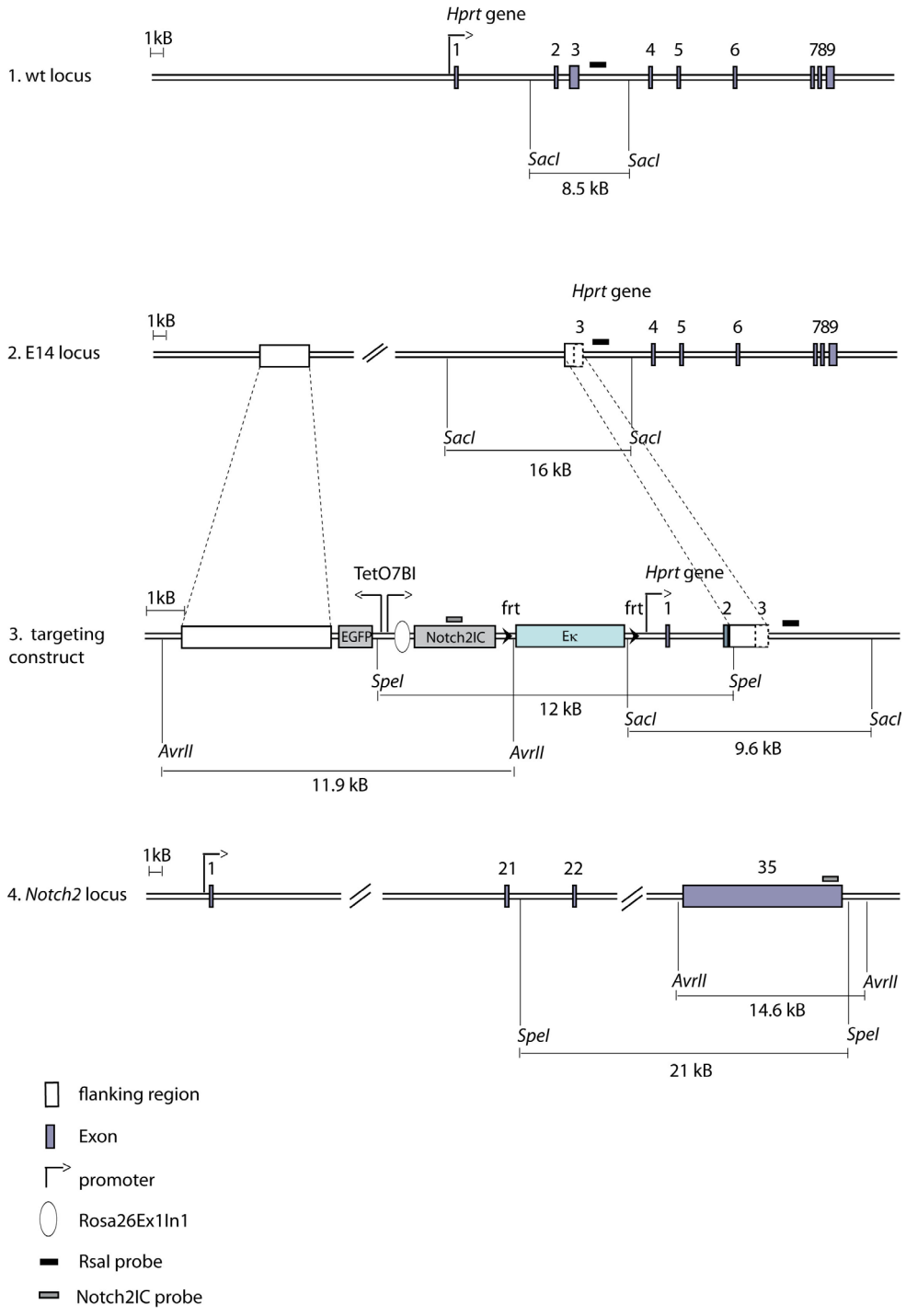


Figure 27 Targeting strategy
(1) The 5' region of the *Hprt* gene was chosen as an integration site for the Tet-regulatable *Notch2-IC* construct. *Hprt* contains nine exons. (2) In the E14TG2a ES cells (E14) the *Hprt* promoter and exon/ intron 1 and 2 are deleted. (3) The targeting construct contains homologous arms (flanking regions, white bars) for the E14 *Hprt* locus and additionally the *Hprt* promoter and exon/ intron 1 and 2 to complement the deleted regions of the E14 *Hprt* locus. The EGFP-BITetO7-Notch2-IC-Eκ construct was cloned in between the upstream homologous arm and the *Hprt* promoter. The dotted lines indicate the sites of homologous recombination between the E14 *Hprt* locus and the targeting construct. The restriction enzyme sites (*SacI, AvrII, SpeI*) and the fragment sizes resulting from restriction, Southern blotting and probing with the *RsaI* probe (binding downstream of *Hprt* exon 3) as well as the Notch2 probe are depicted in this scheme. (4) The endogenous Notch2 locus is sketched to reveal the Southern blot fragments obtained with the Notch2 probe on the endogenous locus.

We decided to place the targeting construct 5' of the murine X-linked *Hprt* gene since this is a commonly used locus for transgene expression apart from the *Rosa*26 locus that is already engaged by the R26STOPrtTA strain (Figure 27 (1)). In 1987, Hooper *et al.* described an embryonic stem (ES) cell line that is Hprt-deficient due to the loss of the *Hprt* promoter and exon 1 and 2 (Bronson et al., 1996; Hooper et al., 1987). A schematic overview of this locus is given in Figure 27 (2). *Hprt* codes for the Hypoxanthine-guanine-phosphoribosyl-transferase that is an important enzyme in the salvage purine nucleotide synthesis pathway. Hprt deficient cells cannot grow in HAT (hypoxanthine aminopterin thymidine) medium due to toxicity of aminopterin in the absence of Hprt. Targeting vectors were developed that allow the insertion of transgenes into the 5' region of the *Hprt* gene in these ES cells by homologous recombination, concurrently reconstituting the *Hprt* gene. Hence selection in HAT (hypoxanthine aminopterin thymidine) medium only allows homologously recombined clones to grow. The described construct was flanked by *Hprt*-homologous arms. The upstream homologous arm contains the *Hprt* upstream homologous region and the downstream homologous arm contains the exon 3, preceeded by the human *Hprt* promoter and exon 1 and 2. The targeting construct will thus be placed upstream of the *Hprt* gene.

3.3.3 ES cell targeting and screening

The generation of a transgenic mouse strain requires three different steps: (i) Cloning of the targeting construct, (ii) Transfection of murine embryonic stem cells with the construct and screening for homologous recombination, (iii) Injection of correctly targeted ES cell clones into blastocystes that will be implanted into foster mothers. Chimeric offsprings are subsequently crossed to achieve germline transmission.

After cloning and sequencing, the linearized Notch2-IC construct (strategy described in 3.3.2, Figure 27 (3)) was transfected into the Hprt deficient ES cell line E14TG2a (129/SV background) (Tsuda et al., 1997). Transfection resulted in 19 HAT-resistant colonies. These colonies were picked and expanded for further analysis.

To verify the correctness of homologous recombination, three different Southern blot strategies were performed (Figure 27 and Figure 28). They are designed to prove the correct integration of the targeting construct into the locus at the 5' and 3' ends as well as to prove the complete and single integration of the inserted construct. Restriction enzymes, probes and resulting fragments are depicted in Figure 27, Southern blot results in Figure 28. In Figure 28A the Southern blot with *SacI* digest of clones (1A-D) and the parental E14TG2a ES cell line is shown. Hybridization with the RsaI probe resulted in 9.6 kb fragment in the targeted clones proving the correct 3' integration of the targeting vector. The 16 kb fragment, arising in the non-targeted E14TG2a ES cells results from hybridization of the *RsaI* probe with the deleted *Hprt* allele. Since *Hprt* is located on the X-chromosome and the E14TG2a cell line is derived from a male, the untargeted and undeleted wildtype allele, which would give rise to an 8.5 kb fragment, could neither be detected in the targeted ES cell clones nor in the parental cell line. Digest with *AvrII* and probing the DNA with a Notch2 probe resulted in an 11.9 kb fragment, revealing that all four clones tested were integrated correctly at the 5' end and showing the single integration of the construct as there were no further hybridization signals on the blot (Figure 28B and data not shown). The 14.6 kb fragment appearing in lanes with homologously recombined clones as well as with the parental ES cell lines results from hybridization of the Notch2 probe with the endogenous *Notch2* locus (Figure 27 (4) and Figure 28B). To control the integrity of the construct internally, the Notch2 probe was hybridized to *SpeI* digested DNA (Figure 28C). All four clones showed the expected 12 kb fragment. The additional 21 kb fragment that is also present in the untargeted E14TG2a cells again derives from the endogenous *Notch2* locus. We concluded that all four clones showed the right integration of our construct into the *Hprt* locus.

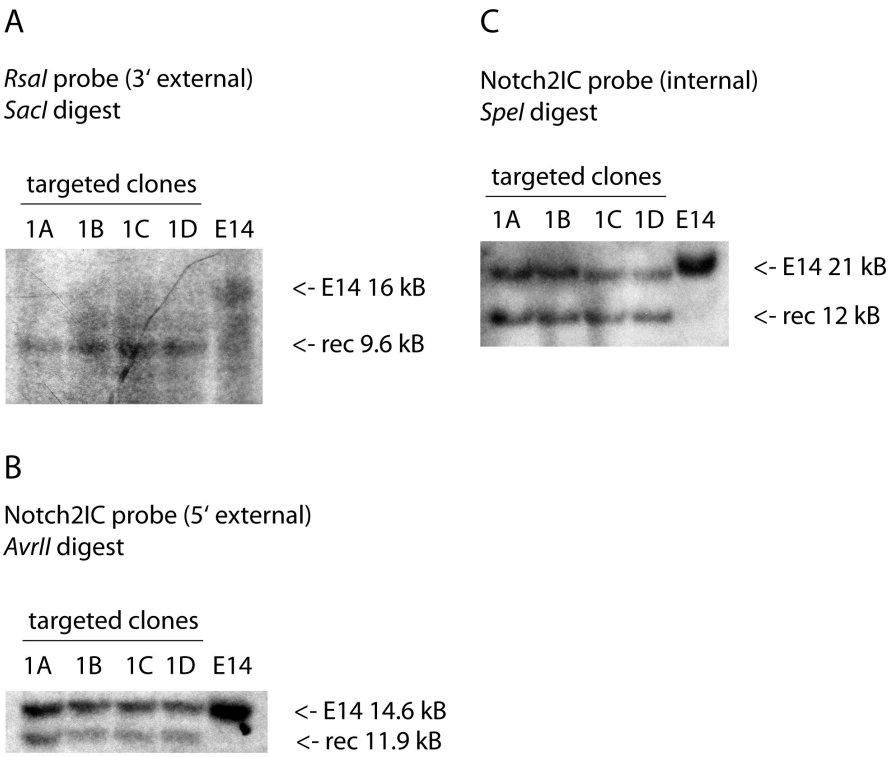


Figure 28 Verification of correct target integration via Southern blot analysis
ES cell DNA from clones 1A-D was subjected to restriction enzyme digest, Southern blotting and probing with the indicated probes. (A) After *SacI* digestion and incubation with the *RsaI* probe (3' external fragments of 16 kb and 9.6 kb can be detected, corresponding to the deleted untargeted allele in E14 cells and the targeted, respectively. (B) After *AvrII* digestion, the Notch2 probe recognizes a 14.6 kb fragment deriving from the endogenous Notch2 locus in all ES cells and an 11.9 kb fragment in the recombinant ES-cell clones, reflecting the complete integration of the targeting vector at the 5´end. (C) The Notch2 probe detects fragments of 21 kb in the case of the endogenous locus and 12 kb for the integrated Notch2-IC after digestion with *SpeI*.

These four clones were further subjected to sequencing of the *Notch2-IC* transgene to screen for clones that are free of mutations. For this purpose, the transgene was amplified in two parts from the genomic ES cell DNA by PCR. Five different oligonucleotides were designed that allow sequencing of the whole inserted *Notch2-IC* cDNA amplified as a PCR product (Figure 29). The obtained sequences were subjected to BLAST analysis to compare them to the original sequence. All four clones showed the correct sequences (data not shown).

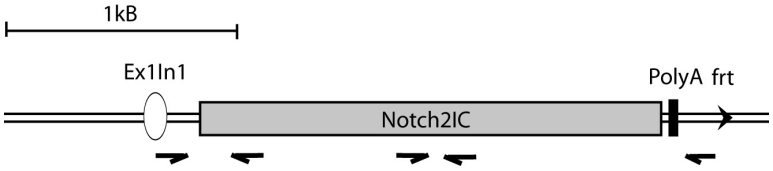


Figure 29 Sequencing of the Notch2-IC DNA from targeted ES cell clones
The scheme shows the *Notch2-IC* cDNA and its flanking regions. It was sequenced with five different primers indicated as arrows. The scale is indicated by the bar.

Clones 1C and 1D were chosen for injection into blastocystes. These two were further tested for EGFP expression in an *in vitro* assay. ES cells were transfected with a construct expressing rtTA under the control of the CAG (CMV enhancer, chicken beta actin) promoter and cultured in medium supplemented with or without doxycycline (Dox) directly after transfection. 16 hours later, cells were harvested and EGFP expression was analyzed via flow cytometry. Both clones showed expression of EGFP in up to 17% of the cells which approximately reflects the number of transfected cells (Figure 30). Only a few EGFP expressing cells (0.1-0.2%) could be detected in transfected clones without Dox (0ng/ml). Hence, the bidirectional TetO7 promoter is functional in ES cells and can be activated by Dox-bound rtTA. In the absence of Dox the leakiness of the TetO7 promoter is very low and can be neglected.

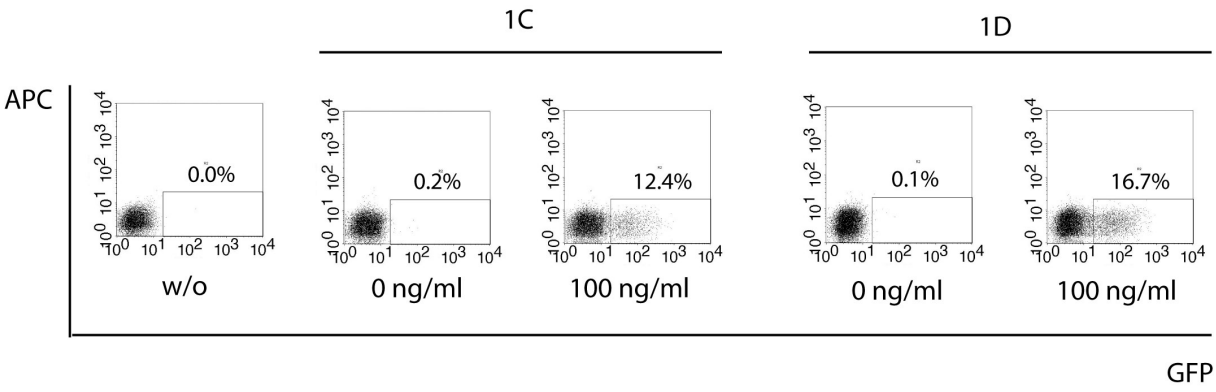


Figure 30 Functional testing of targeted ES cells
Flow cytometry analysis to determine the expression levels of EGFP in the targeted ES cell clones 1C and 1D. Cells were cultured in medium supplemented with 100 ng/ml Dox after transfection with a CAGGS-rtTA containing plasmid. Untransfected cells from clone 1C (w/o) and transfected, but untreated (0ng/ml) cells from clone 1C and 1D served as a control.

3.3.4 Injection into blastocysts

Clone 1C and 1D were prepared for injection into C57BL/6 derived blastocystes that were implanted into foster mothers at the Institute of Developmental Genetics of the Helmholtz Center Munich (HMGU). The blastocystes of clone 1C gave rise to five chimeras (all male), the blastocystes of clone 1D to three chimeras (one female, two male) with an average chimerism of 70%. Theses chimeras are now crossed to C57BL/6 mice for germline transmission.

4 Discussion

The activation and expansion of B cells during an adaptive immune response is necessary to obtain antibody producing plasma and memory B cells. This process is mediated by certain receptors, among them CD40, the BCR and Notch. CD40 is an important costimulatory receptor, whereas the BCR is required for specific recognition of antigen and for the survival of resting B cells. The Notch receptor, commonly involved in differentiation processes, has recently been associated with activation of B cells during an immune response. Notch signaling enhances proliferation of B cells and the production of $IgG1^+$ plasma cells when activated together with CD40 and the BCR. There is evidence that deregulation of these activation processes can lead to lymphoma development. In the case of CD40 and Notch aberrant expression of the receptors is a hallmark of certain B cell lymphomas and was suggested to be involved in the pathogenesis of the diseases. Recently we provided the experimental proof that deregulated CD40 signaling provided by a LMP1/CD40 fusion protein promotes B cell lymphomagenesis. Aberrant expression of the BCR has not been described for B cell lymphomas, however, it was speculated whether its tonic, ligand-independent survival signal is important for the initiation and maintenance of lymphomas. Notch2 is expressed on certain B cell tumors, however, experimental proof for its oncogenic potential in B cells is missing. Interestingly, these three receptors, CD40, the BCR and Notch, are mimicked by an oncogenic, predominantly B cell specific virus, the Epstein-Barr virus (EBV). Virus infected B cells are immortalized *in vitro* by a certain expression program, where LMP1 (mimicking a constitutively active CD40), LMP2A (mimicking a constitutively active BCR) and EBNA2 (mimicking a constitutively active Notch receptor) are produced among a few other viral gene products.

4.1 Constitutive CD40 signaling selectively activates the noncanonical NF-κB pathway and the MAPK Erk and Jnk

CD40 ligation results in the activation of numerous signaling pathways that trigger physiological outcomes like enhanced survival, proliferation, germinal center formation, CSR, SHM and memory B cell development. *In vitro*, both CD40 and the LMP1/CD40 fusion protein have been shown to result in MAPK and NF-κB activation (Gires et al., 1997; Hatzivassiliou et al., 1998). *In vivo*, constitutive CD40 signaling via LMP1/CD40 resulted in B cell expansion and promoted lymphomagenesis (Homig-Holzel et al., 2008). Here we show

that constitutive activity of CD40 in B cells *in vivo* selectively activates the noncanonical NF-κB pathway and the MAPK Jnk and Erk.

Fractionation experiments revealed that LMP1/CD40-expressing B cells have higher levels of p52, p50 and RelB in the nucleus than control B cells, but normal levels of p65 and c-Rel, indicating that LMP1/CD40 exclusively activates the noncanonical NF-κB pathway, consisting of RelB heterodimers. Unlike p52, whose processing from p100 is tightly regulated, p50 is constitutively processed from p105 and normally dimerizes with p65 or cRel. However, in the absence of p65 and c-Rel it is able to form homodimers that either act as transcriptional repressors of canonical target genes due to the absence of a transactivation domain or bind to Bcl3 that mediates gene transcription. However, we could not detect elevated levels of Bcl3 in the nucleus of LMP1/CD40-expressing B cells excluding the latter possibilty. Alternatively, RelB has been described to form dimers with both p52 and p50 that activate transcription of noncanonical target genes, thus elevated p50 levels might correlate with the formation of RelB/ p50 heterodimers in LMP1/CD40-expressing B cells (Derudder et al., 2003). The canonical NF-κB pathway on the other hand may even be dampened in LMP1/CD40-expressing B cells. Examination of whole cell extracts revealed that IκBα is less phosphorylated in LMP1/CD40 than in control cells. This is not due to constant degradation and resynthesis as investigated by cycloheximide treatment of the cells. Furthermore the cells are less stimulated in the canonical NF-κB pathway by external α-CD40 triggering than control cells. Canonical NF-κB signaling is known to be rapidly induced and counter-regulated by -among other factors- upregulation of its own inhibitor, IκBα, whereas noncanonical NF-κB signaling reacts slowly and lacks this tight regulation (Hoffmann and Baltimore, 2006). This phenomenon might account for the preferential activation of the noncanonical pathway by the constantly signaling LMP1/CD40. Deletion of TRAF2 or 3 that bind to the cytoplasmic tail of CD40 has been shown to result in activation of noncanonical NF-κB signaling and the disruption of TRAF3 binding to NIK seems to be crucial for the activation of this pathway by BAFF-R signaling (Gardam et al., 2008; Grech et al., 2004; Sasaki et al., 2008; Xie et al., 2004). In Western Blot experiments, TRAF2 was slightly decreased in LMP1/CD40 B cells, however, TRAF3 levels appeared normal (data not shown). Until now, the role of the noncanonical NF-κB pathway during LMP1/CD40 induced lymphomagenesis is not clear. In mouse models, chronic activation of the noncanonical NF-κB pathway is not sufficient to induce lymphomagenesis: TRAF2 deletion in B cells leads to chronic activation of the noncanonical NF-κB pathway, expansion of B cells but not to lymphomagenesis. Only a mutant form of p52 (p80HT) has been proven to be tumorigenic,

whereas constitutive activity of wildtype p52 was not (Wang et al., 2008; Zhang et al., 2007). However, it is known that in certain human lymphomas the noncanonical NF-κB pathway is activated, either by mutations as found in multiple myelomas (MM) or through chronic activation of upstream signaling pathways (Annunziata et al., 2007; Keats et al., 2007). Interestingly, in some cases, MM cells show a strong upregulation of CD40. Since the noncanonical NF-κB pathway alone is not tumorigenic, one may speculate that other pathways being activated by (LMP1/) CD40 further shift the balance to lymphomagenesis.

This shift might be mediated by MAPK. We found the MAPK Jnk and Erk to be activated upon LMP1/CD40 signaling, whereas Erk activity accounted for the enhanced survival of LMP1/CD40-expressing B cells observed *in vitro*. To speculate, Erk could provide the additional hit to render LMP1/CD40-expressing B cells prone to lymphomagenesis. In fact, the Ras-Raf-Mek-Erk cascade is active in about 40% of all tumors and has been associated with proliferation and survival of hematopoetic cells (McCubrey et al., 2007; Wan et al., 2004; Weinberg, 2007). Moreover, Erk was found to be constitutively active in primary and HRS cell lines, which was attributed to CD40, CD30 and RANK signaling (Kuppers, 2009; Zheng et al., 2003). Tumor cell proliferation was abrogated by treating the cells with the Mek1/2 inhibitor UO126, supporting the idea that Erk is an important player in lymphoma maintenance.

Tumor cells from LMP1/CD40 induced lymphomas on the other hand do not show a common pattern of signal activation as seen in premalignant cells. This and the long onset of tumor initiation suggest that other mutations have to occur in LMP1/CD40-expressing B cells to ultimately transform cells. These mutations may additionally render the cells less dependent on the LMP1/CD40 induced signaling pathways. However, tumor cells do not lose LMP1/CD40 expression, suggesting that they still require its signaling (data not shown). The LMP1/CD40 transgenic mouse strain provides a model system to study the contribution of the signaling pathways in premalignant cells to tumor initiation and to study second hits that further promote lymphomagenesis. Importantly, the long tumor latency makes it well comparable to human tumor formation, where tumors commonly arise late in life.

4.2 Constitutive CD40 signaling renders B cells less dependent on BCR signals via Igβ, but requires expression of CD19

B cells are constantly selected for the expression of a functional BCR during their lifetime. Even in a resting state, B cells require the expression of the BCR for survival. Upon activation, the BCR serves as an antigen binding molecule that allows only antigen specific B

cells to expand and differentiate. However, it is discussed whether the B cell receptor is also required for the development and maintenance of B cell lymphomas. Indeed, most B cell lymphomas still express the BCR and its coreceptor CD19 (Kuppers et al., 2005). Only one B cell lymphoma entity, HL, has downmodulated all BCR components. Since HL cells show high expression of CD40, CD30 and RANK, the loss of the tonic survival signal from the BCR might be rescued by the aberrant activation of other receptors. Thus it appeared interesting to us to investigate whether aberrant CD40 activation renders B cells independent of tonic BCR signaling. In addressing these questions, our studies reveal that LMP1/CD40-expressing B cells are less dependent on Igβ but strictly dependent on CD19 signaling.

4.2.1 The influence of Igβ on LMP1/CD40-expressing B cells *in vivo*

The current study shows that deletion of a signaling component of the B cell receptor, Igβ, in peripheral, LMP1/CD40-expressing B cells results in abrogation of B cell expansion by LMP1/CD40, but rescues a substantial amount of Igβ deleted B cells that are otherwise not able to survive in the periphery. Thus LMP1/CD40-expressing B cells are less sensitive to the loss of Igβ than wildtype B cells.

Deletion of Igβ in LMP1/CD40-expressing B cells in the periphery does not result in splenomegaly and expansion of peripheral B cells as observed for LMP1/CD40 Igβ proficient mice. However, B cells numbers in LMP1/CD40//Igβ$^{fl/\Delta}$ mice are significantly higher than in Igβ$^{fl/\Delta}$ mice and are comparable to control mice. Furthermore, unlike in Igβ$^{fl/\Delta}$ mice, most of the B cells from LMP1/CD40//Igβ$^{fl/\Delta}$ mice are deleted (GFP^{+}) cells, suggesting that they are not counterselected. These findings reveal that B cells in the peripheral organs are not independent but less dependent on Igβ mediated BCR signals when they express LMP1/CD40, suggesting that the survival signal delivered by the BCR can be complemented to a certain degree by constitutive CD40 signaling. The nature of this survival signal is not known so far. In part, it might be mediated by the constitutive activation of the noncanonical NF-κB pathway. Recently, a publication by Stadanlick *et al.* claimed that BAFF-R induced p100 processing to p52 is dependent on p100 production by the BCR and thus both pathways are interconnected in providing survival signals to resting B cells (Stadanlick et al., 2008). Indeed, a fractionation experiment showed that p100 is reduced in the cytoplasm of LMP1/CD40//Igβ$^{fl/\Delta}$ B cells, but p52 is still increased in the nucleus in comparison to wildtype cells (data not shown). Thus processing of p100 to p52 by LMP1/CD40 signaling in the absence of a functional BCR might rescue B cells from cell death to a certain extent.

Further investigations will require intensive studies of the signaling pathways in LMP1/CD40//Igβ$^{fl/\Delta}$ B cells that might account for the rescue of Igβ deficient B cells by LMP1/CD40 signaling.

Interestingly, LMP1/CD40//Igβ$^{fl/\Delta}$ B cells in the periphery express low levels of IgM and IgD on their surface whereas the few remaining GFP^{+} Igβ$^{fl/\Delta}$ B cells express only slightly reduced levels of IgD. Ig expression on the surface requires the expression of Igα and Igβ and Kraus *et al.* observe that Ig is only weakly expressed on the surface of a fraction of peripheral Igα deficient B cells; however, it is still expressed on most B cells that only harbor a mutation in the cytoplasmic tail of Igα (Kraus et al., 2004). In accordance with this finding, GFP^{+} Igβ$^{fl/\Delta}$ B cells still express Ig; however in contrast, GFP^{+} LMP1/CD40//Igβ$^{fl/\Delta}$ B cells do only weakly. These observations suggest that LMP1/CD40//Igβ$^{fl/\Delta}$ B cells have a longer lifespan than Igβ$^{fl/\Delta}$ B cells. While Igβ$^{fl/\Delta}$ B cells are eliminated before the Ig receptor complexes are completely internalized, LMP1/CD40//Igβ$^{fl/\Delta}$ B cells survive due to the signals provided by LMP1/CD40, resulting in the visualized downmodulation of the Ig complex from the cell surface.

As in Igβ$^{fl/\Delta}$ mice, MZ B cells (CD21^{high}CD23^{low}) are more strongly reduced than Fo B cells (CD21^{int}CD23^{+}) in LMP1/CD40//Igβ$^{fl/\Delta}$ mice. This is in accordance with previous studies showing that MZ B cells are preferentially lost when BCR signaling is impaired (Martin et al., 2001). Thus mainly Fo B cells deficient for Igβ are rescued by LMP1/CD40 signaling.

The finding that CD40 signals can rescue BCR negative B cells to a certain extent might explain how premalignant GC B cells with downmodulated BCR components can develop into Hodgkin lymphoma cells in EBV negative cases. HRS cells express increased levels of CD40, CD30 and RANK on their surface but show downregulation of most B cell specific genes, among them components of the BCR. Since CD40 is involved in B cell activation, germinal center formation and B cell differentiation, it might provide survival signals to GC B cells with downregulated BCR expression if it is activated aberrantly. In EBV positive cases of HL on the other hand, expression of viral genes might be able to both substitute for the BCR and to transform cells. The fact that all HL that show crippled mutations in their BCR are EBV positive and express LMP2A as a viral BCR analogue suggests that this rescue only functions partially (Kuppers, 2005). Thus only downmodulation of BCR components can be rescued without EBV encoded proteins, however, loss of the BCR due to crippled mutations cannot.

So far we could show that premalignant LMP1/CD40 B cells are less dependent on tonic BCR signaling via Igβ, however, it will be interesting to study whether malignant cells of LMP1/CD40 mice do so as well. By the establishment of tumor derived cell lines we might be able to knock-down Igβ or to treat the cells with Syk inhibitors and assess proliferation and survival. If these cells are less dependent on BCR signals as well, therapeutic strategies targeting the BCR in B cell lymphomas might not be suitable for CD40 related malignancies since CD40 has the capability of complementing BCR mediated survival.

4.2.2 The influence of CD19 on LMP1/CD40-expressing B cells *in vivo*

Unlike Igβ, the effect of CD19 deficiency on B cell survival cannot be rescued by LMP1/CD40 expression, arguing for a prominent, BCR independent role of CD19 in CD40 mediated effects. Expression of LMP1/CD40 in $CD19^{-/-}$ B cells *in vivo* induces activation of the cells characterized by cell surface markers, but does not lead to B cell expansion, resulting in a similar B cell compartment as seen in $CD19^{-/-}$ mice.

LMP1/CD40//$CD19^{-/-}$ mice show a dramatic reduction of B cells in spleen, lymph nodes and peritoneal cavity compared to LMP1/CD40//$CD19^{+/-}$ mice, resulting in similar B cells numbers as found in $CD19^{-/-}$ mice, indicating that constitutive CD40 signaling completely depends on CD19. This met us with surprise since we discovered that LMP1/CD40-expressing B cells *in vivo* are less dependent on Igβ signaling than control B cells. Thus CD19 might exert a different function apart from serving as a coreceptor of the BCR in constitutive CD40 signaling.

To ensure that the effects observed in the peripheral compartments are not due to a defect in B cell development, we analyzed the different B cell populations present in the bone marrow. Development of LMP1/CD40//$CD19^{-/-}$ B cells in the bone marrow seems relatively normal, a minor reduction of immature B cells can be observed for both LMP1/CD40//$CD19^{-/-}$ and LMP1/CD40//$CD19^{+/-}$ B cells, arguing against a defect in B cell development induced by CD19 deficiency. LMP1/CD40//$CD19^{-/-}$ mice furthermore show a lack of recirculating mature B cells as has been observed in LMP1/CD40//$CD19^{+/-}$ mice. This might be due to retention of LMP1/CD40 activated B cells in the periphery by expression of homing factors like CXCR5 that target B cells to the B cell zone in follicles (Forster et al., 1996).

Immature B cells emerging from the bone marrow that enter the spleen are called transitional B cells. Among the three transitional B cell subsets in the spleen, T1 B cells are reduced in LMP1/CD40//$CD19^{+/-}$ as well as in LMP1/CD40//$CD19^{-/-}$ mice. The reduction of T1 B cells

might be due to less influx of transitional B cells from the bone marrow or to an accelerated T1 differentiation to T2 B cells. Since CD19$^{-/-}$ mice show lower numbers of T1 B cells as well, a survival and/ or developmental defect might additionally lead to the pronounced reduction of T1 B cells in LMP1/CD40//CD19$^{-/-}$ mice. It has been shown that the first B cell subset to receive noncanonical NF-κB signaling by BAFF-R stimulation is the transitional T2 subset. BCR induced p100 production, delivering the substrate for BAFF-induced p100 processing to p52, is not induced in T1, but in T2 B cells (Stadanlick et al., 2008). Thus the constitutive activity of the noncanonical NF-κB pathway in T1 B cells in LMP1/CD40-transgenic mice might account for the reduction of T1 B cells in LMP1/CD40//CD19$^{+/-}$ and LMP1/CD40//CD19$^{-/-}$ mice. The 'unnatural' p52/RelB signal might turn T1 into T2 B cells, resulting in a reduction of T1 B cells.

With respect to mature peripheral B cell subsets, LMP1/CD40//CD19$^{-/-}$ mice display a drastic reduction of Fo and MZ B cells compared to LMP1/CD40//CD19$^{+/-}$ mice. CD19 deficiency in mice leads to a reduction of Fo B cells of about 40-50%; a similar reduction can be observed in LMP1/CD40//CD19$^{-/-}$ mice. Thus constitutive CD40 signaling is completely dependent on CD19 signals in the expansion of Fo B cells and cannot compensate for the defect induced by CD19 deficiency. The loss of CD19 in LMP1/CD40 mice leads to a reduction of MZ B cells that is not as strong as observed in CD19$^{-/-}$ mice. However, these cells cannot be found in the marginal zone in splenic sections of LMP1/CD40//CD19$^{-/-}$ mice (data not shown), hence they might be MZ B cells only by high expression of CD21 but not by localization, suggesting that constitutive CD40 signaling cannot rescue the defect in the MZ B cell compartment induced by CD19 deficiency. Surprisingly, the activation status of splenic B cells from LMP1/CD40 mice is not altered when CD19 is deleted; these B cells still upregulate activation markers like CD95, MHC class II and ICAM-1. Nevertheless, the cell size of LMP1/CD40//CD19$^{-/-}$ B cells is reduced as compared to LMP1/CD40//CD19$^{+/-}$ B cells.

Peripheral B cells of the peritoneal cavity are strongly reduced in LMP1/CD40//CD19$^{-/-}$ mice as compared to control mice, affecting both the B1 and B2 compartment. B1 B cells require strong signals from the BCR –possibly by self antigen- to develop and survive (Martin et al., 2001). This cell type is reduced not only in LMP1/CD40//CD19$^{-/-}$, but also in CD19$^{-/-}$ and LMP1/CD40//CD19$^{+/-}$ mice. It has been discussed that CD19 deficiency leads to B1 B cell loss because these cells require constant triggering of the BCR for their survival or alternatively because they require CD19 for their capacity of self renewal (Fuentes-Panana et al., 2006; Krop et al., 1996; Martin and Kearney, 2001). LMP1/CD40 signaling cannot rescue this phenotype. The loss of B2 B cells in the PC is only observed in LMP1/CD40//CD19$^{-/-}$

mice. This phenotype might be due to the impairment of BCR signals by CD19 deletion and the upregulation of CD95 (Fas) by LMP1/CD40 expression that renders them more prone to Fas-induced apoptosis. Indeed, it is known that B2 B cells in the PC are -unlike B1 B cells- especially prone to Fas-induced apoptosis (Masuda et al., 1997).

4.2.3 A new role for CD19 in B cell lymphomagenesis

The current study demonstrates that CD19 is strictly required for CD40 induced B cell expansion and lymphomagenesis. Expression of LMP1/CD40 in $CD19^{-/-}$ B cells does not promote survival or proliferation in the main B cell population *ex vivo*. *In vivo*, CD19 deficiency in LMP1/CD40-expressing B cells reduces their life span compared to control B cells.

The expansion of B cells in LMP1/CD40//$CD19^{+/-}$ mice was attributed to an intrinsic capacity of enhanced survival and spontaneous cell division. Since we do not observe an expansion of B cells in LMP1/CD40//$CD19^{-/-}$ mice, we were interested whether this is reflected in their ability to survive and proliferate. Unlike splenic LMP1/CD40//$CD19^{+/-}$ B cells, LMP1/CD40//$CD19^{-/-}$ B cells do not proliferate *in vitro* and are impaired in their survival. This implicates that LMP1/CD40//$CD19^{-/-}$ B cells have an intrinsic defect in their proliferation and survival due to an inability to respond to constitutive CD40 signals. Of note, there is a relative increase of living cells at day 3 to 5 in culture which might be explained by a small pool of LMP1/CD40//$CD19^{-/-}$ B cells that react to the constitutive CD40 signal *in vitro,* whereas the majority of the cells does not and dies. Determination of this population requires characterization of the cells by staining of cell surface markers.

To expand *in vitro* to *in vivo* studies, Irmgard Förster developed a method called "BrdU pulse chase assay" that made it possible to track labeled cells over time *in vivo*. This assay revealed that peripheral B cells are relatively stable and survive for several weeks to months before they die and are renewed from the bone marrow (Forster and Rajewsky, 1990). Within this assay, we wanted to evaluate whether the lack of expansion of LMP1/CD40//$CD19^{-/-}$ B cells *in vivo* is due to reduced generation, longevity or the lack of proliferation. The obtained results suggest that peripheral LMP1/CD40//$CD19^{-/-}$ B cells have a high turnover rate and thus a reduced longevity. They are strongly labeled with BrdU during the pulse period but decline rapidly during the chase. BrdU labeling of peripheral B cells during the pulse likely stems from the influx of BM derived B cells (that have incorporated BrdU almost to 100% by division in the BM) since peripheral B cells do not show a higher percentage of cells in the S-phase than control B cells as measured by DNA-content (data not shown). This strong

labeling might thus indicate that they are relatively unstable and constantly refilled from the bone marrow. It is in contrast to $CD19^{-/-}$ B cells that exhibit a similar decline to $CD19^{+/-}$ B cells, albeit it has been shown that they have a slight survival disadvantage that is revealed after long times of labeling and tracking (Otero et al., 2003). Hence, LMP1/CD40-expressing B cells *in vivo* are highly susceptible to the loss of CD19. Interestingly, this survival defect is not as prominent *in vitro*, arguing for a role of the microenvironment in the induction of cell death of LMP1/CD40//$CD19^{-/-}$ B cells *in vivo*. Astonishingly, LMP1/CD40//$CD19^{+/-}$ B cells show a higher percentage of BrdU labeled B cells in the spleen but not in the peripheral blood and show enhanced survival in the blood but not in the spleen. This phenotype might be due to a defect in negative selection of LMP1/CD40-expressing B cells at the T1/ T2 B cell stage, induced by the abnormally high noncanonical NF-κB signaling, resulting in more BrdU labeled B cells passing the transitional stage. However, since this selection defect would result in an accumulation of non-selected, autoreactive B cells in the spleen, these cells might become anergic and die. The non-autoreactive B cells passing this stage on the other hand survive and are able to recirculate to the blood and lymph nodes. These might indeed have a prolonged life span which is detected by the BrdU assay in the blood but masked in the spleen. To finally prove this hypothesis, blockage of BM influx will be applied to exclude newly emerging cells from the bone marrow that have to pass the transitional stage. This requires treatment of mice with a α-IL7 receptor antibody that blocks B cell development in the bone marrow (Sudo et al., 1993).

These data are in line with the observation that CD19 deficiency in LMP1/CD40-expressing B cells abrogates lymphoma development. From the age of 12 to 19 months about 70% of LMP1/CD40//$CD19^{+/-}$ mice develop lymphomas whereas none of the LMP1/CD40//$CD19^{-/-}$ mice does. Our data point out that in the case of aberrant CD40 activation CD19 is required for lymphomagenesis. It will be interesting to study whether LMP1/CD40-expressing lymphoma cells are still dependent on CD19 expression either by downregulating CD19 via siRNA *in vitro* or by treating diseased mice with αCD19 antibodies coupled to Genistein that inhibits CD19 signaling (Uckun et al., 1995).

4.2.4 CD19 deficiency impairs Erk activation in LMP1/CD40-expressing B cells

The examination of signaling pathways shows that CD19 deficiency in LMP1/CD40 transgenic mice does not result in a failure to activate the noncanonical NF-κB pathway but in impaired Erk activation.

LMP1/CD40//CD19$^{-/-}$ B cells exhibit high levels of overall and nuclear p52 and high levels of nuclear RelB and p50 as seen in LMP1/CD40//CD19$^{+/-}$ B cells. P65 and c-Rel levels are similar to control B cells and IκBα is less phosphorylated as observed in LMP1/CD40//CD19$^{+/-}$ B cells. Thus as CD19 proficient, CD19 deficient LMP1/CD40-expressing B cells selectively activate components of the noncanonical NF-κB pathway. However, unlike in LMP1/CD40//CD19$^{+/-}$ B cells, we observed a strong reduction of p100 in LMP1/CD40//CD19$^{-/-}$ and CD19$^{-/-}$ B cells. The publication by Stadanlick *et al.* describes that p100 production is mediated by tonic BCR signals in primary B cells from the T2 B cell stage on (Stadanlick et al., 2008). Thus the abrogation of CD19 might diminish tonic BCR mediated signaling to produce p100. However, since LMP1/CD40//CD19$^{-/-}$ B cells still exhibit increased levels of p52, p100 has to be produced through another pathway induced by LMP1/CD40 in this case. It will be interesting to study whether CD19 deficiency alone and in combination with LMP1/CD40 (that delivers p100) abrogates a B cell's response to BAFF-R signaling. Activation of the BAFF receptor leads to the preferential activation of the noncanonical NF-κB pathway and requires p100 as a substrate for processing to p52.

The investigation of the MAPK Erk and Jnk in LMP1/CD40//CD19$^{-/-}$ B cells revealed that Jnk is constitutively activated as in LMP1/CD40//CD19$^{+/-}$ B cells whereas the activation of Erk is abolished. Thus the loss of Erk activity might account for the impaired expansion of B cells in LMP1/CD40//CD19$^{-/-}$ mice. To confirm this speculation, LMP1/CD40 transgenic mice have to be crossed to conditionally Erk deficient mice (Hatano et al., 2003). At present it is unclear whether CD19 is only required for the constitutive activity of CD40 provided by LMP1/CD40 or whether it is also required for ligand-triggered stimulation of the CD40 receptor on B cells. Supporting the latter hypothesis, CD19$^{-/-}$ B cells do not respond to TD antigens *in vivo* and they do not show Erk activation after CD40 stimulation for 24 hours *in vitro* as compared to wildtype B cells (Engel et al., 1995; Rickert et al., 1995) (data not shown). Indeed, it was discussed by Gärdby *et al.* whether CD19$^{-/-}$ B cells cannot respond to TD antigens because they have a defect in CD40 signaling (Gardby et al., 2001). The question remains how Erk exerts its function in driving expansion of LMP1/CD40-expressing B cells *in vivo*. It is known to promote both survival and proliferation of cells. Hence, it will be interesting to analyze which antiapoptotic signals are diminished in LMP1/CD40-expressing B cells upon CD19 deletion and whether cell-cycle regulating proteins are affected.

So far CD19 has been described as a coreceptor of the BCR. Our data suggest that it might also function as a coreceptor for CD40. Consequently, we analyzed whether CD40 signals are

similarily transduced through CD19 as BCR signals are. As a coreceptor of the BCR, CD19 exerts its function via PI3K predominantly since mutation of Tyr 482 and 513 which have been shown to bind PI3K in the CD19 cytoplasmic tail results in a very similar phenotype to the complete knock out (Wang et al., 2002). Furthermore, the effects of CD19 deficiency can be rescued by Pten deficiency, which results in constitutive PI3K activity (Anzelon et al., 2003). Moreover, it was shown that BCR induced activation of Erk is dependent on PI3K and PLCγ (Jacob and Kelsoe, 1992). Therefore we tested whether Erk activation in LMP1/CD40-expressing B cells is dependent on PI3K as well. Treatment of primary LMP1/CD40//CD19$^{+/-}$ B cells with two different PI3K inhibitors, LY294002 and Wortmannin revealed that Erk phosphorylation is abolished upon inhibition of PI3K whereas Jnk phosphorylation is not. Concomitantly, LMP1/CD40-expressing B cells were found to depend on PI3K in their enhanced survival *in vitro*, similar to their dependence on Erk. The kinase Akt is described as the main readout of PI3K activity; LMP1/CD40 signaling, however, does not constitutively activate Akt in B cells. It has been shown that certain PI3K p110 subunits activate Akt or Erk preferentially, thus it will be interesting to study if a certain subunit is responsible for the activation of Erk by LMP1/CD40 (Denley et al., 2008). The activation of Erk by LMP1/CD40 is thus a CD19 and PI3K dependent process that is required for expansion of B cells *in vivo*. Currently, we intend to investigate whether LMP1/CD40, CD19 and associated PI3K are found in lipid rafts to further support the assumption of CD19's role as a coreceptor of CD40.
To set a link between LMP1/CD40 and CD19, we analyzed the phosphorylation status of the PTK Lyn. Former studies showed that CD40 activation of B cells leads to the phosphorylation of the PTKs Lyn, Fyn and Syk (Ren et al., 1994; Uckun et al., 1993). We hypothesized that constitutive CD40 signaling *in vivo* engages CD19 via Lyn that itself is known to bind CD19. Indeed, Lyn is constitutively phosphorylated in LMP1/CD40-expressing B cells. Hence, Lyn provides a possible link between CD40 and CD19, similar in its function to link the BCR and CD19.

These findings constitute the following model (Figure 31). LMP1/CD40 activates the MAPK Jnk and the noncanonical NF-κB pathway without the need for CD19 expression. In contrast, the activation of Erk by constitutive CD40 signaling is dependent on CD19. We propose that LMP1/CD40 activates Lyn via an unknown mechanism, presumably in a raft-dependent manner. Lyn associates with and activates CD19 that then binds PI3K and thus recruits it to the signalosome. PI3K activation ultimately leads to the phosphorylation of Erk1/2, either directly or indirectly through recruitment of Vav1. Interestingly, activation of Erk by CD40

has been shown to be mediated by Lyn and Syk in dendritic cells (Vidalain et al., 2000). According to Vidalain *et al.*, CD40 activates Erk via Lyn and Syk within lipid rafts and results in cytokine production.

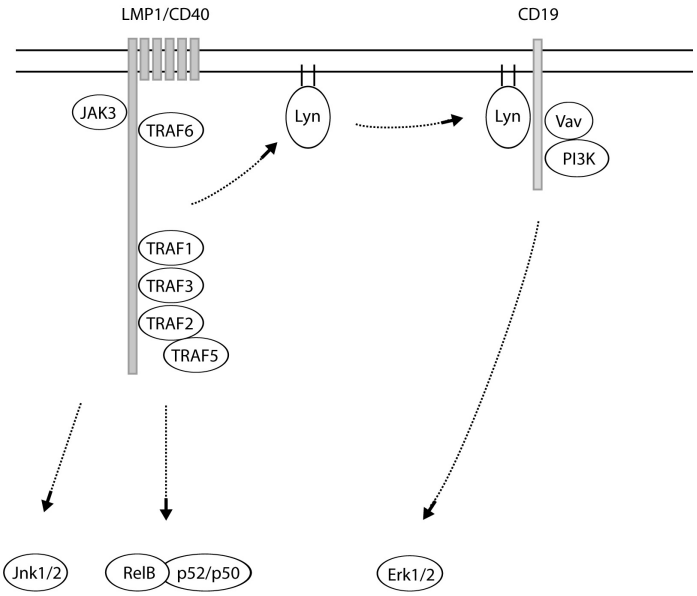


Figure 31 Working model
LMP1/CD40 activates Jnk and the noncanonical NF-κB pathway without the need for CD19. Its activation of Erk however is dependent on CD19 expression. LMP1/CD40 further activates Lyn, a protein tyrosin kinase that is usually involved in proximal BCR signaling. Lyn is known to associate with the cytoplasmic tail of CD19 upon activation. This results in recruitment of PI3K. Via an unresolved mechanism, PI3K is required for the activation of Erk in LMP1/CD40-expressing B cells. This activation might be responsible for promoting survival and proliferation.

Besides the BCR, CD19 might serve as a coreceptor for a variety of B cell activating receptors integrating different signals at a proximate level. If this status is maintained in already established tumor cells, the inhibition of CD19 signaling can be a specific therapy to target (aberrantly) activated B cells by promoting cell death. To test this hypothesis in a human disease, B-CLL serves as a possible candidate. Malignant cells of B-CLL express CD40, the BCR and CD19 and are known to activate both PI3K and Erk. Activation of CD40 by CD40L expressing T cells is thought to protect these cells from Fas mediated apoptosis (de Totero et al., 2004). Ablation of CD19 in B-CLL cells will show whether the described scenario accounts for these tumor cells as well. Indeed, blocking CD19 signaling by Genistein-coupled α-CD19 antibodies has been proposed as a therapeutic approach of B cell precursor lymphomas in 1995 by Uckun *et al.* (Uckun et al., 1995).

4.3 The generation of a Tet inducible Notch2-IC transgenic mouse strain

Notch has been associated with oncogenic and growth-promoting potential in various cancers (Koch and Radtke, 2007). It correlates with a poor prognosis in breast cancer and medulloblastoma. Notch1 is known to be involved in T cell tumorigenesis. In a few cases, Notch1 is fused to the TCRβ locus through a chromosomal translocation, resulting in a truncated Notch1-IC. In most of the cases it is aberrantly activated via insertions or frameshift mutations. However, the chromosomal translocation did not only lead to the discovery of human Notch, Notch1-IC overexpression in BM reconstituted or transgenic mice also resulted in a T-ALL model system (Beverly et al., 2005; Pear et al., 1996). In B cells, aberrant expression of Notch has been found in HL, B-CLL and Multiple Myeloma (MM) (Houde et al., 2004; Hubmann et al., 2002; Jundt et al., 2004; Kapp et al., 1999). A former member of our lab showed that expression of Notch1 or 2 in a B cell line promotes proliferation but concomitantly triggers apoptosis (Kohlhof et al., 2009). Thus Notch expression alone might not be able to induce lymphomagenesis in B cells unless the induction of apoptosis is counteracted by survival inducing pathways like CD40 signaling. A similar scenario is found in HRS cells of Hodgkin lymphomas where Notch1 and 2 as well as CD40 are highly expressed.

We aimed to establish a mouse model to study the effects of Notch2-IC signaling in B cells *in vivo* and to investigate whether it has oncogenic potential when aberrantly activated alone or together with constitutive CD40 signals. If Notch2-IC expression indeed promotes lymphomagenesis we will be able to deprive established tumor cells from Notch expression to test its requirement for maintenance of the tumor as we set *Notch2-IC* under a Tet regulatable promoter. Since this promoter is bidirectional, *EFGP* was cloned on the other side to be able to track Notch2-IC- expressing B cells via EGFP fluorescence. *In vitro*, we could show that GFP can be expressed from the bidirectional TetO7 promoter of our construct in targeted embryonic stem cells and we assume that Notch2-IC is concomitantly expressed. A member of our lab recently provided evidence that the R26STOPrtTA strain we received from Wei Hsu is applicable for Dox regulated gene expression in B cells *in vivo* (S. Feicht, personal communication). We assume that crossing the Tet-Notch2-IC strain to the R26STOPrtTA and a B cell specific Cre strain will result in a Tet controllable, B cell specific expression of Notch2-IC. In the offspring of these crossings we will study the effects of Notch2-IC on B cell development and tumorigenesis. Since the TetO7 promoter does not promote very strong transgene expression, we decided to place a frt-flanked B cell specific enhancer (Eκ) downstream of the Notch2-IC construct. This might result in leakiness; however, it also

enables us to study different Notch2-IC expression levels. The Tet-Notch2-IC mouse strain can be further crossed to the LMP1/CD40 mouse strain to analyze how constitutive Notch activation influences constitutive CD40 signaling regarding the B cell phenotype and in triggering lymphomagenesis. This might generate a suitable model for B-CLL or HL where CD40 and Notch are known to be expressed concomitantly and suggested to be involved in the phenotype of the tumor cells. In HL, signals from the Notch receptor might play a role in downmodulating B cell specific genes, since Notch is known to block B cell development when expressed in B cell progenitors (Witt et al., 2003). Thus combination of Notch2-IC and LMP1/CD40 expression in GC B cells *in vivo* might result in Hodgkin-like lymphomas, providing a tool to study the human disease in a murine model.

4.4 Mechanisms of B cell activation and lymphomagenesis

During T cell dependent immune reactions, B cells are activated through two major signals: binding of specific antigen to the B cell receptor and CD40 receptor costimulation through T cell contact (Figure 32A). These activated B cells are able to form germinal centers where somatic hypermutation and class switch recombination take place. Highly affine, positively selected B cells then differentiate into plasma or memory B cells. Deregulated signals from CD40 and other potentially costimulatory receptors such as Notch have been implicated in B cell lymphomagenesis. Our lab could show recently that the constitutive activity of CD40 in B cells leads to premalignant expansion of B cells *in vivo* and promotes B cell lymphomagenesis supporting its potential role in human B cell tumors such as Hodgkin lymphomas or B-CLL (Figure 32B). We could further show that the constitutive activity of CD40 in B cells leads to the selective activation of the noncanonical NF-κB pathway as well as the MAPK Erk and Jnk. *In vitro* studies provided evidence that Erk is critically required for the improved survival of B cells promoted by constitutive CD40 activity. In contrast, B cell lymphomas, developing in these mice, did not reveal a consistent pattern of activated signaling pathways. These results imply that second hits are required to initiate transformation of aberrantly CD40 activated cells. It has long been discussed whether the initiation and/ or maintenance of B cell tumors requires expression of a functional BCR: almost all B cell tumors express the BCR, however, one entity, Hodgkin lymphoma, shows a downregulation of BCR components. Resting, mature B cells strictly depend on the tonic survival signal provided by the BCR. If this is also true for pre- and malignant B cells is not known so far, but might provide a tool to specifically target B cell tumors. Ablation of the signaling domains of the BCR, Igα or Igβ, in

mature B cells results in loss of the peripheral B cell pool (Figure 32C). Deletion of CD19, described as a coreceptor of the BCR, leads to a reduction of perpheral B cell numbers but not to a complete loss. During premalignant B cell expansion due to constitutive CD40 signals this observation is reversed: aberrantly activated B cells are less dependent on BCR signals, however, they fully depend on CD19 (Figure 32B).

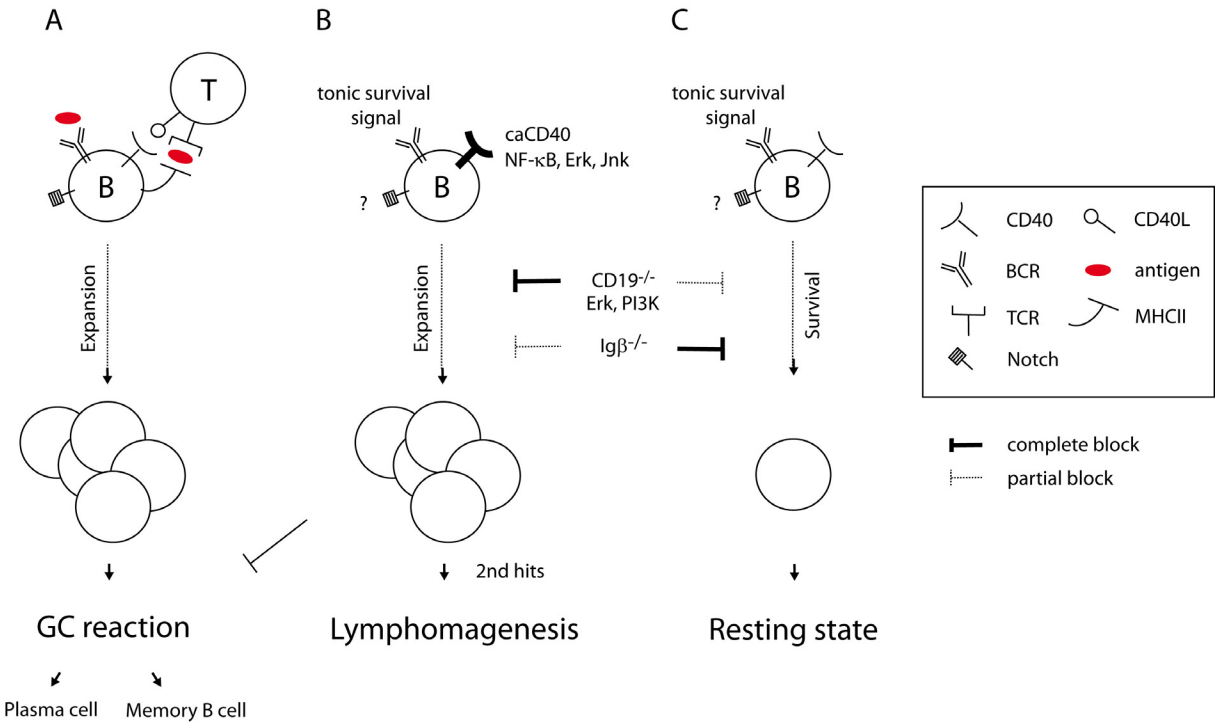


Figure 32 CD40 signaling in B cell activation and lymphomagenesis
(A) B cell activation during a T cell dependent immune response requires (i) signaling through the BCR by antigen binding and (ii) interaction with activated T helper cells. T cells recognize specific B cells through binding of their TCR complex to the antigen presented on MHCII on B cells. CD40L that is expressed on activated T cells provides an important costimulatory signal to B cells through CD40 binding. This activation results in B cell expansion and a germinal center reaction, leading to plasma as well as memory B cell differentiation. (B) Constitutive CD40 signaling in B cells results in premalignant B cell expansion and renders B cells prone to lymphomagenesis. The constitutive activity of CD40 results in activation of the noncanonical NF-κB pathway and the MAPK Jnk and Erk. Deletion of CD19 in this model completely blocks premalignant B cell expansion and specifically abrogates Erk activation, whereas deletion of Igβ leads to a less severe reduction of B cells. (C) Resting B cells require the expression of a functional BCR for survival. Ablation of Igβ results in a complete loss of B cells, ablation of CD19 diminishes B cell numbers. B (B cell), T (T cell), GC (germinal center), caCD40 (constitutively active CD40 receptor, achieved by the fusion protein LMP1/CD40), BCR (B cell receptor), TCR (T cell receptor), MHCII (major histocompability complex of the class II), ? (function unknown).

This led us to two conclusions: (i) aberrant activation of B cells through constitutive CD40 signaling partially overcomes the need for tonic BCR signaling and (ii) CD19 has an important, BCR independent function during (aberrant) B cell activation through CD40. Thus

it probably functions as a coreceptor for CD40 as it does for the BCR. The complete ablation of B cell expansion through CD40 by CD19 deletion is accompanied by a loss of Erk activation. We propose that CD19 is required for CD40 mediated Erk activation and survival of B cells *in vivo* and thereby is crucial for rendering the cells prone to lymphomagenesis. The role of Notch2 during B cell activation and lymphomagenesis has not been clarified until now but may be elucidated in future by the transgenic mouse strain for Dox-regulatable Notch2-IC.

5 Summary

B lymphocytes recirculate throughout the body in a resting state for long periods of time. However, upon infection, specific B cells encounter their cognate antigen and become activated. This activation signal leads to engagement of different receptors -among them CD40, the BCR and Notch- that trigger rapid expansion of B cells. It has been speculated that (deregulated) expression of these receptors promotes B cell lymphomagenesis. Thus, if the tight regulation of activation is disturbed, B cells might transform and give rise to lymphomas. In fact, most of the B cell lymphomas are derived from GC or post-GC B cells. Previous work revealed that deregulation of the CD40 receptor signal promotes premalignant B cell expansion and lymphomagenesis. Since most of B cell lymphomas still express the BCR, their survival has been suggested to be strictly dependent on the so called tonic, ligand-independent BCR signal. The Notch receptor is known to be involved in the development of T-ALL and is expressed aberrantly on HL and B-CLL, however, an oncogenic role in B cell tumorigenesis has not been proven experimentally so far.

In the present work, the influence of CD40 and tonic B cell receptor signaling on aberrant B cell activation and lymphomagenesis was investigated. Furthermore, a transgenic mouse strain to study the malignant potential of constitutively active Notch2 in B cells was generated.

The expression of a constitutively active CD40 receptor, LMP1/CD40, in B cells has formerly been shown to trigger B cell expansion and lymphomagenesis *in vivo* (Homig-Holzel et al., 2008). The current investigation of the signaling pathways that are activated in premalignant LMP1/CD40-expressing B cells *in vivo* demonstrates that constitutive CD40 signaling selectively activates the noncanonical NF-κB pathway and the MAP kinases Erk and Jnk. Furthermore, we show that enhanced survival induced by LMP1/CD40 signaling in *ex vivo* isolated B cells is dependent on Erk, but only marginally on Jnk activity. Malignant B cells from LMP1/CD40 transgenic mice on the other hand display an inconsistent pattern of activated signaling pathways. To conclude, constitutive CD40 signaling activates specific signaling pathways that render B cells prone to lymphomagenesis. However, secondary events have to take place to finally initiate lymphomagenesis.

The examination of the role of tonic BCR signaling in LMP1/CD40 induced B cell expansion *in vivo* was pursued in two ways. Ablation of Igβ in mature LMP1/CD40-expressing B cells abolishes tonic BCR signals completely. By that, expansion of the cells is abrogated, but Igβ deficient B cells can be rescued by LMP1/CD40 expression to a certain degree, showing that

constitutive activity of CD40 renders B cells less dependent on tonic BCR signaling. Ablation of CD19 on the other hand was applied to diminish survival signals by the BCR. This investigation revealed that CD19 is strictly required for LMP1/CD40 induced B cell expansion and lymphomagenesis *in vivo*, pointing to a BCR independent role of CD19 during constitutive CD40 signaling. Deletion of CD19 in LMP1/CD40-expressing B cells leads to a reduced life span of B cells in the periphery, suggesting that CD19 is required for the survival of CD40-activated B cells. This survival is most likely mediated by the activation of the MAPK Erk through PI3K that is abolished upon CD19 deletion in LMP1/CD40 expressing B cells. Thus B cells that are activated through constitutive CD40 signaling are less dependent on tonic BCR/Igβ signaling than wildtype B cells but strictly dependent on CD19 expression. Presumably, CD19 exerts a function independently of the BCR in the case of constitutive CD40 signaling. Indeed, it has been speculated whether the irresponsiveness of CD19 deficient B cells to TD antigens *in vivo* is due to impaired CD40 signaling.

Besides from CD40 and the BCR, Notch2 expression is observed in different B cell tumors. It has been speculated whether aberrant activation of the receptor is involved in B cell tumorigenesis. Thus we aimed to generate a Tet regulatable Notch2-IC transgenic mouse line where constitutively active Notch2 can be reversibly expressed in B cells. The established transgenic strain may help to understand the function of Notch in the development of certain B cell malignancies.

6 Zusammenfassung

B-Lymphozyten als zellulärer Bestandteil des adaptiven Immunsystems zirkulieren lange Zeit als ruhende Zellen durch den Körper. Bei einer Infektion kommen einige wenige B-Zellen in Kontakt mit spezifischen Antigenen und werden aktiviert um eine Immunreaktion gegen den Erreger aufzubauen. Während dieser Aktivierung erfahren B-Zellen verschiedenste Signale, die durch Rezeptoren vermittelt werden und eine rasche Expansion der Zellen auslösen. Einer dieser Rezeptoren ist der CD40-Rezeptor, der eine Schlüsselrolle bei der T-Zell-abhängigen Aktivierung von B-Zellen spielt. Vorangegangene Arbeiten deckten auf, dass aberrante CD40 Signale zur Expansion von B-Zellen führen und die Entstehung von B-Zell-Lymphomen fördern. Dies weist darauf hin, dass die Aktivierung von B-Zellen während einer Immunreaktion streng reguliert werden muss, um aberrante Expansion und Tumorentstehung zu verhindern. Tatsächlich entstammen die meisten B-Zell-Lymphome dem Keimzentrum, das sich durch die Aktivierung und Expansion von B-Zellen während einer T-Zell-abhängigen Immunantwort bildet. Die meisten B-Zell-Lymphome exprimieren zudem den B-Zell-Rezeptor, dessen Liganden-unabhängige (tonische) Signale für das Überleben ruhender B-Zellen unabkömmlich sind, weshalb spekuliert wird, ob dieses Signal auch bei der Entstehung oder sogar Aufrechterhaltung von B-Zell-Tumoren eine Rolle spielt. Notch, ein häufig in Zell-Differenzierungsprozessen involvierter Rezeptor, induziert Leukämien in T-Zellen und ist in dem Großteil aller T-ALL Patienten mutiert. Notch1 und 2 werden auf HL und B-CLL B-Zellen exprimiert, allerdings wurde bisher nicht erforscht, ob Notch Expression eine Rolle bei der Entstehung dieser Tumore spielt.

In der vorliegenden Arbeit wurde der Einfluss eines aberranten CD40-Signals und des tonischen B-Zell-Rezeptor-Signals auf B-Zell-Aktivierung und Lymphomentstehung untersucht. Zudem wurde ein Mausstamm generiert, in dem der Einfluss von deregulierten Notch-Signalen auf die Enstehung von B-Zell-Lymphomen untersucht werden kann.

Die Expression eines konstitutiv aktiven CD40-Rezeptors, in Form des Fusionsproteins LMP1/CD40, führt zur B-Zell-Expansion und Lymphomentstehung *in vivo* wie von Cornelia Hömig-Hölzel gezeigt werden konnte. In der vorliegenden Arbeit wird beschrieben dass konstitutive CD40-Signale in prä-malignen B-Zellen selektiv den nicht-kanonischen NF-κB Signalweg und die MAP-Kinasen Erk und Jnk aktivieren. Die Aktivität von Erk scheint insbesondere für das verbesserte Überleben der Zellen *in vitro* notwendig zu sein. Maligne B-Zellen der LMP1/CD40-transgenen Mäuse zeigen dagegen kein durchgängiges Aktivierungsmuster der beschriebenen Signalwege. Die lange Tumorlatenzzeit wie auch der

Verlust des prä-malignen Aktivierungsmusters in transformierten Zellen lassen darauf schließen, dass weitere Mutationen notwendig sind, um die Zellen letztendlich zu transformieren.

Um zu untersuchen, ob LMP1/CD40-induzierte Aktivierung, Expansion und Lymphomentstehung abhängig von tonischen B-Zell-Rezeptor-Signalen sind, wurden zwei verschiedene Strategien verfolgt. Zum einen wurde Igβ, eines der beiden Signalmoleküle des B-Zell-Rezeptors, zum anderen CD19, ein Ko-Rezeptor des B-Zell-Rezeptors, in LMP1/CD40-exprimierenden B-Zellen deletiert. Die Deletion von Igβ resultiert in einem völligen Verlust des tonischen B-Zell-Rezeptor-Signals, die Deletion von CD19 vermindert dieses womöglich. Die Untersuchungen ergaben, dass LMP1/CD40-exprimierende B-Zellen weniger abhängig von tonischen BCR-Signalen als Wildtyp-Zellen sind. Im Gegensatz dazu ist die LMP1/CD40-induzierte Expansion und Tumorentstehung von B-Zellen komplett von CD19 abhängig. Deletion von CD19 in LMP1/CD40-exprimierenden B-Zellen führt zu einer verringerten Lebensspanne der Zellen *in vivo*, was darauf hinweist, dass CD19 für das Überleben CD40-aktivierter B-Zellen notwendig ist. Dieses Überlebenssignal könnte durch die Aktivierung der MAPK Erk vermittelt werden, die in LMP1/CD40-exprimierenden B-Zellen ohne CD19 und bei PI3K Inhibition stark vermindert ist. Demnach sind LMP1/CD40-exprimierende B-Zellen stärker von CD19 als vom tonischen B-Zell-Rezeptor-Signal abhängig. Möglicherweise hat CD19 bei konstitutiver CD40 Stimulation von B-Zellen eine BCR-unabhängige Funktion. Interessanterweise wurde diskutiert, ob CD19 defiziente Mäuse aus diesem Grund nicht auf T-Zell-abhängige Antigene reagieren können.

Neben CD40 und dem B-Zell-Rezeptor exprimieren einige B-Zell-Lymphome übermäßig Notch1 und/ oder Notch2. Um zu untersuchen ob diese aberrante Expression von Notch Rezeptoren eine Rolle bei der Entstehung oder Aufrechterhaltung von B-Zell-Tumoren spielt, wurde ein Mausstamm generiert, in dem ein konstitutiv aktives Notch2-Molekül reversibel in B-Zellen exprimiert werden kann.

7 Material

7.1 Plasmids

pHprt-Notch2-IC-Ekappa

This contruct constitutes the Hprt targeting vector that encodes for the *Notch2-IC* transgene which is under the control of the bidirectional TetO7 promoter (TetO7BI). This bidirectional promoter contains minimal CMV promoter regions and 7 repeats of the E.coli Tet operon structure in both transcribable directions. *EGFP* was targeted to the other side of this bidirectional promoter to allow tracking of Notch2-IC-expressing cells via the fluorescence signal. A frt-flanked Eκ enhancer was further placed downstream of the EGFP-TetO7BI-Notch2-IC. This construct was inserted into a targeting cassette allowing the homologous recombination into the murine *Hprt* locus via two homologous arms (3.8 kB and 4 kB). The 3.8 kB arm is located upstream of the Hprt gene whereas the 4 kB arm is within the Hprt gene, containing intron 2 and exon 3. A 3.6 kB construct is located upstream of the 4 kB arm that complements the endogenous *Hprt* locus by providing the promoter, exon 1, part of intron 1 and exon 2 that are lacking in *Hprt* deficient ES cells used for targeting.

Blueiiks

This vector was used for subcloning. The multiple cloning site was exchanged with a fragment containing suitable cloning restriction sites for the targeting vector cloning strategy (*NotI-AscI-SfiI-SacI-PacI-SbfI-XbaI-BamHI-EcoRI-NsiI-MluI*).

BSAsc LMP1 (Cornelia Hömig-Hölzel)

This vector contains LMP1 and bovine polyA.

LMP1rosa26neu (Cornelia Hömig-Hölzel)

This vector encodes for LMP1, RFP and Neo^R, flanked by homologous arms of the *Rosa26* locus.

pEBNA-SVH-GL-EmCAG-rtTA-tTR (Georg Bornkamm, modified by Hella Kohlhof)

This vector contains the bidirectional promoter TetO7BI, with EFGP cloned in one and Luc in the other direction.

pgem7zf-frt (provided by Ursula Zimber-Strobl)
This pGEM-based vector contains frt sites.

BC230A 2-10-02 (Georg Bornkamm)
This vector contains the B cell specific enhancer Eκ.

pTracer-CMV-Notch2cDNA vector (Shimizu et al., 2000); provided by Freddy Radtke)
The described vector provides the full length *Notch2* cDNA.

Targeting vector pMP9 (provided by Klaus Rajewsky)
This targeting vector for the *Hprt* locus contains the 5' upstream homologous region and the human *Hprt* promoter, exon 1 to 2 as complementing regions and exon 3 as the 3' homologous region of the *Hprt* locus. *NotI* and *MluI* can be used to clone the gene of interest in between the homologous arms.

p141CAG-3SIP (Georg Bornkamm)
This vector contains a CAG promoter and was used as an expression vector of rtTA2^{S}-M2 in ES cells.

pRTS-1 (pRTS-GL-SVH) (rtTA corrected) (Georg Bornkamm)
This is a Tet-on expression plasmid containing the Dox sensitive variant rtTA2^{S}-M2.

7.2 Bacteria

XL-1blue (*Escherichia coli*)
Genotype: F´:Tu10 proA+B+ laclq D (lacZ) M15/recA1 end A1 gyr A96 (Nalr) thi hsd R17 (rk-mk+) glu V44 rel A1 lac.

7.3 Cell lines

E14TG2a (Hooper et al., 1987), provided by Ralf Kühn)
This 129/SV derived male embryonic stem cell line carries a deletion of the Hprt promoter, Exon 1 and 2.

Embryonic feeder cells

EF cells were prepared by from pSV2neo, PEP-IL4 C57BL/6 embryos and used for ES cell culture only until passage 3.

7.4 Mouse strains

LMP1/CD40^{flSTOPP} (Homig-Holzel et al., 2008)

Balb/C mouse strain that is transgenic for the chimeric *LMP1/CD40* gene placed into the *Rosa26* locus. A loxP flanked STOP cassette upstream of the *LMP1/CD40* gene prevents expression of the gene unless Cre is expressed and leads to the deletion of the STOP cassette.

CD19-Cre (Rickert et al., 1997)

The Cre-recombinase is placed into the *CD19* locus in this C57BL/6 mouse strain, thereby being regulated by the CD19 promoter in its expression but destructing the *CD19* gene.

Igβ$^{fl(GFP)}$ (Casola et al., 2006)

C57BL/6 mouse strain with loxP flanked exons 5 and 6 of the *Igβ* gene and a downstream IRES-*GFP*.

Igβ$^{\Delta c}$ (N. Uyttersprot)

C57BL/6 mouse strain with deleted exons 5 and 6 of the *Igβ* gene.

CD21-Cre (Kraus et al., 2004)

C57BL/6 mouse strain with a randomly integrated BAC clone with Cre controlled in its gene expression by the CD21 promoter.

R26STOPrtTA (Yu et al., 2005)

129/SV mouse strain with the rtTA2^{S}-M2 that is preceeded by a loxP flanked STOP cassette and placed into the *Rosa26* locus.

C57BL/6 (Charles River Laboratories)

This mouse strain was used for blastocyst injection and for germline transmission crossings.

7.5 Primer

Primer name	Primer sequence (5'-3')
Ex1Fw1 LMP1	AGG AGC CCT CCT TGT CCT CTA
CD40 PCR3	CTG AGA TGC GAC TCT CTT TGC CAT
Rosa fw1 (60)	CTC TCC CAA AGT CGC TCT G
Rosa rev2 (62)	TAC TCC GAG GCG GAT CAC AAG C
CD19c	AAC CAG TCA ACA CCC TTC C
CD19d	CCA GAC TAG ATA CAG ACC AG
Cre 7	TCA GCT ACA CCA GAG ACG G
LMP1/CD40del fw	GTT GCA ATA CCT TTC TGG GAG TTC
LMP1/CD40del rev	GGG CTC CTC CAG TCC AGT C
Robr2631	TCC CGA CAA AAC CGA AAA TC
3ArM02	TTC CTT GAC CCT GGA AGG T
Robr2638	AAG CAC GTT TCC GAC TTG AG
Notch2IC fw	CGAATCGAATTCCCTGCAGGGGTCATCATGGCCAAGCGGAAGCAAGC
Notch2IC rev	GGAGTGGAATTCCCTGCAGGTCATGCATACACCTGCATGTTGCT
pA_EGFP seq	ACCATTATTATCATGACATTAACCT
EGFP_TetO7BI seq	CGATGTTGTGGCGGATCTTGAAGTT
TetO7BI seq	CGAGCTACTTTCGCTGAC
Rosa26Ex1In1 seq	TAGCATCTGTAGGGCGCAGTAGT
Rosa26SA_Notch2IC seq	CCAGGACAACATGGGCCGATGTC
Notch2IC seq	TCCTCACACGTATGTCTCCGATG
Notch2IC_bpA_frt seq	TCACCTCCAGGGCGAGCACC
Ekappa 1 seq	GGCATAAGGACTGGGGAGTT
Ekappa 2 seq	CTTTGGCTGGACTGGGCTAA
Ekappa 3 seq	AGGGAGGTTTTAGCCCAGT
Ekappa 4 seq	CACACTGCTTTGGTCAAGGA
Notch2IC genomic 1 seq	CAAACTCTTCGCGGTCTTTC
Notch2IC genomic 2 seq	GGGATGTGATCATGGGAGAG
Notch2IC genomic 3 seq	TCTTGACTTCTGCGCTCTCA
Notch2IC genomic 4 seq	GAATTCGGATCCAGACATGA
Notch2IC genomic 5 seq	GTTCTGCCTGAGGAGGAGTG

7.6 DNA probes

RsaI (Tsuda et al., 1997)

A 250 bp fragment was isolated from the RsaI plasmid (kindly provided by U. Zimber-Strobl) using the enzymes *BamHI* and *HindIII*.

Notch2 (F. Hampel)

A 319 bp fragment was isolated from the pSG5_N2IC plasmid using the enzymes *XmaI* and *StuI* (kindly provided by Franziska Hampel).

IgH (Gao et al., 2000)

A 1.6 kb fragment was isolated from the IgH plasmid (kindly provided by Stefano Casola) using the enzymes *EcoRI* and *HindIII*. The probe spans the J_H3-4 region of the mouse IgH locus.

7.7 Antibodies

Antigen	Ab ID	Source	kDa	Dilution	Dilutant
pErk	Cell Signaling	rabbit	44, 42	1:1000	TBST , 5% BSA
Erk	Cell Signaling	rabbit	44,42	1:1000	TBST , 5% BSA
pJnk	Cell Signaling	rabbit	46, 54	1:1000	TBST , 5% BSA
Jnk	Cell Signaling	rabbit	46, 54	1:1000	TBST , 5% BSA
pp38	Cell Signaling	rabbit	38	1:1000	TBST , 5% BSA
P38	Cell Signaling	rabbit	38	1:1000	TBST , 5% BSA
pAkt (Ser)	Cell Signaling	rabbit	60	1:1000	TBST , 5% BSA
pAkt (Thr)	Cell Signaling	rabbit	60	1:1000	TBST , 5% BSA
Akt	Cell Signaling	rabbit	60	1:1000	TBST , 5% BSA
pIκBα	Cell Signaling	mouse	40	1:1000	TBST , 5% MP
IκBα	Santa Cruz	rabbit	40	1:1000	TBST , 5% BSA
p52p100	Cell Signaling	Rabbit	52, 100	1:1000	TBST , 5% BSA
RelB	Cell Signaling	rabbit	60	1:1000	TBST , 5% BSA
cRel	Santa Cruz	mouse	75	1:500	TBST , 5% BSA
p65	Santa Cruz	rabbit	65	1:1000	TBST , 5% BSA
Bcl3	Santa Cruz	rabbit	60	1:500	TBST , 5% BSA
p50p105	Santa Cruz	goat	50, 100	1:500	TBST , 5% BSA
α-Tubulin	Cell Signaling	mouse	55	1:1000	TBST , 5% BSA
pLyn	Cell Signaling	rabbit	53, 56	1:1000	TBST , 5% BSA
Lyn	Cell Signaling	rabbit	53, 56	1:1000	TBST , 5% BSA
H3	Cell Signaling	mouse	17	1:1000	TBST , 5% BSA

7.8 Small chemical inhibitors

SP600125 (AG Scientific)
The lyophylized powder was solved in DMSO in a stock concentration of 10 mM and used in a working concentration of 2.5 µM

UO126 (Cell Signaling)
A 10 mM stock concentration was prepared in DMSO and diluted to 10 µM as a working concentration.

LY294002 (Cell Signaling)
The lyophylized powder was solved in DMSO in a stock concentration of 10 mM and used in a working concentration of 20 µM.

Wortmannin (Cell Signaling)
The lyophylized powder was solved in DMSO in a stock concentration of 10 mM and used in a working concentration of 0.1 µM.

7.9 Enzymes

Restriction endonucleases were purchased from New England BioLabs and MBI Fermentas.
Taq DNA polymerase was purchased from Invitrogen Life Technologies.

7.10 Software

Adobe Photoshop CS3
Adobe Illustrator CS3
Clone Manager 9
CELLQuest Becton Dickinson
Endnote
Microsoft Excel
Microsoft Word
OpenLab Improvision
TINA

8 Methods

8.1 Mice

8.1.1 Mouse breedings

LMP1/CD40//CD19$^{-/-}$ mice were generated by crossing LMP1/CD40^{flSTOP} (Balb/C) and CD19-Cre (C57BL/6) mice. LMP1/CD40//Igβ$^{\Delta/\Delta}$ mice were generated by crossing LMP1/CD40^{flSTOP} (Balb/C), Igβ$^{fl(GFP)/\Delta c}$ (C57BL/6) and CD21-Cre (C57BL/6) mice. Analyses were thus performed on a mixed background. Mice were analyzed at 8-16 weeks of age unless stated otherwise. Mice that were monitored for lymphoma development were kept under observation and palpated regularly to detect lymphoma development. Animals that were obviously sick were sacrificed and analyzed. All animals were sacrificed and analyzed at an age of 19 months. All mice were bred and maintained in specific pathogen-free conditions and the experiments were performed in compliance with the German animal welfare law and have been approved by the institutional committee on animal experimentation.

8.1.2 Isolation of primary lymphocytes

Mice were killed by CO_2 gassing for two minutes prior to dissection. Cells from the peritoneal cavity were isolated by rinsing the cavity with medium (1x RPMI 1640, containing 5% (v/v) FCS, 1% (v/v) Penicillin-Streptomycin, 1% (v/v) non-essential aminoacids, 1% (v/v) sodiumpyruvate, 1% (v/v) L-Glutamin, 50 µM β-Mercaptoethanol (Gibco)) and draining it with a syringe. Spleen and inguinal lymph nodes were taken out as whole organs; bone marrow cells were prepared from the femur by rinsing the cavity with medium. Single cell suspension from spleen and lymph nodes were prepared by passing the tissues through a cell strainer (Becton Dickinson). Single cell suspensions from spleen and bone marrow were depleted from erythrocytes by 3' lysis with a hypotonic buffer (1:9 mixture of 170 mM Tris/HCl, pH 7.65, and 155 mM NH_4Cl).

Isolation of splenic B cells was performed by CD43 depletion of non-B cells by Magnetic Cell Separation (MACS) according to the manufacturer's protocol (Miltenyi). This resulted in a purity of 85 to 95% B cells.

8.1.3 Flow cytometry

Single-cell suspensions prepared from various lymphoid organs were surface stained with combinations of FITC, PE, PerCP and APC conjugated monoclonal antibodies. Antibodies to B220, CD19, CD5, CD21, CD23, CD43, CD95, ICAM-1, AA4.1, IgD and IgM were purchased from BD Biosciences. The monoclonal antibody to CD54 was kindly provided by J. Mysliwietz (Helmholtz Center Munich). All analyses were made with a FACSCalibur™ (BD Biosciences) and results were analyzed using CELLQuest™ software. Data were analyzed from $3x10^4$ viable lymphocyte-gated cells as determined by forward and side scatter and TOPRO-3 (Molecular Probes) staining. TOPRO-3 is a fluorescent dye that binds DNA and thus only stains dead cells that are permeable for the dye to diffuse into the nucleus.

8.1.4 *In vitro* cultures of primary lymphocytes

MACS purified B cells were cultured for up to 5 days in 96-well plates ($5x10^5$/well). The percentage of living cells was determined by staining and excluding dead cells by TOPRO-3 that binds to DNA and analyzed with a FACSCalibur™. For proliferation assays splenic cells ($5x10^6$/ml) were labeled by incubation in serum-free RPMI containing 5-(and 6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; final concentration 5 µM; Molecular Probes) for 5 min at 37°C. CFSE binds to proteins on the inner membrane of cells and is thus transferred equally to each daughter cell upon cell division. CFSE-labeled cells were cultured for up to 5 days in 96-well plates ($5x10^5$/well) in B cell medium with 10% FCS (v/v) and analyzed by flow cytometry. For treatment with small chemical inhibitors B cells were cultured with 2.5 µM SP600125 (AgScientific), 10 µM UO126 (Cell Signaling), 20 µM LY294002 (Cell Signaling), 0.1 µM Wortmannin (Cell Signaling) or DMSO (Calbiochem) in B cell medium from 1 hour to five days. SP600125 is a reversible ATP-competitive inhibitor of the kinase Jnk thus directly inhibiting its function to phosphorylate substrates (Bennett et al., 2001). UO126 inhibits the kinase activity of the MAPKK Mek1/2 and therefore inhibits the activation of Erk by its MAPKK. LY294002 and Wortmannin specifically inhibit the catalytic activity of PI3K by binding to the ATP-binding pocket of the kinase.

For Western blot analysis MACS purified B cells were left untreated for 1-2 hours before addition of stimuli or isolation of proteins. For stimulation experiments, resting B cells were stimulated in B cell medium with 1% FCS from 5 min. up to 24 hours with agonistic anti-CD40 antibodies (2.5 µg/ml; eBioscience (HM40-3)). To verify IκB-α degradation assays, B cells were treated with 10 µM cycloheximide (Sigma) 30 minutes before stimulation with

anti-CD40 antibodies. Cycloheximide blocks protein synthesis and thereby also re-synthesis of IκB-α.

Primary tumor cells were frozen in RPMI, 10% DMSO (v/v), 20% FCS (v/v). Since all tumor cells are $CD43^+$ and lyse during MACS sorting with CD19-beads we were not able to purify them by magnetic beads as we did for premalignant cells. Therefore after thawing, cells were cultured for two days as described above in order to obtain a more pure tumor cell population, before Western-Blots extracts were prepared.

8.1.5 *In vivo* 5-Bromo-2'-deoxyuridine (BrdU) assay

Mice were fed with 0.8 mg/ml BrdU (Sigma) in the drinking water for 14 days, water was exchanged every 2-3 days. Mice were bled at day 7 and 14 of and at day 42 and 70 during the assay. Lymphocytes were isolated from the blood by Pancoll gradient centrifugation (PAN). For this purpose, 50 µl of Heparin-charged blood were carefully placed on top of 800 µl Pancoll and spinned down for 20 minutes at room temperature without brake. The fluffy phase below the plasma layer as the first phase contains the lymphocytes and was transferred into a fresh tube and subsequently washed with PBS/ 1% BSA for BrdU analysis. The spleen was dissected from animals at day 14 and 70 and splenocytes were isolated as described above. BrdU incorporation into the DNA of blood and spleen derived B cells was analyzed by the APC BrdU Flow kit (BD Biosciences).

8.2 ES cell culture

8.2.1 General cell culture techniques

Cell culture was performed under sterile conditions in a laminar flow (Heraeus) in a specific ES cell culture room. Cells were grown in an incubator (Heraeus) at 37°C, 5% CO_2, 95% air humidity. Cells were generally spun down at 900 rpm (250g) for 5 minutes at room temperature.

8.2.2 Thawing and freezing of cells

Cryo-tubes (Nunc) containing cells were stored in liquid nitrogen and thawed at 37°C until the still frozen, major part could be poured into a 50 ml tube (Falcon) filled with medium. After centrifugation (to remove DMSO), cells were plated as described elsewhere. Freezing of

cells was performed by adding 500 µl pre-cooled 2x freezing medium (20% DMSO (v/v), 50% FCS (v/v), 30% medium (v/v)) drop by drop to 500µl cells (generally $1x10^6$ unless stated otherwise) that were put into cryo-tubes. Freezing medium and cells were mixed by inverting the tube shortly and immediately transferred into a pre-cooled freezing box (Nalgene), filled with Isopropanol to allow slow freezing of the cells (-1°C/min) in a -80°C freezer. After one day to two weeks cells were transferred to liquid nitrogen.

8.2.3 Culturing of embryonic fibroblasts

Embryonic stem (ES) cells were cultured on a layer of embryonic fibroblasts (EF cells) to prevent differentiation of the cells. EF cells are dissected from mouse embryos and can be passaged up to 3 times in EF cell medium (DMEM, 10% FCS (v/v), 1% non-essential aminoacids (v/v), 1% L-glutamine (v/v)). Passaging was performed every 3-4 days. For co-culture with ES cells, EF cells can be mitotically inactivated by incubation with Mitomycin C, a *Streptomyces caespitosus* derived cell toxin. Adherently grown EF cells were once washed with PBS, then incubated with 10mg/ml Mitomycin C for 3 hours at 37°C and again washed three times with PBS. They were subsequently trypsinized by incubation with Trypsin plus 1% chickenserum (v/v) (Gibco) at 37°C until the reaction was stopped by adding 2 volumes of medium after 5 minutes. Cells were then pipetted up and down cautiously to obtain a single-cell suspension and transferred into a 15-50 ml tube (Falcon) for centrifugation. Cell counting was performed with a Neubauer chamber and cells were plated on freshly gelatinzed plates (15' incubation with 0.1% gelatine PBS solution (v/v)) in the appropriate cell density in ES cell medium. ES cells were added to the EF cell layer after three hours minimum.

8.2.4 Culturing of embryonic stem cells

E14TG2a ES cells were cultured on a layer of Mitomycin C treated EF cells in E14 medium (mixture provided by PAN containing GMEM medium, 1% Glutamin (v/v), 1% Sodiumpyruvate (v/v), 1% non-essential aminoacids (v/v), 15% ES cell tested FCS (v/v), 100 µM β-Mercaptoethanol; 0.1% LIF (leukemia inhibiting factor) (v/v) was freshly added (Chemicon) to prevent ES cell differentiation). The cells were passaged every other day and fed every day according to the protocol provided by Ralf Kühn. Passaging required trypsinization of cells because ES cells form adherent colonies on the EF cell layer. Trypsinization was performed by washing the cells once with PBS, incubating them with

Trypsin (w/o Phenolred, provided by PAN) for 2-3 minutes at 37°C and stopping the reaction by 2 volumes of medium. Single cells were obtained by cautious pipetting. After centrifugation the cells were counted with the Neubauer chamber and plated on an EF cell layer in the appropriate cell density.

8.2.5 Transfection of ES cells

ES cells were transfected to generate stable ES cell clones and to transiently transfect the generated stable ES cell clones for transgene expression analysis. In both cases, ES cells were grown to a density of about $1x10^7$ cells/ 10 cm and fed four hours before transfection to obtain highly proliferating cells. ES cells were then trypsinized as described above and resuspended in transfection medium. $7x10^6$ ES cells in 700 µl transfection medium were transfected with 20 µg linearized plasmid DNA in 10 µl transfection medium in the case of stable transfection and with circular plasmid DNA in the case of transient transfection. First, the DNA was transferred to a transfection cuvette (Gene Pulser cuvette, 0.4 cm elektrode; Biorad), then the cells were added. Cells and DNA were incubated for 10-20 minutes on ice before transfection was performed with an electroporator (Gene Pulser, Biorad) at 230 V, 500 µF. Transfected cells were left at room temperature for 10 minutes and were then plated on gelatinzed plates without an EF cell layer. As a control, 10^3 transfected and non-transfected cells were plated on gelatinized plates with normal medium to monitor transfection efficiency and cell loss during transfection.

8.2.6 Selection and expansion of stably transfected ES cell clones

After transfection with the targeting vector (pMB9_Tet_N2IC) ES cells were grown in ES cell medium for 36 hours. Subsequently, the medium was replaced by HAT (Hypoxanthine, Aminopterin, Thymidine) selection medium (Sigma). In contrast to the manufacturer's protocol, HAT medium was diluted to 0.5x from the stock solution. This medium allows only Hprt proficient cells to grow, thus selecting clones with a homologous recombination of the targeting vector into the *Hprt* locus. Aminopterin is a toxin that blocks the "de novo" nucleotide synthesis pathway; addition of hypoxanthine and thymidine however allows Hprt proficient cells to switch to the "salvage" pathway. HAT medium was renewed every day and was replaced by HT (Hypoxanthine, Thymidine; prepared as HAT) medium at day 5 after transfection (Sigma). After 5 days, non-homologously recombined cells had died (as

controlled by non-transfected cells treated with HAT), thus selection had occurred. To let selected colonies grow under less stringent conditions and thus faster, HAT can be replaced by HT that lacks Aminopterin. Cells were kept in HT medium that was exchanged every day until day 9 after transfection. At that day, colonies appeared to be nicely formed and appropriate for picking and transfer to 96-well plates. Medium was exchanged by PBS and colonies were picked with a 200 µl tip in a laminar front flow under a stereomicroscope (Nikon) and transferred in 25 µl to a 96 round-well plate. To each well containing a colony, 25 µl of Trypsin were added and the plate was incubated at 37°C for 2 minutes. 50 µl HT medium were added to stop the reaction and single cells were obtained by pipetting up and down ten times. These suspensions were transferred to a 96 well plate (flat bottom) containing a Mitomycin C treated EF cell layer and 100 µl HT medium. Cells were cultured in the 96 well plates for two days (with medium change each day) before passaging to three 96 wells. Two of these plates contained EF cells to expand the ES cell clones for freezing and later injection into blastocysts whereas the other one was only gelatinized to expand the ES cell clones for DNA preparation and subsequent Southern blot analysis. One of the two EF cell containing ES cell clone 96 well plates was frozen by trypsinization and addition of 10% final DMSO (v/v) in ES cell medium, whereas the other one was passaged to a 24 well plate after two days. At the same day, the 96 well plate for DNA preparation was passaged onto three plates to have two backups. These cells were grown to full density for about 5 days to have enough cells for DNA extraction and Southern Blotting. ES cell clones on the 24 well plate were trypsinized after two days and then frozen in cyro-tubes as described before. These cells could then be stored in liquid nitrogen until injection.

8.2.7 Induction of transgene expression in transiently transfected ES cell clones

Correctly targeted ES cell clones were chosen for testing transgene expression. For this purpose, cells were transfected with the pRT_CAG_rtTA plasmid, containing the Tet-inducible rtTA variant rtTA2s-M2. After transfection, the cells were plated in medium containing Doxycyline (Dox) (a Tetracycline derivative) to activate the transactivator rtTA2s-M2. As a control, non-transfected and transfected ES cells were grown without Dox to monitor leaky expression. After 16 hours, the cells were trypsinized and gene expression from the bidirectional TetO7 promoter was monitored by analyzing EGFP expression by flow cytometry.

8.2.8 Culturing of stable ES cell clones for injection into blastocysts

After verification of correctly integrated DNA by Southern Blot and testing for transgene expression, two clones were chosen for injection into blastocysts. For this purpose, cells were thawed and cultured on mitotically inactive EF cells. They were fed each day and trypsinized at day 3 for passaging. This procedure brought the cells into a proliferative phase after 36 hours where they were again trypsinized, suspendend, and injected into blastocysts (in cooperation with the Institute of Developmental Genetics at the Helmholtz Center Munich). Blastocysts were derived from pregnant C57BL/6 mice to obtain visible chimerism in the offspring. Since E14TG2a ES cells give rise to brown mice, chimeras of black C57BL/6 and brown 129/SV cells can be identified by coat color.

8.3 Standard methods of molecular biology

8.3.1 DNA techniques

8.3.1.1 Plasmid isolation from bacteria

Plasmids were isolated from bacteria with the Jetstar Kit (Genomed) for cloning or with the Endotoxin-free Qiagen Maxi Kit (Qiagen) for transfection into embryonic stem (ES) cells. The latter plasmid was linearized with *SgfI* according to the manufacturer's protocol overnight at 37°C and purified again by Phenol-Chloroform extraction. 1 volume of Phenol/Chloroform/Isoamylalcohol was added to the restriction mix and the tube was inverted (ratio of components: 25:24:1, Roth). After centrifugation (9000xg, 5 minutes) the upper phase (containing genomic DNA) was saved and 1 volume of Chloroform/Isoamylalcohol (24:1) was added and the tube inverted. After the same centrifugation procedure 1/10 volume 3 M NaAc and 2.5 volumes 100% EtOH (v/v) were added to the upper phase and the tube was inverted each time. To precipitate DNA efficiently, the tube was kept at -20°C for 30 minutes. DNA was then spinned down by high speed centrifugation for 20 minutes. The pellet was washed and at the same time the tube was sterilized by filling it completely with 70% EtOH (v/v). DNA could be stored in this way until the day of transfection or centrifuged as described before and the DNA pellet dried in the ES cell lamina flow. The dried pellet was solved in 100 µl transfection buffer (RPMI w/o phenolred, Gibco) by incubating it at room temperature for 1 hour and its density was subsequently measured to determine its concentration.

8.3.1.2 DNA isolation from ES cells in 96-well plates

ES cells were grown to confluency, washed twice with PBS and then treated with lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5 % Sarcosyl (w/v), 1 mg/ml Proteinase K) at 56°C overnight. During this procedure, the 96-well plate was wrapped into plastic foil and put into a humid chamber to prevent drying out. Subsequently it was cooled down at room temperature for one hour. To precipitate DNA, 100 µl 100% Ethanol (v/v) were added and incubated for one hour at room temperature. After a short spin down by centrifugation the liquid was decanted and the DNA was washed with 70% Ethanol (v/v) three times before drying at room temperature. 30 µl of the restriction buffer mix was added directly.

8.3.1.3 DNA isolation from ES cells and primary lymphocytes

ES cells from 10 cm plates or primary lymphocytes were lysed over night in 500 µl cell lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5 % Sarcosyl (w/v), 1 mg/ml Proteinase K). 170 µl of a saturated NaCl solution was added and the sample was centrifuged at 9000xg. The supernatant was transferred to a new reaction tube and 1 volume of Phenol/Chloroform/Isoamylalcohol was added and the tube was inverted (ratio of components: 25:24:1, Roth). After centrifugation (9000xg, 5 minutes) the upper phase (containing genomic DNA) was saved and 1 volume of chloroform was added and the tube inverted. After centrifugation the DNA was precipitated with 2 1/2 V 100% Ethanol (v/v) and 1/10 V 3 M NaAc (v/v). DNA was spun down by centrifugation, washed three times with 70% Ethanol (v/v) and the dried pellet was resolved in TE buffer by shaking the tube for 4 hours at 37°C.
Alternatively DNA was precipitated with 2 1/2 V 100% Ethanol (v/v) and 1/10 V 3 M NaAc (v/v) directly without former Phenol-Chloroform purification.

8.3.1.4 DNA isolation from mouse tails

A small piece of the mouse tail was lysed at 56°C for 1-2 hours in 500 µl lysis buffer (Tris/HCl pH 8, (100 mM); EDTA (5 mM); SDS (0.2 %) (w/v); NaCl (200 mM); 100µg/ml Proteinase K). 170 µl of a saturated NaCl solution was added and the sample was centrifuged at 10000xg. The supernatant was transferred to a new reaction tube and 600 µl of 100% Isopropanol (v/v) were added. After reversion of the tube and precipitation of DNA, the

sample was centrifuged at 10000g. The supernatant was removed and the DNA pellet washed with 70% Ethanol (v/v) to remove salt from the solution. After drying the pellet the DNA was solved in 100µl TrisHCl buffer by shaking at 37°C (modified from Laird et al., 1991).

8.3.2 Restriction digest of plasmid DNA

Restriction digests of plasmid DNA were performed according to manufacturer's protocols for cloning purposes.

8.3.3 Restriction digest of genomic DNA

Genomic DNA from ES cells and primary splenic lymphocytes was restricted by specific enzymes for the subsequent performance of a Southern blot. The restriction buffer mix (1mM Spermidin, 1mM DTT, 100 µg/ml BSA, 50 µg/ml RNase, 1x buffer, 50 U of the respective enzyme) was either added to undissolved ES cell DNA (96 well) directly or to DNA from either ES cells or primary lymphocytes dissolved in TE buffer. Digestion took place at 37°C for 16 hours.

8.3.4 Ligation of plasmid DNA fragments

Ligation of restricted plasmid DNA fragments was performed with the T4 DNA ligase overnight at 16°C according to manufacturer's protocols for cloning purposes (NEB).

8.3.4.1 Polymerase chain reaction (PCR) (Mullis, 1983)

Amplification of specific DNA by PCR was used for three purposes: to clone the *Notch2-IC* cDNA from the pTracer_CMV_Notch2cDNA vector to establish the targeting construct; to clone the *Notch2-IC* cDNA from the genomic DNA of targeted ES cells for sequencing and to detect specific regions in the DNA of mice for genotyping. The latter was applied to determine the genotype of transgenic mice by analyzing tail DNA.

The following reaction mixture was applied in different variations:

Reaction sample	1x	Reaction cycle		
H_2O	18.4-19.15µl	Starting temperature	94°C	5’
Taq Buffer (10 x)	2.5µl	Cyclic denaturation	94°C	45’’
$MgCl_2$ (50 mM), $MgSO_4$ (25 mM)	1-2µl	Cyclic hybridization	55-63°C	45’’
dNTP-Mix (10 mM)	0.5µl	Cyclic elongation	72°C	1-3’
Primer sense	0.1-0.25µl	Final elongation	72°C	10’
Primer antisense	0.1-0.25µl	# cycles	29-33	
TaqPol (5 U/ml), Pwo, Taq:Pfu (100:1)	0.15-0.75µl			
DNA (ca. 5 ng)	1.5µl			
DMSO (100 %)	0.25µl			

The respective oligonucleotides (primers) are shown in 8.5.

8.3.4.2 Cloning

Cloning of the pHprt-EGFP-TetO7BI-Notch2-IC-Ekappa vector was performed as follows: The Blueiiks was used for subcloning of the construct before integration into the MP9 targeting vector. To obtain suitable restriction sites, the multiple cloning site of the Blueiiks (BKS) vector was exchanged with a fragment containing NotI-AscI-SfiI-SacI-PacI-SbfI-XbaI-BamHI-EcoRI-NsiI-MluI restriction sites (BKS_Oligo). First of all the endogenous *Rosa2*6 exon 1 and intron 1 with its splice donor and acceptor was excised from the LMP1rosa26neu vector by SacI and PacI restriction digest and inserted into the Blueiiks vector (BKS_Oligo_rosa). Secondly, the polyA stretch was cut out by BamHI and XbaI from the BSAsc LMP1 vector and used as a transcriptional termination sequence for the Notch2-IC cDNA (BKS_Oligo_rosa_bpA). Eµ was first subcloned into the $pGEM7_zFRT_2$ vector containing two frt sites. The sequence in between these two frt sites was exchanged by BamHI and ClaI restriction with a suitable cloning sequence containing ClaI-BlnI-PmeI-BamHI restriction sites. Eµ was inserted in between the two frt sites by BlnI and PmeI restriction from the pEBNA-SVH-GL-EmCAG-rtTA-tTR vector ($pGEM7_zFRT_2$_Oligo_Em). Then the frt-Eµ-frt sequence was inserted into the BKS_oligo_rosa_bpA vector by EcoRI and NsiI restriction (BKS_Oligo_rosa_bpA_Em). Subsequently, bpA-EGFP-TetO7BI was restricted from the pEBNA-SVH-GL-EmCAG-rtTA-tTR vector by AscI and SfiI and inserted into the BKS (BKS_Oligo_rosa_bpA_Em_pAEGFPBITetO7). In the next step, Notch2-IC

was derived from the pTracer-CMV-Notch2cDNA vector by PCR using the primers described in 7.5 that contained restriction sites for SbfI at their 3' ends (Notch2ICfw and Notch2ICrev). This PCR derived construct was first sequenced to exclude PCR-derived mutations and then inserted into the targeting BKS vector by SbfI restriction (BKS_Oligo_rosa_bpA_Em_pAEGFPBITetO7_Notch2IC). The obtained construct was digested with NotI and MluI and inserted into the MP9 Hprt targeting vector (pHprt_Notch2IC). After this step it was decided to replace the Eμ enhancer with the Eκ enhancer to secure B cell specificity since Eμ was shown to be active not only in B cells but also in T cells. The Eκ enhancer was derived from the BC230A 2-10-02 vector by SacI and KpnI digest and subcloned into a BKS vector containing the restriction sites BlnI-SacI-KpnI-PmeI that were inserted as an oligo. Ek was then inserted into the pHprt_Notch2IC targeting vector by BlnI and PmeI digest, replacing the Eμ (pHprt_Notch2IC_Ekappa). The construct in this vector was then sequenced after Maxi-preparation with the Endofree Maxi Kit (Quiagen). The targeting vector was linearized with SgfI for transfection into ES cells and homologous recombination with the endogenous *Hprt* locus.

Cloning of the rtTA expression plasmid for transient transfection into targeted ES clones was performed by inserting rtTA2^{S}-M2 into the pCAG-3SIP vector after EcoRI digest.

8.3.4.3 Agarose-Gelelectrophoresis

PCR and restriction digest derived products were separated on a 0.8-2% Agarose gel (w/v) in a gelelectrophoresis chamber (Peqlab) with TAE buffer (40 mM Tris/HCl, 20 mM Acetate, 1 mM EDTA, pH 8.5) at 80-100 V for 1-2 hours.

8.3.5 Southern Blot analysis (Southern et al., 1997)

Enzymatic restriction of genomic DNA was performed with the according enzymes for 16 hours. A specific restriction buffer (1 mM spermidin, 1 mM DTT, 100 μg/ml BSA, 50 μg/ml RNAse, enzyme specific buffer, 50 U of the enzyme) was used to improve restriction efficiency of genomic DNA. Restricted DNA was subsequently separated on a 0.8% agarose gel together with a standard 1kB DNA ladder (Invitrogen) overnight. After recording the separated DNA and standard together with a ruler, the gel was incubated in 0.25 N HCl for 25 minutes to denaturate and fragment DNA. To equilibrate the gel for DNA transfer it was

subsequently incubated in alkaline transfer buffer (0.4 M NaOH, 0.6 M NaCl) for 40 minutes after short rinsing in water. Blotting of DNA from the gel to a nylon membrane (Immobilon™ Ny+ membrane, Millipore) was performed overnight from top to bottom by capillary pressure of the transfer buffer. After transfer, slots were marked on the membrane with a soft pencil and the membrane was incubated in 2xSSC (0.3 M NaCl, 0.03 M NaCitrate, pH 6.5) for 40 minutes to neutralize it. DNA was then cross-linked to the membrane by baking at 80°C for 1 hour. Before incubation with the radioactively labeled probe, the 2xSSC-wetted membrane was pre-hybridized in pre-warmed hybridization buffer without the probe (1 M NaCl, 50 mM Tris, pH 7.5, 10% (w/v) dextran sulfate, 1% (w/v) SDS, 250 µg salmon sperm DNA/ml (Sigma)) for 6 hours at 65°C to prevent unspecific binding. Labeling of the probe (50 ng) was performed with the "Random Prime Labeling Kit" (GE Healthcare) with 50 µCi α32-dCTP (Hartmann-Analytic) according to manufacturer's protocols (GE-Healthcare). The labeled fraction was prepared by using the G50 sephadex columns according to manufacturer's protocols (GE-Healthcare). Before incubation with the membrane in the hybridization buffer, the probe was heated to 100°C for 5 minutes and quenched on ice for 2 minutes. Incubation with the membrane took place overnight at 65°C. Subsequently the membrane was washed two to three times for 10 minutes in washing buffer (0.2xSSC, 0.5% SDS (w/v)) at 58°C to wash off unbound probe. After each washing step, radioactivity of the blot was measured by a radioactivity detector and washing was stopped when radioactivity reached 30 counts. Radioactivity was visualized by radiosensitive films (Biomax MS PE Applied Biosystems 35x43 cm, KODAK). Exposure times varied from 6 hours to 2 days at -80°C in a Biomax cassette.

8.3.6 Protein detection

8.3.7 Protein isolation

Protein was isolated from B cells after purification by MACS separation and after resting of cells in medium containing 1% FCS for 1-2 hours. Cells were washed once with ice-cold PBS before protein extraction.

For whole cell extracts, 20 µl of an 2xNP40 lysis buffer (10 mM Tris pH 7.4, 300 mM NaCl, 4 mM EDTA, 2% NP40 (v/v), freshly added phosphatase inhibtors (Halt phosphatase inhibitor cocktail, Pierce) and protease inhibitors (Mini Complete, Roche)) were added to the cell pellet of $5x10^6$ B cells without resuspension and shaked on a vortexer at 4°C for 20

minutes. Cell debris and DNA was separated from the protein supernatant by centrifugation at maximum speed for 15 minutes at 4°C.

For B cell fractionation, $2x10^7$ B cells were incubated in 100µl buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA, 1 mM DTT, 1x complete protease inhibitors (Roche)) for 15 minutes on ice. After addition of 6.75 µl 10% NP40 and shaking for 5 minutes in at 4°C, nuclei were spinned down at 10000xg (max. speed) for 15 min and the supernatant (cytoplasmic fraction) was saved. Nuclei were washed once with buffer A before lysis in 40 µl buffer C (20 mM Hepes pH 7.9, 0,4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1x complete protease inhibitors (Roche)). After shaking at 4°C for 30 minutes and centrifugation at 10000xg for 15 minutes, the supernatant (nuclear fraction) was saved. Protein samples were subsequently frozen at -80°C.

8.3.8 Western Blot analysis

Protein concentration of protein samples was measured by using a Bradford reagent (DC protein assay; Bio-Rad) and a bovine serum albumin standard slope. 5x Laemmli buffer (300 mM Tris pH 6.8, 7.5% SDS (w/v), 50% Glycerin (v/v), 0.01% bromphenol blue, 1% β-Mercaptoethanol) was added to the samples to a concentration of 1x before heating them at 70°C for 10 minutes. Denatured proteins were loaded onto a SDS polyacrylamide gel together with a protein standard (Benchmark™, Invitrogen) and separated according to molecular weight by discontinuous SDS-Polyacrylamide gelelectrophoresis (PAGE) (Laemmli, 1970). Electrophoresis was carried out in Laemmli running buffer (25 mM Tris base, 0.2 M glycine, 0.1% SDS) at 20-40mA, using a Bio-Rad electrophoresis chamber. Discontinuous PAGE is characterized by a stacking gel ((5% (v/v) acrylamide, 0.625 mM Tris pH6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.006% (w/v) TEMED),) and a resolving gel (12% (v/v) acrylamide, 3.75mM Tris pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.004% (w/v) TEMED). In the stacking gel SDS-loaded and thereby negatively charged proteins are focused at the separation line, in the resolving gel proteins are separated according to their molecular weight.

Separated proteins in the resolving gel were transferred to a polyvinylidenfluoride (PVDF) membrane (Immobilon™ P membrane, Millipore) by using the Bio-Rad mini tank blotting chamber. This transfer was accomplished in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% Methanol (v/v)) for 2-3 hours at 250 mA in the coldroom. To verify transfer efficiency and equal loading the membrane was incubated with Ponceau S that stains proteins unspecifically (Bio-Rad). The Ponceau S band according to the size of the proteins detected

by specific antibodies is shown as a loading control. Before incubation with specific antibodies, the membrane was incubated with TBS (0.1 M Tris/HCl pH 7.5, 0.1 M NaCl) containing 0.02% Tween (v/v) and 5% milk powder (w/v) for one hour with moderate shaking to block unspecific protein binding sites on the membrane. Binding of the primary antibody in a TBST 5% BSA (w/v) solution (unless stated otherwise in chapter 7) was performed over night at 4°C while rolling. Incubation with the secondary antibody was performed for 1-3 hours at room temperature in TBST 1% milk powder (w/v) after 3 times 5 minutes of washing the membrane in TBST. After the very same washing procedure, the membrane was incubated with the ECL™ detection reagent by GE Healthcare for one minute. Chemiluminescent signals were detected by exposition with photosensitive films (CEA RP new) using a Cawomat 2000 IR processor (both Ernst Christiansen). Stripping of membranes for re-probing with specific antibodies was performed at 56°C for 30 minutes in stripping buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS (w/v), 100 mM β-Mercaptoethanol).

9 Supplementary data

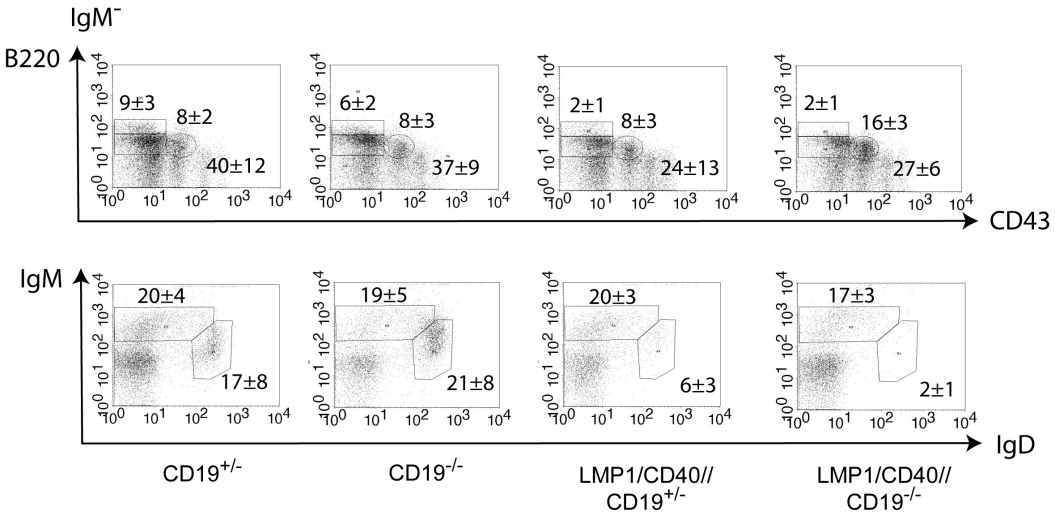


Figure S1 B cell development in the BM in LMP1/CD40//CD19$^{-/-}$ mice
Shown are FACS dot plots from a representative experiment characterizing B cell populations in the BM. Numbers indicate the mean percentages and SD of gated populations from at least three experiments. Pre ($IgM^-B220^{low}CD43^-$) pro (IgM^-B220^{low} $CD43^+$) and mature, recirculating IgD^+ B cells ($IgM^-B220^{high}CD43^-$) (upper panel), immature (IgM^+IgD^-) and mature, recirculating (IgM^+IgD^+) B cells (lower panel).

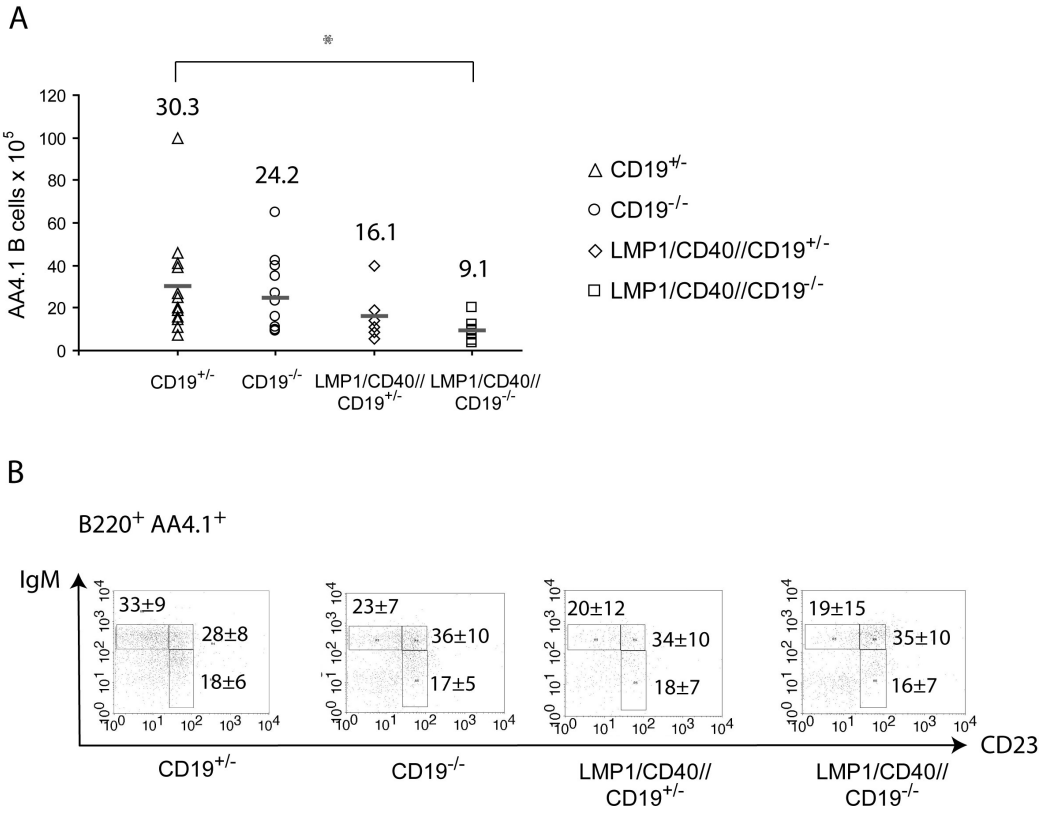


Figure S2 LMP1/CD40//CD19$^{-/-}$ mice show a reduction of transitional B cells
(A) Calculation of total B220^{+}AA4.1^{+} transitional B cells in the spleen from the indicated genotypes. Each symbol represents a single mouse. Mean percentages are shown as horizontal bars. *P<0.05, calculated by the two-tailed student's t test. (C)Transitional B cells were characterized by staining splenic cells with TOPRO3, α-B220, α-AA4.1, α-IgM and α-CD23 in a flow cytometry analysis. T1 (B220^{+}AA4.1^{+}IgM^{high}CD23^{-}), T2 (B220^{+}AA4.1^{+}IgM^{high}CD23^{+}) and T3 (B220^{+}AA4.1^{+}IgM^{low}CD23^{+}) B cells can be differentiated. Numbers indicate the mean percentages and SD of gated populations from at least five independent experiments.

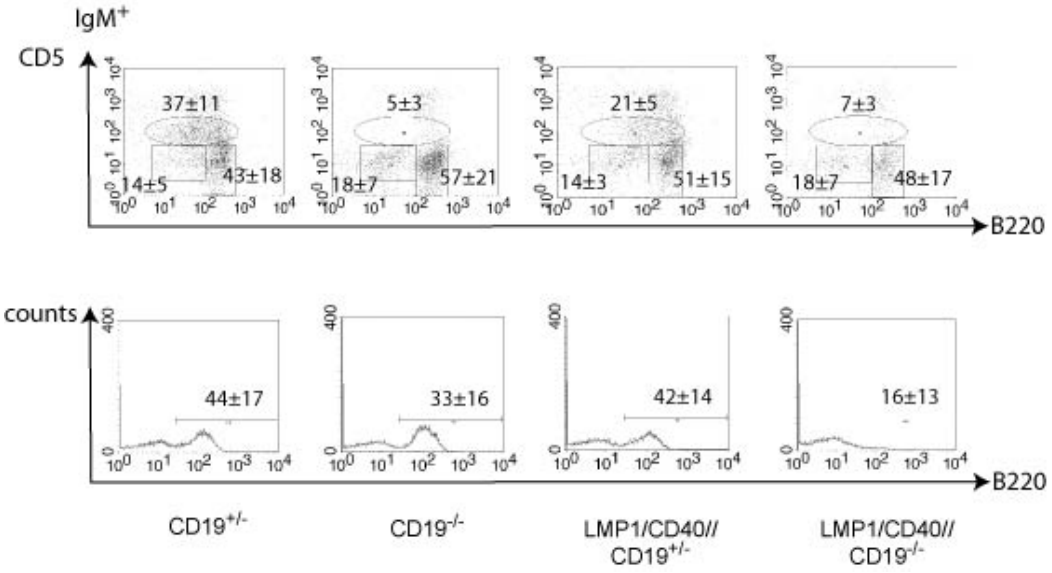


Figure S3 Loss of B cells in the PC of LMP1/CD40//CD19$^{-/-}$ mice
Flow cytometry analysis of peritoneal B cell subsets. The staining in the upper panel resolves B1a ($IgM^+CD5^+B220^{low/+}$), B1b ($IgM^+CD5^-B220^{low}$) and B2 ($IgM^+CD5^-B220^+$) cells. Percentages of $TOPRO3^-B220^+$ B cells are shown in a histogram (lower panel). Numbers indicate mean values and SD of percentages of gated populations from at least three different experiments.

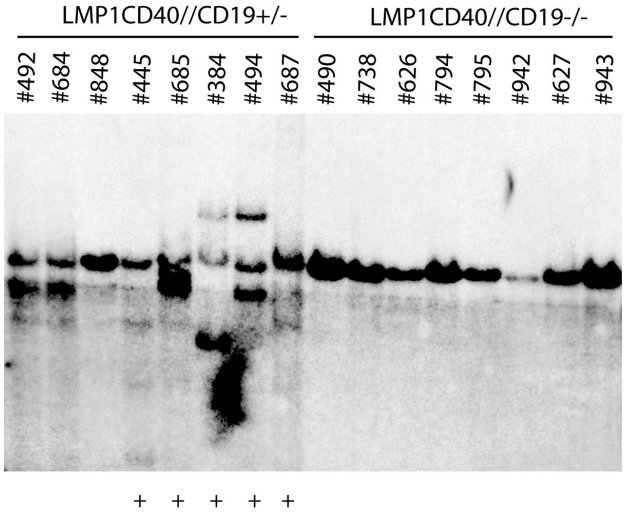


Figure S4 Southern Blot analysis testing the clonality of lymphomas
Lymphomas were scored by Southern blot analysis examining IgH configuration with an IgH specific probe. Genomic DNA was prepared from splenic cells of the indicated genotypes and digested with *EcoRI*. (+) mono- or oligoclonal.

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05/2006

1st Method's Seminar of the SFB 684 "Molecular mechanisms of normal and malignant hematopoesis"

Title of the talk: "Transgenic mouse models"

Poster presentations

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04/2009

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"Generation of two conditional transgenic mouse strains for Notch2-IC"

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5th B Cell Biology Forum of the Study Group "Biology of B Lymphocytes" of the German Society for Immunology, Bad Bevensen, Germany

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03/2006

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"Cooperation of CD40 and the B cell receptor in B cell proliferation and survival"

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"A new role for CD19 in B cell activation and lymphomagenesis"

Publication

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