Latent Epstein-Barr virus infection and the germinal center reaction

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Contents

List of figures **5**

List of tables **5**

List of abbrevations **6**

1 INTRODUCTION **8**

1.1 B lymphocytes and the humoral immune response **8**

1.2 B cell development **8**

1.3 T cell dependent immune response **9**

1.4 Germinal center B cell malignancies **13**

1.5 Epstein-Barr virus **13**

1.6 EBV and the germinal center reaction **15**

1.7 LMP1 and CD40 ..... **16**

1.8 LMP2A and the B cell receptor **19**

2 AIM OF THE PROJECT **21**

3 RESULTS **23**

3.1 Comparison of LMP1 and CD40 signaling in B cells in vivo **23**

3.1.1 Generation of a transgenic mouse line expressing a conditional CD40/LMP1 23

3.1.2 B cell specific expression of the CD40/LMP1 transgene in a CD40-/- background ... 25

3.1.3 Lymphoid compartment characterization of CD40/LMP1+//CD40-/- mice 27

3.1.4 CD40/LMP1 restores normal serum immunoglobulin titers in CD40-/- mice 29

3.1.5 CD40/LMP1 substitutes CD40 in germinal center formation 29

3.1.6 CD40/LMP1 substitutes CD40 in the production of high affinity antibodies 31

3.1.7 CD40/LMP1+//CD40-/- mice somatically mutate their Ig genes comparable to wild type 32

3.1.8 The higher percentage of germinal center B cells in immunized CD40/LMP1+ mice is not a result of a higher proliferation rate 33
3.1.9 CD40/LMP1+//CD40-/- mice do not show signs of autoimmunity 34
3.1.10 LMP1 signaling induces cytokine-independent class switch recombination 35

3.2 The influence of constitutive active CD40 signaling on germinal center B cells *in vivo* 37
3.2.1 Constitutive CD40 signaling is not compatible with the GC reaction 38
3.2.2 LMP1/CD40//Cγ1-cre mice show an age dependent increase of splenic weight and accumulation of aberrant B cells 39
3.2.3 The B cell accumulation in the spleens of LMP1/CD40//Cγ1-cre mice can be classified as B cell lymphoma by histology 41
3.2.4 The Ig genes in the aberrant B cell population are not somatically mutated 43

3.3 Modeling of EBV and the germinal center reaction *in vitro* 43
3.3.1 EREB2-5 cells gain a memory B cell phenotype upon EBNA2 inactivation 44
3.3.2 Generation of the expression vectors coding for LMP1, LMP2A and EBNA1 45
3.3.3 EBNA2 independent expression of LMP1 and LMP2A in a human B cell line 47
3.3.4 EBNA2 independent LMP1 and LMP2A expression induces a GC B cell phenotype *in vitro* 49
3.3.5 LMP1 induces expression of mature IgG transcripts, which is abrogated by co-expression of LMP2A 50
3.3.6 LMP2A down modulates AID protein expression 52

3.4 Does EBNA2 independent expression of LMP1, LMP2A and EBNA1 elicit transforming capacity in human B cells? 53
3.4.1 Co-expression of LMP1 and LMP2A prolongs B cell survival in vitro 53
3.4.2 Co-expression of LMP1 and LMP2A in EREB cells cultured without estrogen prolongs proliferation in vitro 54
3.4.3 LMP1 and LMP2A co-expression is not sufficient to maintain immortalization of B cells *in vitro* 56

4 DISCUSSION 57

4.1 The influence of a CD40 ligand regulated LMP1 signaling on B cells in vivo 57

4.2 The influence of constitutive active CD40 signaling on germinal center B cells *in vivo* 60

4.3 Modeling of EBV latent infection *in vitro* 62

4.4 A new scenario of EBV latent infection 66

5 SUMMARY 69

6 ZUSAMMENFASSUNG 71

7 MATERIAL 73

7.1 Plasmids 73

7.2 Bacteria 74

7.3 Cell lines 74

7.4 Mouse strains 74

7.5 Primer 75

7.6 DNA probes 76

7.7 Antibodies 77

7.8 Software 78

8 METHODS 79

8.1 Mice 79

8.1.1 Generation of the transgenic mouse line CD40/LMP1 79

8.1.2 Mice crossings 79

8.1.3 Mouse immunizations 80

8.1.4 Preparation of primary lymphocytes from mice 80

8.1.5 In vivo BrdU assay 81

8.1.6 Isolation of germinal center B cells and analysis of somatic hypermutation 81

8.2 Cell culture 81

8.2.1 Primary murine B cells 81

8.2.2 Bone marrow derived dendritic cells from the mouse 82
8.2.3 EREB2-5 cell line..... 82
8.2.4 Primary human B cells 83

8.3 Fluorescence-activated cell sorting (FACS) 83

8.4 Immunohistochemistry 84

8.5 Immunofluorescence 84

8.6 Enzyme-linked immunosorbent assay (ELISA) 84

8.7 RNA isolation and analysis 85

8.8 Western blot analysis 87

8.9 Southern Blot analysis..... 88

REFERENCES 90

Acknowledgements..... 98

Curriculum Vitae 100

Publications and presentations 102

List of figures

Figure 1.1. T cell-dependent immune response 12
Figure 1.2. LMP1 as a functional homologue of CD40 18
Figure 1.3. LMP2A mimics the B cell receptor 20
Figure 3.1. Generation of a transgenic mouse line conditionally expressing CD40/LMP1 24
Figure 3.2. B cell specific expression of CD40/LMP1 25
Figure 3.3. CD40/LMP1 expression in non-B cells 26
Figure 3.4. B and T cell distribution in the spleen 27
Figure 3.5. CD40/LMP1+//CD40-/- mice show normal B cell subset distributions 28
Figure 3.6. CD40/LMP1 can rescue the class switch recombination deficiency in CD40-/- mice 29
Figure 3.7. CD40/LMP1 is able to substitute for CD40 in germinal center formation 30
Figure 3.8. CD40/LMP1+//CD40-/- mice show a higher percentage of germinal center B and plasma cells upon immunization 31
Figure 3.9. CD40/LMP1 substitutes CD40 in the production of class switched and high affinity antibodies 32
Figure 3.10. CD40/LMP1+//CD40-/- B cells do not show a higher proliferation rate in the germinal center 34
Figure 3.11. CD40/LMP1+//CD40-/- mice do not have anti-cardiolipin antibodies 34
Figure 3.12. LMP1 signaling induces cytokine-independent class switch recombination to IgG1 36
Figure 3.13. LMP1/CD40//Cγ1-Cre mice 37
Figure 3.14. Constitutive CD40 signaling is not compatible with the GC reaction 38
Figure 3.15. LMP1/CD40//Cγ1-cre mice show an increase of splenic weight 40
Figure 3.16. Representative histological analyses of one 24 weeks old LMP1/CD40//Cγ1-cre and one wild type control mouse 42
Figure 3.17. Schematic representation of the generation of lymphoblastoid cell lines (LCLs) and the EREB2-5 cell system 44
Figure 3.18. EREB2-5 cells up-regulate the memory B cell marker CD27 upon EBNA2 inactivation 45
Figure 3.19. pRT-1 expression vectors 46
Figure 3.20. Estrogen independent expression of LMP1 and LMP2A 48
Figure 3.21. CD10, CD77 and CD38 expression in EREB-LMP1/2A cells 50
Figure 3.22. RT-PCR for mature IgG1 and IgA1 transcripts in EREB cells 51
Figure 3.23. AID protein expression 53
Figure 3.24. Co-expression of LMP1 and LMP2A prolongs B cell survival after EBNA2 inactivation 54
Figure 3.25. Co-expression of LMP1 and LMP2A sustains proliferation upon EBNA2 inactivation 55
Figure 3.26. Morphology of EREB-LMP1/2A cells in the absence of estrogen 56
Figure 4.1. A new scenario of EBV latent infection 68

List of tables

Table 3.1. Frequency and range of mutations in a 500 bp-long region in the intron downstream of the rearranged $V_HD_HJ_H4$ joints of splenic GC B cells of two independent experiments 33
Table 3.2. RT-PCR for mature Ig transcripts 52

List of abbrevations

a	anti
AID	activation-induced cytidine deaminase
APC	allophycocyanin
APS	ammonium peroxydisulfate
BCR	B cell receptor
BL	Burkitt lymphoma
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
COOH	carboxy-terminus (protein)
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	copy DNA
CMV	cytomegalovirus enhancer
Cre	protein recombinase of the phage *P1* ("causes recombination")
CSR	class switch recombination
d	day
DC	dendritic cells
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleic triphosphate
EBER	non-polyadenylated small RNAs of EBV
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ES cells	embryonic stem cells
et al.	"et alii"
FACS	Fluorescence-associated Cell Sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FO B cell	follicular B cell
GC	germinal center
HL	Hodgkin lymphoma
HRP	Horseradish peroxidase
HRS cells	Hodgkin-Reed/Sternberg cells
h	hour
Ig	immunoglobulin
Igα/β	immunoglobulin-associated signaling molecule 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	kilodalton
l	liter
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LP	leader protein

LPS	lipopolysaccharid
loxP	locus of crossing-over (x) of phage *P1*
m	months
M	molar
MACS	Magnetic Cell Separation
MAPK	mitogen activated protein kinase
MHC	Major Histo Combatibility
min.	minute
mg	milligrams
MOPS	4- morpholinopropanesulphonic acid
mRNA	messenger RNA
MZ B cells	marginal zone B cells
µCi	microcurie
µF	microfaraday
N	amino-terminus (protein)
NF-κB	nuclear factor κB
NK cells	natural killer cells
nm	nanometer
NP-CGG	4-hydroxy-3-nitrophenylacetyl chicken gammaglobulin
n.s.	non-specific
OD	optical density
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PI	propidium iodide
qPCR	quantitative real-time poly chain reaction
RBP-Jκ	recombination signal binding protein J kappa
RIP	receptor interacting protein
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
RT	room temperature
SAS	splice acceptor site
SRBC	sheep red blood cells
SDS	sodium dodecyl sulfate
sec.	second
SHM	somatic hypermutation
STAT	signal transducer and activator of transcription
TEMED	N,N,N`,N`-Tetramethylenediamine
TD	T cell-dependent
TdT	Terminal deoxynucleotidyl Transferase
TE	Tris EDTA
TI	T cell-independent
TRAF	tumor necrosis factor receptor-associated factors
TRADD	tumor necrosis factor receptor associated death domain protein
U	units
UV	ultraviolet light
V	volt
v/v	volume per volume
w	weeks
w/o	without
wt	wild type
w/v	weight per volume

1 Introduction

1.1 B lymphocytes and the humoral immune response

B and T lymphocytes are part of the adaptive immune system. In vertebrates, the adaptive immune system has developed in addition to the innate immune system to improve the defense mechanisms against invading pathogens. Thus, species with an adaptive immune response have large repertoires of T and B cell antigen receptors and antibodies, which increase the possibility to recognize any antigen encountered throughout life. The T and B cell antigen receptors are products of site-specific somatic recombination, which allows an immense repertoire which varies from individual to individual from one species. Additionally, B cells are able to modify their antibodies in a secondary diversification pathway during the immune response to gain more specificity for a certain antigen.

1.2 B cell development

In mammals, B cells develop from hematopoetic stem cells in the bone marrow. They are generated via a series of sequential differentiation steps (Rolink *et al.*, 1995), and are released in the periphery upon expression of a functional B cell receptor (BCR) (Hesslein and Schatz, 2001). In the pro-B cells, the earliest B-lineage stage, the rearrangement of the immunoglobulin (Ig) heavy chain segments occurs via somatic recombination. D_H to J_H joining at the early pro-B cell stage is followed by V_H to D_H joining at the late pro-B cell stage. These genetic processes depend on two enzymes encoded by the recombination-activated genes *rag-1* and *rag-2*. The terminal deoxynucleotidyl transferase adds N-nucleotides at the rearrangement joints, which contributes to the diversity of the B cell receptor (Janeway, 2005). The successful rearrangement of the Ig heavy chain leads to the surface expression of the pre-BCR complex and the so-called pre-B cell stage. The pre-BCR complex consists of the rearranged immunoglobulin heavy chain (IgH) of class μ, a surrogate Ig light chain, which is composed of VpreB and λ5, and the two Ig-associated signaling molecules Igα (CD79A) and Igβ (CD79B). Pre-BCR signaling promotes the rearrangement of the Ig light chain in small pre-B cells. Once a light chain replaces the surrogate light chain to form a mature BCR on the cell surface, the cell is defined as an immature B cell (Bossy *et al.*, 1991).

During development in the bone marrow, B cells undergo several selection processes to guarantee that only B cells which have gained a functional BCR non reactive for self antigens enter the peripheral B cell pool. B cells which fulfill these criteria leave the bone marrow and transit to the spleen (so-called transitional B cells), where they undergo further maturation steps to become mature B cells (Chung *et al.*, 2003). These mature B cells can be subdivided into follicular and marginal zone (MZ) B cells. Follicular B cells represent the major population of circulating B cells in adults. They continuously pass the peripheral lymphoid tissues through the blood stream. In the follicles of the spleen or the lymph nodes they receive survival signals from follicular dendritic cells, which help them to become relatively long-lived B cells (Kosco and Gray, 1992). MZ B cells do not circulate and are resident at the border between the white and the red pulp of the spleen, next to the marginal sinus. MZ B cells have a restricted antigen specificity and seem to play a crucial role in humoral immune responses against blood-borne pathogens (Martin and Kearney, 2002).
Follicular and MZ B cells are referred to as B2 cells, in contrast to the B1 cells, which are mainly produced in the fetal liver and are the predominant population in the peritoneal cavity. They show self-replenishing capacity and are the major source of constitutively expressed IgM. Like MZ B cells, B1 cells express a restricted primary antibody repertoire, generally respond quickly to antigen and are independent of T cells (Martin *et al.*, 2001).

1.3 T cell dependent immune response

B cells are able to elicit T cell-independent (TI) and T cell-dependent (TD) immune responses. B cell activation by many antigens, especially monomeric proteins, requires both binding of the antigen by the BCR and interaction with antigen-specific T helper cells (TD immune response). However, some microbial antigens, such as bacterial polysaccharides, can induce antibody production in the absence of helper T cells (TI immune response). In contrast to the TD immune response, the TI immune response induces only limited isotype switching and does not induce affinity maturation and generation of memory B cells.

Follicular B cells are the B cells mainly involved in the TD immune response. B cells are referred to as naïve B cells until they encounter cognate antigen through their BCR. Cross-linking of the BCR by a TD antigen leads to the internalization, processing and presentation of the antigen through the major histocombatibility (MHC) class-II-antigen-complex on the B cell surface. CD4+ T helper cells which have specificity for the same antigen and have been previously activated by antigen-presenting cells can recognize the MHC-class-II-molecule

and activate the B cells. This activation is mediated by crosslinking of the CD40 receptor on B cells by the CD40 ligand (CD40L) and by the release of cytokines. The activated B cells start to proliferate and take either part in the germinal center reaction or migrate to extrafollicular foci, where they differentiate into low affinity antibody-secreting plasma cells, providing a prompt, but rather unspecific immune response (reviewed in MacLennan *et al.*, 2003) (Figure 1.1).

Germinal center (GC) formation is the signature of a TD immune response, and essential for high affinity immune responses. GC are sites were B cell expansion, Ig class switch recombination, somatic hypermutation, selection, apoptosis, plasma cell and memory B cell formation take place (Wolniak *et al.*, 2004). GC appear within the follicles of secondary lymphoid tissues at the border between the B and T cell zone. The integrity of the follicles with a proper positioning and retention of B, T and follicular dendritic cells is critical for GC formation. In the initial phase of the GC reaction proliferation of B cells takes place. GC B cells then undergo class switch recombination (CSR) and somatic hypermutation (SHM) of the Ig genes, which lead to secondary diversification of antibodies. In both processes the activation-induced cytidine deaminase (AID) plays an essential role (Revy *et al.*, 2000; Muramatsu *et al.*, 2000). AID deaminates cytosines on the Ig locus, which lead to uracil:guanin mismatches, and a removal of uracil by uracil-N-DNA glycosylase (UNG) or by alternative pathways, including base-excision repair and mismatch repair, leading to CSR or SHM (de Yebenes and Ramiro, 2006).

The class switch recombination (CSR) involves the replacement of the μ constant region gene with downstream γ, α or ε constant regions. This allows the generation of antibodies with different constant regions but the same antigen specificity. The various Ig isotypes mediate different effector functions and operate in distinct places. IgM antibodies are produced early in immune responses, before CSR and SHM takes place. Thus, IgM antibodies often show relative low affinity, but confer high overall avidity because of its pentameric structure, which makes it especially effective in activating the complement system. The later humoral immune response is dominated by IgG, IgA or IgE antibodies. The main function of IgG is to opsonize pathogens for engulfment by phagocytes and to activate the complement system. IgA is the principal isotype in secretions and mainly acts as a neutralizing antibody. IgE antibody is mainly bound by receptors on mast cells and basophils, and antigen binding triggers them to release toxic products and histamine. The type of immune response determines which isotypes are generated and at what frequencies. T cells and cytokines released by them are

involved in regulation of isotype expression. *In vitro*, B cells can be triggered to mediate CSR by stimulation with lipopolysaccharid (LPS) or CD40L and specific cytokines, which determine the isotype of the antibodies. Thus, IL4 induces switching to IgG1 and IgE, γ-interferon (INF) to IgG2a and TGFβ to IgA (Esser and Radbruch, 1990). In a variety of TD immune responses, IgG1 is the dominant isotype, mediated by type 2 T helper cells producing mainly IL4.

The somatic hypermutation process introduces individual point mutations into the V regions of the Ig heavy and light chain genes, which increases the BCR diversification and may alter the affinity for the antigen (Liu *et al.*, 1997). The point mutations are acquired stepwise as a B cell proliferates in the GC, with a rate of 1 to 2 mutations per generation. All in all, V-region genes accumulate mutations at a rate of about one base pair change per 10^3 base pairs per cell division, whereas other somatic cells show rates of around one base pair change per 10^{10} base pairs per cell division. The mutations are not completely randomly distributed throughout the V region, but there are certain "hotspots", indicating a preference for characteristic short motifs of four to five nucleotides (Janeway, 2005).
In contrast to CSR, which can also take place in the extrafollicular differentiation, SHM is restricted to GC B cells (Jacob *et al.*, 1993; Jacob and Kelsoe, 1992). Since the nucleic acid changes in the Ig genes can also lead to a loss of specificity for the antigen or even a gain of self-reactivity, a tight selection process must take place in the GC. Thus, only cells carrying a BCR with a high affinity for the specific antigen are positively selected and receive survival signals through their BCR and CD40. In contrast, low affinity B cells fail to receive maintenance signals, go into apoptosis and are ingested by tangible body macrophages (Wolniak *et al.*, 2004).
High affinity B cells ready to leave the GC either differentiate to antibody producing plasma cells or to long-lived memory B cells. GC-derived plasma cells primarily migrate to the bone marrow where they persist for long periods producing circulating high affinity antibodies (Slifka and Ahmed, 1998). Memory B cells are long-lived B cells, suggested to reside in secondary lymphoid tissues in the periphery and are the main effector cells in secondary immune responses (McHeyzer-Williams and McHeyzer-Williams, 2005).

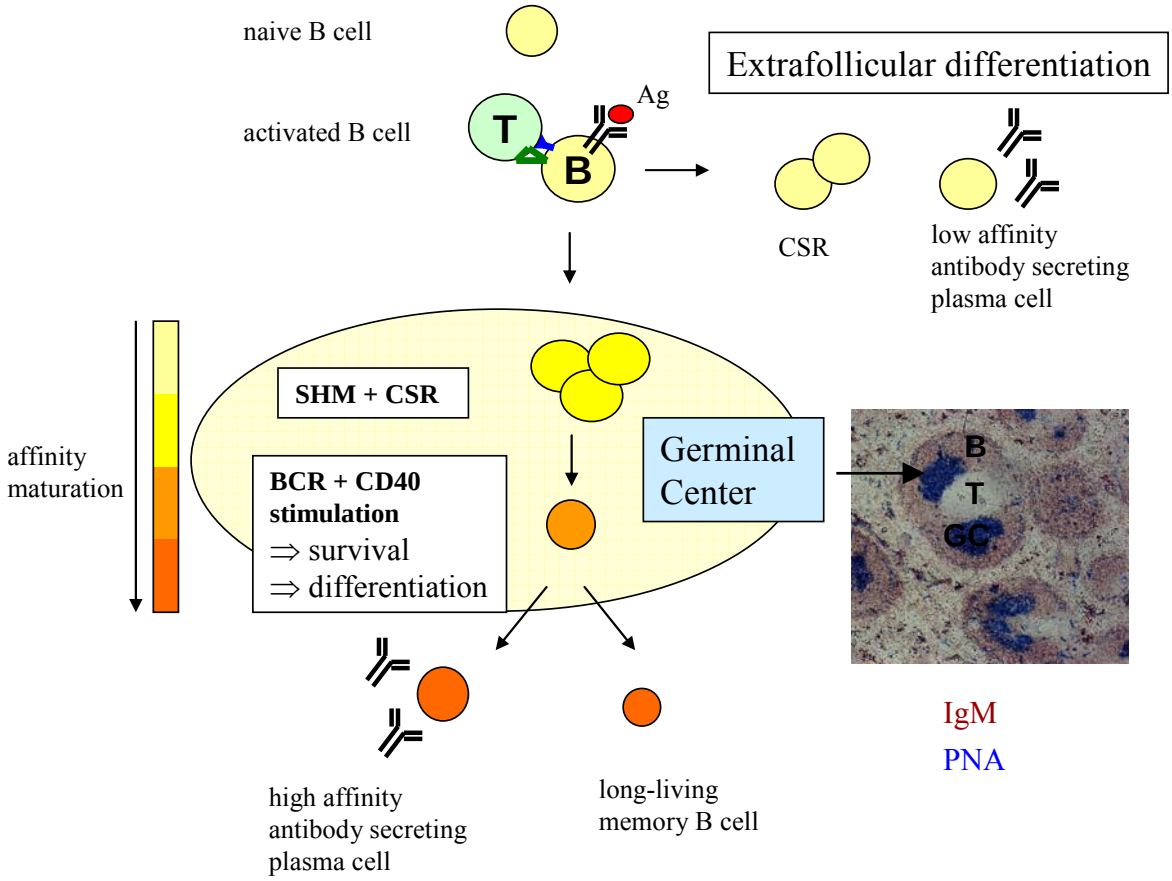

Figure 1.1. T cell-dependent immune response

A naïve B cell which encounters cognate antigen (Ag) and interacts with an antigen specific T helper cell via the MHC-class II and the CD40 receptor, becomes activated and enters either the extrafollicular or germinal center differentiation pathway. In the extrafollicular foci, clonal B cell expansion and differentiation into plasma cells takes place. These plasma cells are short lived and secrete low affinity antibodies that may be switched or unswitched. In the germinal center reaction expansion of B cells, somatic hypermutation (SHM) and class switch recombination (CSR) take place. Through CD40 and B cell receptor (BCR) stimulation B cells harboring high affinity receptors are provided with survival signals and differentiate either into high affinity plasma or memory B cells. During the germinal center reaction B cells gain B cell receptors with a higher affinity through somatic hypermutation processes; the affinity maturation is indicated by the color.
A section of a murine spleen at the peak of germinal center formation after immunization is shown. In blue are the peanut agglutinin (PNA) stained GC B cells and in red the naïve IgM^+ B cells depicted, whereas the unstained region in the follicle reflects the T cell zone. Original magnification, x 50.

1.4 Germinal center B cell malignancies

Most types of human B cell lymphoma derive from GC B cells or their descendents (Kuppers *et al.*, 1999; Shaffer *et al.*, 2002; Stevenson *et al.*, 2001). During the GC reaction extensive proliferation, hypermutation and recombination processes take place, which all increase the risk of malignant transformation. Both CSR and SHM generate DNA breaks, eliciting a certain risk for genetic lesions. Thus, several B cell malignancies harbor translocations that place proto-oncogenes under control of the Ig heavy or one of the light chain loci, which are likely to have occurred as an error of CSR or SHM. The indication that a lymphoma originates from a GC B cell is based on the presence of SHM and the gene expression profile. Several types of B cell lymphoma express GC signature genes, including follicular lymphomas, Burkitt's lymphomas and a subgroup of diffuse large B cell lymphomas (DLBCLs). Some of these even show ongoing SHM.

The characteristic of Burkitt's lymphoma (BL) is the chromosomal translocation of c-myc to one of the Ig loci, which induces the deregulation of this oncogene. The phenotype of Burkitt's lymphoma cells is remarkable similar to GC centroblasts ($CD10^+CD77^+CD38^+$), and they express somatically mutated V region genes, and sometimes even show ongoing hypermutation (Klein *et al.*, 1995; Chapman *et al.*, 1995).

In Hodgkin's lymphoma (HL) of the classical type it is not as evident as in BL that the malignant cells originate from GC or post-GC B cells. The cellular origin of HL has long been unclear, since they do not resemble any normal haematopoietic cell type. Single-cell molecular analyses revealed that the malignant cells had rearranged Ig V genes, which also were mutated (Kuppers *et al.*, 1994). Interestingly, many of these cells showed nonsense mutations or deleterious deletions in their Ig genes (Kanzler *et al.*, 1996). GC B cells that acquire so-called "crippled mutations" are normally deleted by apoptosis, since a B cell without a functional BCR is not able to survive (Lam *et al.*, 1997). It has been recently shown that all HL cases with crippled mutations are Epstein-Barr-virus (EBV) positive, indicating that EBV helps these cells to survive and contribute to tumorigenesis (Brauninger *et al.*, 2006).

1.5 Epstein-Barr virus

Epstein-Barr virus (EBV) is a human γ-herpesvirus, which preferentially infects B lymphocytes (reviewed in Rickinson and Kieff, 2001). It is an extremely successful virus, since more than 90% of the world's population are infected by it. The viral DNA is usually

carried as circular DNA or episomes, and persists a lifetime in resting memory B cells of the host. Although persistent EBV infection mostly takes an asymptomatic course, there are several malignancies derived from lymphoid and epithelial tissues strongly associated with EBV, including Burkitt's lymphoma, Hodgkin's lymphoma, post-transplant or immunoblastic lymphoma, T and NK cell lymphoma, nasopharyngeal carcinoma and gastric carcinoma (Kuppers, 2003; Young and Rickinson, 2004). However, in most cases the mechanisms how EBV contributes to tumor development are still elusive.

The *in vitro* infection with EBV leads to the transformation of primary B cells. In these so called Lymphoblastoid Cell Lines (LCLs) eleven out of about 80 viral genes are expressed and referred to as latent genes. These are the six EBV nuclear antigens EBNA 1, -2, -3A, -3B, -3C and –LP (Epstein-Barr viral Nuclear Antigens), three membrane proteins LMP1, LMP2A and B (Latent Membrane Proteins), two small nonpolyadenylated RNAs EBER1 and –2 (Epstein-Barr viral encoded RNAs), and highly spliced Bam A rightward transcripts, or BARTs. Beside this so-called latency III or growth program, where all latent genes are expressed, other latencies with a more restricted viral gene expression exist.

Apart from latency 0 in resting cells, EBNA1 is expressed in all EBV-positive proliferating cells (Hochberg *et al.*, 2004). EBNA1 is essential for the replication of the circular viral DNA before mitosis and for its distribution into progeny cells during cell division.

EBNA2 together with EBNA-LP are the first viral proteins expressed in EBV infected B lymphocytes. EBNA2 is the key regulator of viral gene expression, stimulating transcription of all EBNA and LMP genes during latency III. EBNA2 also modulates the transcriptional activity of several cellular genes. It interacts with the cellular DNA binding recombination signal binding protein J kappa (RBP-Jκ, also referred to as CBF1 and CSL) to modulate gene expression (Grossman *et al.*, 1994; Henkel *et al.*, 1994; Zimber-Strobl *et al.*, 1994). Since the cellular protein Notch interacts with RBP-Jκ as well, EBNA2 has been considered to be a functional equivalent of activated Notch.

EBNA-LP interacts with EBNA2 and co-activates transcription, whereas the proteins of the EBNA3 family repress this transcriptional activation.

LMP1 and LMP2A are ligand-independent receptors, sharing functional properties with CD40 and BCR, respectively. The functional homologies of LMP1 versus CD40 and LMP2A versus BCR will be discussed later in detail.

LMP1, EBNA2, EBNA3A and 3C have been shown to be essential for B cell transformation *in vitro* (Cohen *et al.*, 1989; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993).

EBV infection of B cells *in vivo* occurs by oral transmission and is usually asymptomatic, although it can be presented as Infectious Mononucleosis (IM) if primary infection is delayed until adolescence. Infected B cells expressing the immunogenic viral proteins are recognized and eliminated by cytotoxic T cells. Nevertheless, the virus has been shown to persist in memory B cells with restricted or even no gene expression, thus escaping immune surveillance (Babcock *et al.*, 1998). It is still a controversial question whether EBV directly infects memory B cells and expression of viral genes is then down-regulated upon pressure of cytotoxic T cells, or whether naive B cells are infected and proceed to develop via normal B cell differentiation processes into memory B cells.

1.6 EBV and the germinal center reaction

Thorley-Lawson and colleagues have stated that EBV infected B cells have to pass the germinal center to get access to the memory B cell compartment (Thorley-Lawson, 2001; Thorley-Lawson and Babcock, 1999). This model is based on EBV gene expression studies in tonsillar B cells of healthy persons. They could show that only B cells with a naïve phenotype express the genes of the latency III program, whereas B cells with a GC phenotype express the more restricted latency II program, where only EBNA1 and the two membrane proteins LMP1 and LMP2A can be detected (Babcock *et al.*, 2000; Babcock and Thorley-Lawson, 2000; Joseph *et al.*, 2000). It has been proposed that LMP1 and LMP2A provide survival and proliferation signals to pass the germinal center without negative selection, and allow the EBV-infected B blasts to become resting memory B cells (Thorley-Lawson, 2001). However, there are experimental data not compatible with the hypothesis that EBV uses the germinal center reaction to establish persistence in memory B cells. Thus, it has been shown that LMP1 expressed in B cells of transgenic mice blocks GC formation (Uchida *et al.*, 1999). In addition, Kurth and colleagues showed that in infectious mononucleosis (IM) EBV directly infects both naive and memory B cells, but that most of the EBV infected B cells derived from clones harboring somatic mutations (Kurth *et al.*, 2000). They could detect differences in morphology and EBV gene expression patterns within members of one EBV+ memory B cell clone, implying another mechanism to establish persistence in memory B cells than passing through a GC reaction.

Even though the data on latent EBV infection and GC reaction are still controversial, it is evident that EBV positive lymphomas like Burkitt's Lymphoma (BL) and Hodgkin's Lymphoma (HL) derive from GC B cells or at least show GC like phenotypes. In contrast to

immunoblastic lymphomas, which show the latency III expression pattern and occur in immunocompromised individuals, the viral gene expression is very much restricted in BL and HL. In BL, only EBNA1 and two non-coding viral RNAs are expressed, whereas in HL EBNA1, LMP1 and LMP2A are expressed. As already mentioned, the characteristic Reed-Sternberg (HRS) cells of HL often have highly mutated immunoglobulin genes, which are not able to encode a functional BCR (Kanzler *et al.*, 1996). Cells without a functional BCR usually undergo negative selection. It is speculated that expression of LMP1 and LMP2A provide the pre-tumor or tumor cell with the essential survival signals which inhibit the elimination during the GC reaction. The survival signals for GC B cells are physiologically provided by the BCR and CD40. These signals might be replaced by the EBV proteins LMP2A and LMP1, which are both expressed in EBV positive HL cells (Herbst *et al.*, 1991; Pallesen *et al.*, 1991; Niedobitek *et al.*, 1997).

1.7 LMP1 and CD40

The viral Latent Membrane Protein 1 (LMP1) and the cellular CD40 receptor are considered to be functional homologues. Both the LMP1 and CD40 cytoplasmic domains interact with a set of cellular signaling molecules called the tumor necrosis factor receptor-associated factors (TRAFs) and activate overlapping signaling pathways, including ERK, JNK, p38/MAPK and NFκB (Figure 1.2). Additionally, they bind to the janus family kinase (JAK) 3, which upon phosphorylation is able to activate the signal transducer and activator of transcription (STAT). Activation of CD40 and expression of LMP1 in B cells exhibits comparable phenotypes, both able to rescue B cells from apoptosis and drive their proliferation (Zimber-Strobl *et al.*, 1996). However, LMP1 and CD40 do not interact with exactly the same sets of molecules, indicating also some differences in their signaling outcome. Thus, both LMP1 and CD40 interact directly with TRAFs 1, 2, 3 and 5, but only CD40 and not LMP1 binds directly to TRAF6 (Ishida *et al.*, 1996; Schultheiss *et al.*, 2001). Conversely, LMP1 but not CD40 binds to the tumor necrosis factor receptor associated death domain protein (TRADD) and receptor-interacting protein (RIP) (Izumi *et al.*, 1999), which is usually associated with death signals. In contrast to other cellular tumor necrosis factor receptors (TNF-R) binding to TRADD, LMP1 recruits TRADD via the TRADD N-terminus but not the TRADD death domain. Consequently, LMP1's association with TRADD does not induce apoptosis, but seems to be crucial for NFκB-activation via recruitment of TRAF2 by the TRADD-binding domain (Kieser *et al.*, 1999).

Moreover, LMP1 signaling does not induce degradation of TRAF2 and TRAF3 as CD40, which might contribute to an enhanced signaling potency of LMP1 (Brown *et al.*, 2001).

The most striking difference between LMP1 and CD40 is that LMP1 constitutively signals independently of ligand, whereas CD40 depends on CD40-ligand for its signaling (Gires *et al.*, 1997). The ligand independency of LMP1 is mediated by aggregation through its six transmembrane spanning domains. In contrast, the CD40 receptor contains an extracellular portion with four cysteine-rich domains which mediate direct ligand binding. The ligand CD40L (CD154, gp39) is mainly expressed on activated T helper cells (Bishop and Hostager, 2003). The biological significance of the CD40-CD40L interaction was first revealed in studies of patients with mutations in the genes for CD40L and CD40 (Hyper-IgM syndrome (HIGM) 1 and 3, respectively), who show defects in immunoglobulin (Ig) class switch recombination (CSR) (Aruffo *et al.*, 1993; Korthauer *et al.*, 1993; DiSanto *et al.*, 1993; Allen *et al.*, 1993). The crucial role of the CD40-CD40L interaction in the T-cell dependent immune response was further supported by studies of CD40 and CD40L knockout mice, which not only show defects in their Ig CSR, but also in their formation of germinal centers (GC), in somatic hypermutation (SHM) of their Ig genes, and establishment of B cell memory (Xu *et al.*, 1994; Kawabe *et al.*, 1994).

In vitro, LMP1 was shown to mimic CD40 in several ways. However, in transgenic mice B cell-specific expression of LMP1 only partially reconstituted the CD40 deficiency (Uchida *et al.*, 1999). LMP1 expression in CD40-/- mice could restore antibody class switching to IgG1 after immunization with T cell dependent antigens, but neither GC formation nor the production of high affinity antibodies. LMP1 expression even blocked GC formation in the presence of the endogenous CD40 receptor, suggesting that LMP1 expression is not compatible with the GC reaction. Recently, it has been shown that the fusion protein of the transmembrane domain of LMP1 and the signaling domain of CD40 (LMP1/CD40), which provides a constitutive active CD40 signal, also blocks the GC reaction (Panagopoulos *et al.*, 2004) (Hömig, 2005). Further it could be shown that a constitutive active CD40 signaling in B cells leads to a splenomegaly with strong accumulation of follicular and marginal zone B cells (Hömig, 2005). These B cells display an activated phenotype, but are impaired in TD immune responses. LMP1/CD40-expressing mice develop lymphoma at high incidence, as do LMP1 transgenic mice (Kulwichit *et al.*, 1998). Thus, it seems that the constitutive activation of B cells by either LMP1 or CD40 leads to a differentiation block which prevents GC formation and ultimately leads to lymphomagenesis in mice. The oncogenic potential of

constitutive LMP1 and CD40 signals was further shown by rodent fibroblast transformation assays (Wang *et al.*, 1985; Hatzivassiliou *et al.*, 2006).

LMP1 is essential for EBV-induced B cell transformation *in vitro* (Kaye *et al.*, 1993), and is expressed in several EBV associated human malignancies, like HL, posttransplant lymphoma and nasopharyngeal carcinoma.

Recently, it has been claimed that even the LMP1 cytoplasmic domain itself regulated by the CD40L (CD40/LMP1) harbors pathogenic features, inducing hyperactivation of B lymphocytes and disordered lymphoid architecture in mice (Stunz *et al.*, 2004). However, in that study CD40/LMP1 was not expressed exclusively in B cells, the site of normal EBV latent infection, but in all antigen presenting cells. Thus, those mice show elevated serum-IL6 most likely responsible for many of observed abnormalities, which seem to be secreted by activated macrophages. Therefore it remained unclear what would be the influence of the LMP1 signaling domain exclusively expressed in B cells.

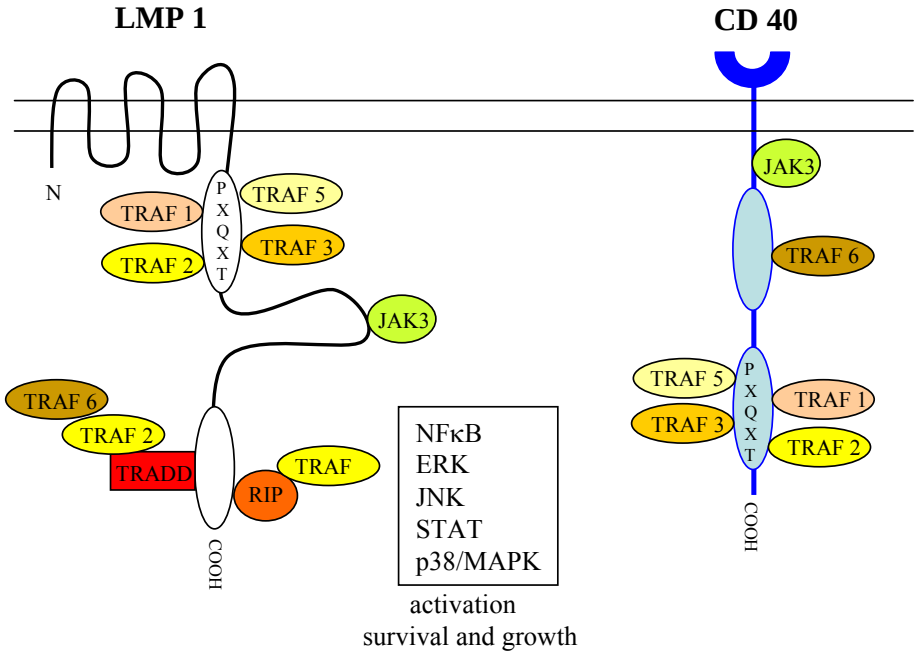

Figure 1.2. LMP1 as a functional homologue of CD40
Both LMP1 and CD40 bind to tumor necrosis factor receptor-associated factors (TRAFs) and activate overlapping signaling pathways, including ERK, JNK, p38/MAPK and NF-κB, which leads to activation, proliferation and survival of B cells.
LMP1, latent membrane protein 1; TRAF, tumor necrosis factor receptor-associated factors; TRADD, tumor necrosis factor receptor associated death domain protein; RIP, receptor interacting protein; JAK, janus kinase; NF-κB, nuclear factor κB; MAPK, mitogen activated protein kinase; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; PxQxT, protein binding motif.

1.8 LMP2A and the B cell receptor

Both the B cell receptor (BCR) and LMP2A contain functional immunoreceptor tyrosine-based activation motifs (ITAMs) (Figure 1.3).

The BCR complex consists of two immunoglobulin heavy chains (IgH) and two immunoglobulin light chains (IgL) associated with the signaling molecules Igα (CD79A) and Igβ (CD79B). IgH and IgL have a variable and a constant region. The variable regions determine antigen specificity of the BCR. Since IgH and IgL lack signaling domains, Igα and Igβ are crucial for BCR signaling. Igα and Igβ are transmembrane molecules, which harbor the ITAMs in their cytoplasmic tails. Through these motifs the interaction with intracellular protein tyrosine kinases, such as Lyn and Syk, and the initiation of several signaling cascades is mediated. In the absence of antigen stimulation, the BCR provides the cell with a tonic signal that has been shown to be essential for the survival of B cells (Lam *et al.*, 1997).

LMP2A is a transmembrane protein which harbors 12 transmembrane spanning domains, through which it is able to self-aggregate and mediate constitutive signaling independent of ligand, as described for LMP1. The amino-terminal cytoplasmic domain of LMP2A contains several tyrosine residues, two of them containing a conserved ITAM motif. The recruitment of Lyn and Syk by LMP2A results in the constitutive phosphorylation of these kinases. LMP2A is able to block signaling after BCR-cross-linking by recruiting Lyn and Syk (Fruehling and Longnecker, 1997). However, LMP2A expression in transgenic mice allows the release of Ig negative B cell in the periphery, indicating that it can mimic BCR signals required for positive selection of B cells in the bone marrow (Caldwell *et al.*, 1998). Further, it helps mature B cells to survive without a BCR and to initiate GC reactions in gut-associated lymphoid tissue (Casola *et al.*, 2004).

LMP2A expression is not essential for EBV-induced B cell transformation *in vitro* (Longnecker *et al.*, 1992). However, its implication in EBV associated tumors has been suggested. Thus, LMP2A can transform epithelial cells *in vitro* (Scholle *et al.*, 2000) and it is consistently expressed in nasopharyngeal carcinoma (Niedobitek *et al.*, 1992; Brooks *et al.*, 1992). Further, it is expressed in post-transplant and EBV associated Hodgkin's lymphoma. In HL, LMP2A may contribute to lymphomagenesis by providing pre-apoptotic Ig crippled cells with BCR-like survival signals. This is in accordance with the observation that only EBV associated HL cells harbor crippled mutations. Additionally, it has been recently shown by three independent groups that EBV can rescue crippled GC B cells from apoptosis *in vitro* (Mancao *et al.*, 2005; Bechtel *et al.*, 2005; Chaganti *et al.*, 2005).

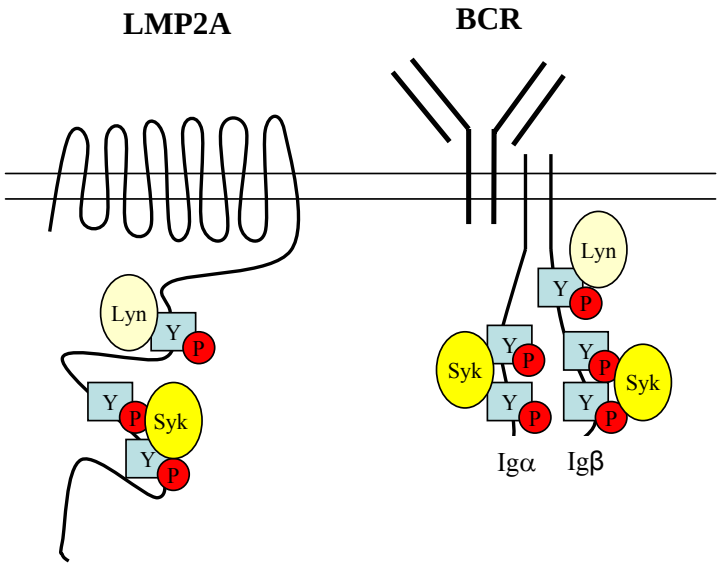

Figure 1.3. LMP2A mimics the B cell receptor
Both LMP2A and BCR interact with intracellular protein tyrosine kinases, such as Lyn and Syk, and initiate several signaling cascades.
BCR, B cell receptor; LMP2A, latent membrane protein 2A; Igα/β, immunoglobulin-associated signaling molecule α and β respectively; Yp; phosphorylated tyrosines of the ITAMs (immunoreceptor tyrosine-based activation motifs).

2 Aim of the project

The Epstein-Barr viral proteins LMP1 and LMP2A share functional properties with the cellular receptors CD40 and BCR, respectively, and therefore elicit unique features to interfere in normal B cell differentiation processes. However, it is still elusive if and how EBV uses B cell differentiation pathways to establish persistence in memory B cells. The current popular hypothesis of Thorley-Lawson and colleagues, which states that EBV infected B cells have to pass a germinal center (GC) reaction to become EBV-positive resting memory B cells, is contrary to the observation that LMP1 expression in B cells of transgenic mice interferes with GC formation. To work out the mechanism how EBV establishes persistence is a prerequisite to understand its role in B cell lymphomagenesis.

The aim of the present PhD thesis is to examine the interplay of EBV and the GC reaction, focusing on the influence of LMP1 and LMP2A.

In the first part, the signaling properties of CD40 and LMP1 should be analyzed and compared *in vivo*. Therefore, transgenic mice should be established, which conditionally express the fusion protein of the extracellular domain of the CD40 receptor and the signaling domain of LMP1 (CD40/LMP1). The influence of the LMP1 signaling domain exclusively expressed in B cells should be elucidated. In CD40 deficient mice it should be analyzed if LMP1 signaling is able to substitute the CD40 signal in the T cell dependent immune response, including class switch recombination, germinal center formation and affinity maturation.

In the second part, another mouse line expressing conditionally a fusion protein of the transmembrane domain of LMP1 and the signaling domain of CD40 (LMP1/CD40), therefore providing constitutive active CD40 signals, should be analyzed. These mice expressing LMP1/CD40 in B cells have previously been shown to be deficient in GC formation, but show an expansion of B cells which ultimately leads to lymphoma development (Hömig, 2005). In the present work, the effect of a constitutive active CD40 signal should be analyzed especially on activated and germinal center B cells. By crossing the LMP1/CD40^{flSTOP} strain to the Cγ1-cre strain, LMP1/CD40 expression will be induced in germinal center B cells. Offspring should be analyzed for germinal center formation upon T cell dependent immunization, and for lymphoma development to study the impact of constitutive CD40 signaling on germinal center B cells and the murine immune system.

In the third and last part, a human B cell system should be established to study the impact of different EBV proteins on the B cell phenotype *in vitro*. To this end, the EBV proteins EBNA1, LMP1 and LMP2A should be expressed either alone or in combination in a naïve human B cell line to analyze their properties to induce germinal center like differentiation and immortalization processes.

3 Results

3.1 Comparison of LMP1 and CD40 signaling in B cells in vivo

Based on several *in vitro* studies the Epstein-Barr-viral protein LMP1 and the cellular CD40 receptor are considered to be functional homologues. However, B cell specific expression of LMP1 in transgenic mice was not able to substitute for CD40 in the TD immune response and even blocked GC formation in the presence of endogenous CD40 (Uchida *et al.*, 1999). Recently, it has been shown that the fusion protein of the transmembrane domain of LMP1 and the signaling domain of CD40 (LMP1/CD40), which provides a constitutive active CD40 signal, also blocked the GC reaction (Panagopoulos *et al.*, 2004) (Hömig, 2005). Thus, it seems that the constitutive activation of B cells by either LMP1 or CD40 leads to a differentiation block which prevents GC formation. In the present work, we wanted to investigate whether the LMP1 signaling domain itself is able to mimic CD40 *in vivo*.

3.1.1 Generation of a transgenic mouse line expressing a conditional CD40/LMP1 transgene

To study LMP1 signaling *in vivo*, we generated a transgenic mouse strain conditionally expressing LMP1 signaling of the ligand-binding and transmembrane domain of CD40 (amino acids (aa) 1-215) and the signaling domain of LMP1 (aa 186-386) (CD40/LMP1) (Figure 3.1A). A single copy of the CD40/LMP1 chimeric gene was inserted into the murine rosa26-genomic locus by homologous recombination in BALB/c-derived embryonic stem (ES) cells (Figure 3.1B). To restrict expression of CD40/LMP1 to specific cell types and to pre-determined stages of B cell development, a loxP-flanked transcription and translation termination site (stop-cassette) upstream of the CD40/LMP1 coding sequence was inserted. After excision of the stop-cassette by the recombinase Cre, the CD40/LMP1 transgene is placed under the transcriptional control of the ubiquitously active rosa26-promoter. Correctly targeted ES cell clones were identified by Southern blot analysis (Figure 3.1C) and used to establish the CD40/LMP1^{flSTOP} inducible mouse strain on the BALB/c genetic background. The generation of the transgenic ES cell clones was described in detail in my diploma thesis (University of Vienna, 2003).

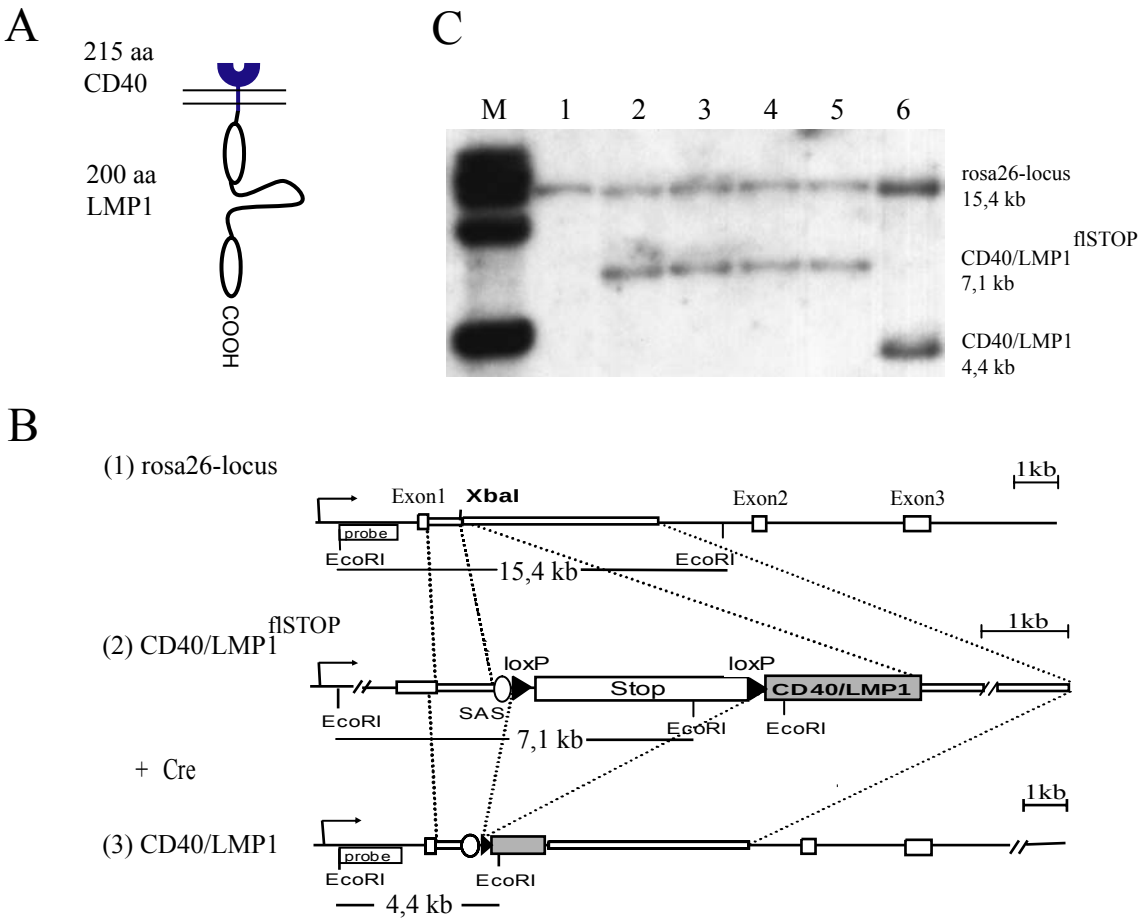

Figure 3.1. Generation of a transgenic mouse line conditionally expressing CD40/LMP1
(A) Schematic representation of the chimeric protein CD40/LMP1. The N-terminal 215 amino acids (aa) of CD40 (receptor binding and transmembrane domain) were fused to the COOH-terminal 200 aa of LMP1 (cytoplasmic domain).
(B) Targeting strategy for the insertion of a conditional CD40/LMP1 allele (CD40/LMP1^{flSTOP}) into the mouse rosa26-locus. The figure shows (1) the wild type rosa26-locus with its 3 exons and the Xba I restriction site in the first intron where the transgene was inserted; (2) the rosa26-locus after homologous recombination of the targeting construct (CD40/LMP1^{flSTOP}); and (3) the rosa26-locus after homologous recombination and deletion of the stop cassette upon Cre-mediated recombination, which leads to the expression of CD40/LMP1 under transcriptional control of the endogenous rosa26-promoter.
The EcoRI recognition sites and the location of the probe for the Southern blot analysis are shown. The expected fragments after EcoRI digestion and hybridization with the labeled probe are indicated. Abbreviations: Cre – Cre recombinase; SAS – splice acceptor site; loxP- locus of crossing over in bacteriophage P1.
(C) Southern blot analysis showing the different alleles after targeting and Cre-mediated recombination of the stop cassette in ES cells. The DNA was digested by EcoRI and hybridized with the labeled probe specific for the rosa26-locus as shown in (B). Lane 1, wild-type ES cells; lanes 2 – 5, ES cell clones showing correct targeting; lane 6, ES cell clone with correct targeting after Cre-mediated deletion of the stop cassette.

3.1.2 B cell specific expression of the CD40/LMP1 transgene in a CD40-/- background

Since EBV latent infection is restricted to B cells, the influence of CD40L-regulated LMP1 signaling was specifically investigated in B cells. Therefore, the CD40/LMP1^{flSTOP} mice were crossed to the CD19-Cre mouse strain to activate expression of the chimeric protein from the pro-pre B cell stage on (Rickert *et al.*, 1997). Un-immunized and immunized CD40/LMP1 expressing mice on a CD40+/+ background were analyzed and did not show any differences to wild type mice (data not shown). Thus, CD40/LMP1 expression in B cells did not have any negative influence on the murine immune system and did not interfere with GC formation. To analyze the properties of LMP1 to mimic CD40 *in vivo*, mice were bred to CD40-/- mice to express CD40/LMP1 on a CD40-deficient background (CD40/LMP1+//CD40-/-).

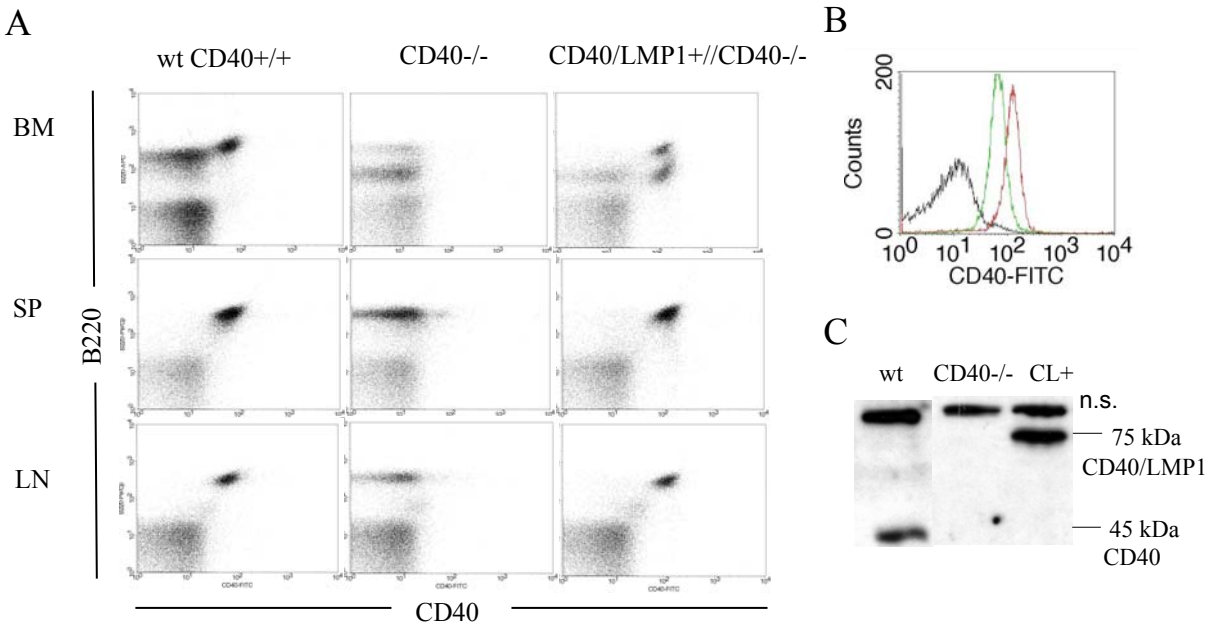

Figure 3.2. B cell specific expression of CD40/LMP1
(A) Cells of bone marrow (BM), spleen (SP) and lymph nodes (LN) were analyzed for the expression of CD40 by flow cytometry. Cells were stained for the mouse CD40 extracellular domain and B220, a B cell marker.
(B) Overlay of the CD40 expression levels in CD40-/- (black line), CD40+/+ (green line) and CD40/LMP1+//CD40-/- (red line) B cells, analyzed by flow cytometry. CD40/LMP1+//CD40-/- B cells show approximately a 2 fold increase in CD40 expression compared to CD40+/+ B cells.
(C) CD40/LMP1 protein expression. Western blots were prepared from lysates of B cells of CD40+/+ (wt), CD40-/- and CD40/LMP1+//CD40-/- (CL+) mice isolated by CD19 MACS separation. The 75 kDa CD40/LMP1 chimeric protein was detected by an anti-LMP1 antibody, and the endogenous 45 kDa CD40 protein by an anti-CD40 antibody. n.s., non specific band, used as a loading control.

FACS analyses of bone marrow and peripheral lymphoid organ cells showed that around 17% of B220$^+$ B cells in the bone marrow (BM) and more than 95% of B220$^+$ B cells in the periphery of CD40/LMP1+//CD40-/- mice stained positive for CD40 and therefore expressed

the transgene (Figure 3.2A). CD40/LMP1 expression already became detectable in B220^{low} pre and immature B cells, whereas the endogenous CD40 is known to be expressed at very low levels in pre-B cells and increases during B cell maturation (Grandien *et al.*, 1996).

An overlay of the FACS staining with the anti-CD40 antibody showed that the CD40/LMP1 expression on B cells was approximately twice the level of CD40 expressed on wild type B cells (Figure 3.2B). Western blot analyses of MACS purified CD19^{+} splenic B cells showed a specific band for the 75 kDa CD40/LMP1 protein only in samples of CD40/LMP1 expressing mice, and the 45 kDa CD40 protein only in the CD40+/+ wild type control (Figure 3.2C).

To show that the transgene is not expressed in dendritic cells (DC), BM cells were isolated from CD40/LMP1+//CD40-/- and control mice (CD40-/- and CD40+/+) and cultivated in the presence of Granulocyte/Macrophage colony stimulating factor (GM-CSF) to select for DC. After seven days, the cells were further stimulated with Lipopolysaccharid (LPS) for 12 hours to induce surface expression of activation markers like CD40, CD80 and MHCclassII on DC. Whereas in both CD40+/+ DC and CD40/LMP1+//CD40-/- DC CD80 expression could be detected after LPS-stimulation, only CD40+/+ DC up-regulated CD40 (Figure 3.3A and B). This indicates that CD40/LMP1 expression is not activated in DC by CD19-Cre. However, around 1% of B220^{-} BM cells showed expression of CD40/LMP1, which were positive for Gr-1, indicating a myeloid origin of these cells (Figure 3.3C).

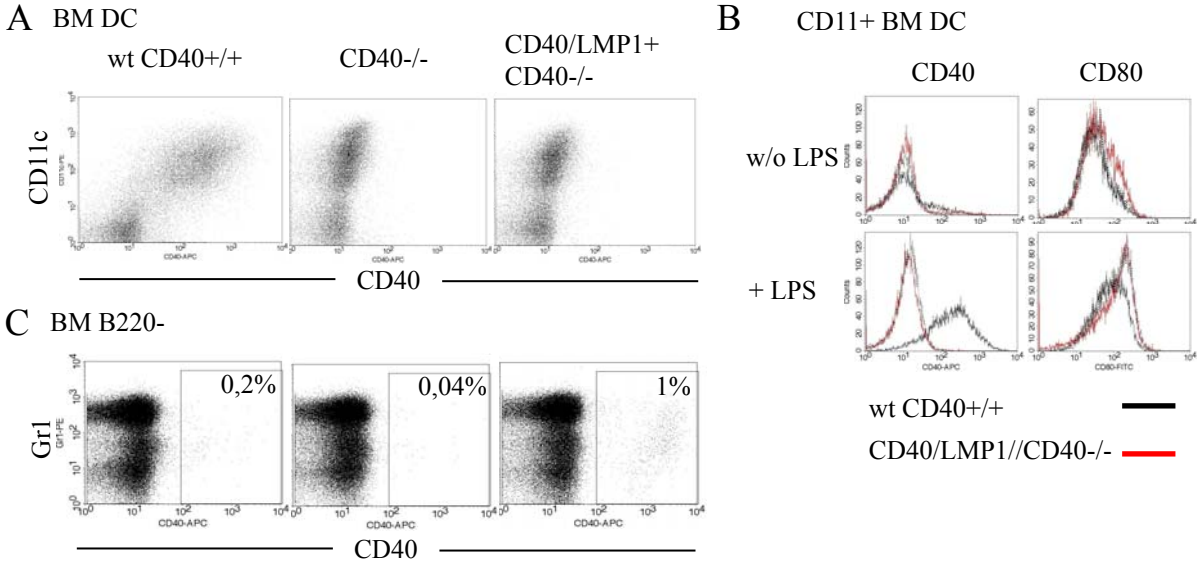

Figure 3.3. CD40/LMP1 expression in non-B cells
(A) Dendritic cells (DC) were enriched from the bone marrow by culturing for 7 days in the presence of GM-CFS. To induce CD40 expression, cells were activated by LPS 12 hours prior to FACS analysis. Cells were gated for living cells (PI negative) and stained for CD11c and CD40.
(B) FACS staining for CD40 and CD80 of CD11c^{+} BM-derived DC after LPS- or without (w/o) stimulation.
(C) Gr1, CD40 co-staining of BM cells gated on B220- and PI-. In BM of CD40/LMP1+//CD40-/- mice around 1% of B220- cells expressed CD40/LMP1.

3.1.3 Lymphoid compartment characterization of CD40/LMP1+//CD40-/- mice

The lymphoid compartments of 8 to 16 weeks old CD40/LMP1+//CD40-/- mice were analyzed and compared to age-matched wt CD40+/+ and CD40-/- control mice (Figure 3.4 and 3.5). The spleens (SP) as well as the inguinal lymph nodes (LN) in CD40/LMP1+//CD40-/- mice showed normal size and weight (Figure 3.4A and data not shown). Total numbers and percentages of $B220^+$ B cells and $CD5^{high}$ T cells in spleen and inguinal lymph nodes were comparable in all three groups of mice analyzed (Figure 3.4B and C).

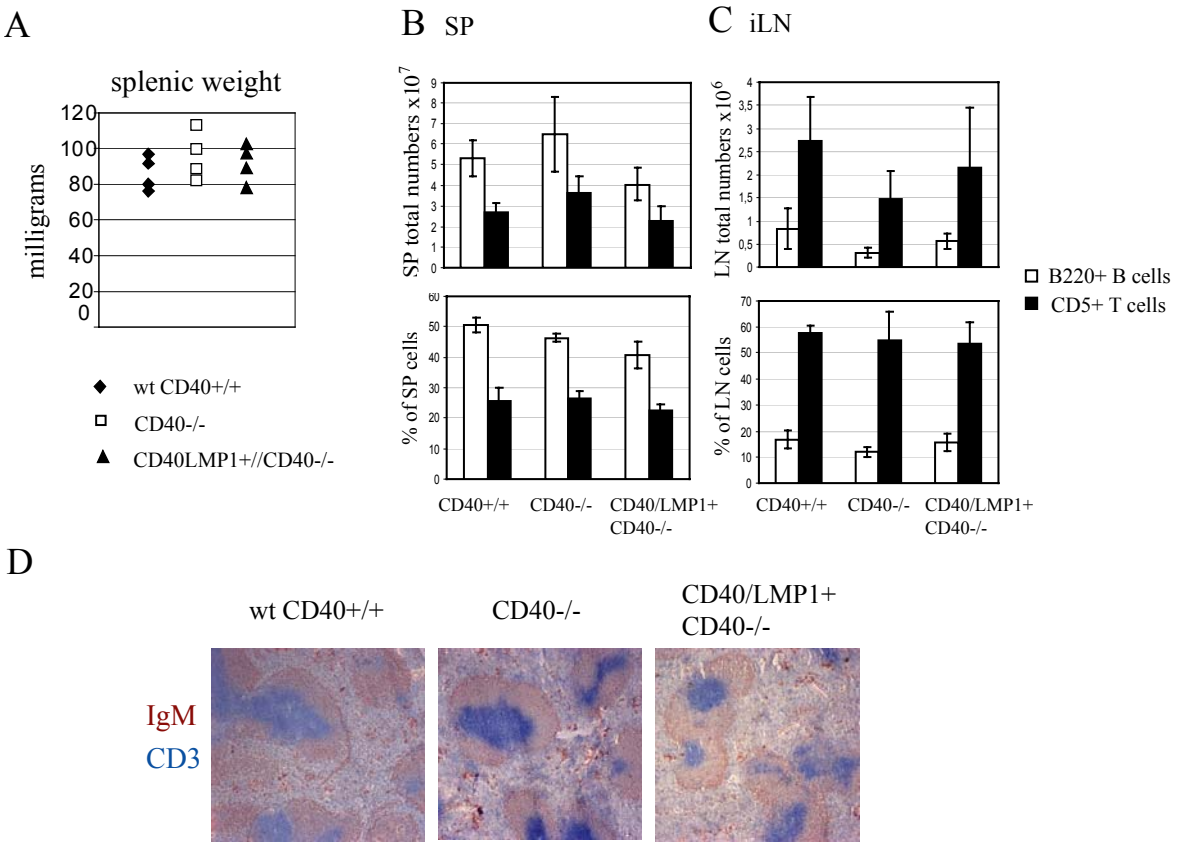

Figure 3.4. B and T cell distributions in the spleen and inguinal lymph nodes are normal in CD40/LMP1+//CD40-/- mice

(A) Splenic weight in milligrams of 4 to 5 wild type (wt CD40+/+), CD40-/- and CD40/LMP1+//CD40-/- mice.

(B) Total numbers and percentages of $B220^+$ B cells and $CD5^+$ T cells in the spleen (SP).

(C) Total numbers and percentages of $B220^+$ B cells and $CD5^+$ T cells in both inguinal lymph nodes (iLN).

Data presented in (B) and (C) are means of four to five mice per group tested in independent experiments.

(D) Immunohistochemical analyses of the splenic structures. Cryosections were stained with anti-IgM specific for B cells (red) and anti-CD3 specific for T cells (blue). Original magnification, x 50.

Immunohistochemical analyses of the spleen revealed that the structure of the follicles with the B and T cell zone was normal in CD40/LMP1+//CD40-/- mice (Figure 3.4D). FACS analyses showed that the CD40/LMP1+//CD40-/- mice had normal percentages of mature IgM^+ IgD^+ B cells in all lymphoid organs and normal percentages of $CD21^+CD23^+$ follicular (FO) and $CD21^{high}CD23^{low}$ marginal zone B (MZB) cells in the spleen (Figure 3.5A and B). Although CD40/LMP1 started to be expressed earlier than the endogenous CD40 in the BM (Figure 3.2A), CD40/LMP1+//CD40-/- mice showed a normal B cell development as revealed by staining against B220 and IgM to distinguish pre/pro ($B220^{low}IgM^-$), immature ($B220^{low}IgM^+$) and recirculating mature B cells ($B220^{high}$, IgM^+) (Figure 3.5C).

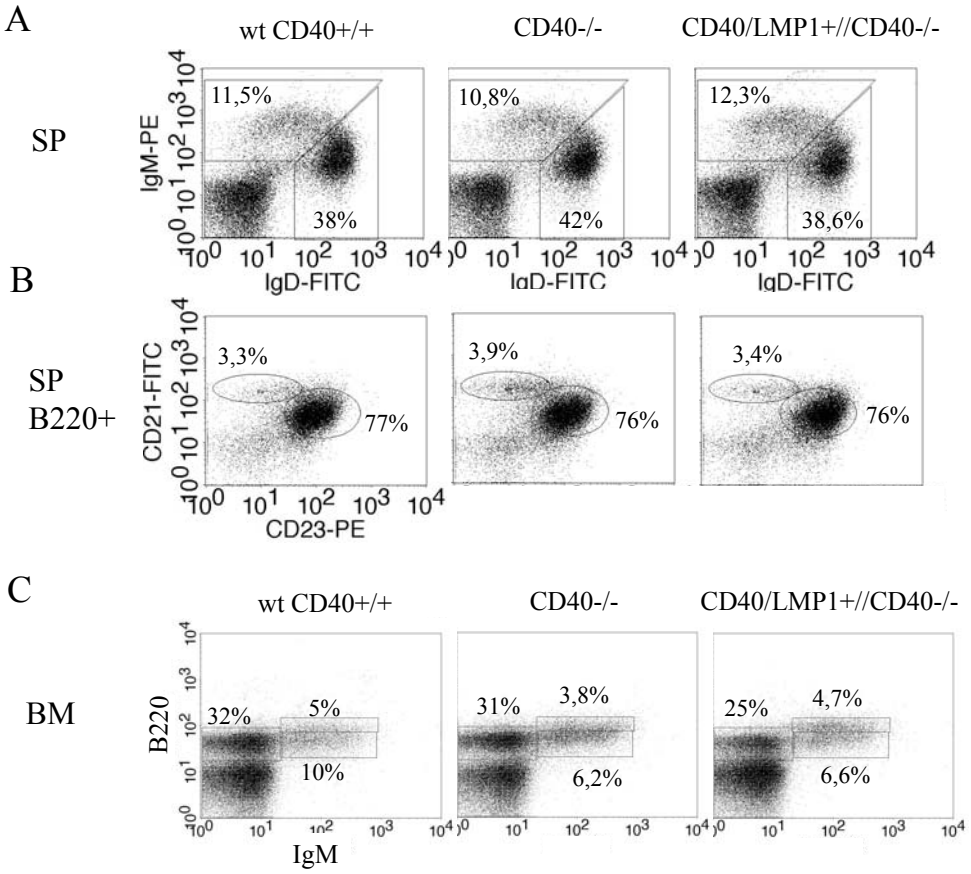

Figure 3.5. CD40/LMP1+//CD40-/- mice show normal B cell subset distributions in the spleen and bone marrow

(A) Lymphocytes of spleens (SP) were analyzed for the expression of IgM and IgD by flow cytometry. Numbers indicate percentages of gated populations, follicular (FO) B cells (IgM^+IgD^+), marginal zone (MZB) and transitional (T) B cells ($IgM^{high}IgD^{low}$).

(B) Flow cytometric analysis of follicular B cells (FO) ($CD21^{int}CD23^+$) and marginal zone B cells (MZB) ($CD21^{high}CD23^{low}$) in the spleen. Numbers indicate percentages of $B220^+$ B cells displaying a MZB or FO B cell phenotype.

(C) Flow cytometry of bone marrow (BM) cells to distinguish pre/pro ($B220^{low}IgM^-$), immature ($B220^{low}IgM^+$) and recirculating mature B cells ($B220^{high}IgM^+$).

3.1.4 CD40/LMP1 restores normal serum immunoglobulin titers in CD40-/- mice

CD40-/- mice show defects in immunoglobulin (Ig) class switching upon T cell-dependent immunization (Kawabe *et al.*, 1994), resulting in decreased IgG1, IgG2a and IgG2b isotype levels and a slight increase in IgM in their total serum Ig titers. To study whether CD40/LMP1 can substitute for CD40 in Ig class switch recombination, total serum Ig titers of un-immunized CD40+/+, CD40-/- and CD40/LMP1//CD40-/- mice were analyzed by Enzyme-linked immunosorbent assay (ELISA) (Figure 3.6). Whereas CD40-/- mice had decreased levels of IgG1, IgG2a and IgG2b in the serum, CD40/LMP1//CD40-/- mice had similar or even higher titers of these isotypes compared to wild type mice. IgM levels were slightly elevated in the CD40-/- mice, but not in the CD40/LMP1//CD40-/- mice. These results show that CD40/LMP1 can rescue the CD40 deficiency in class switch recombination.

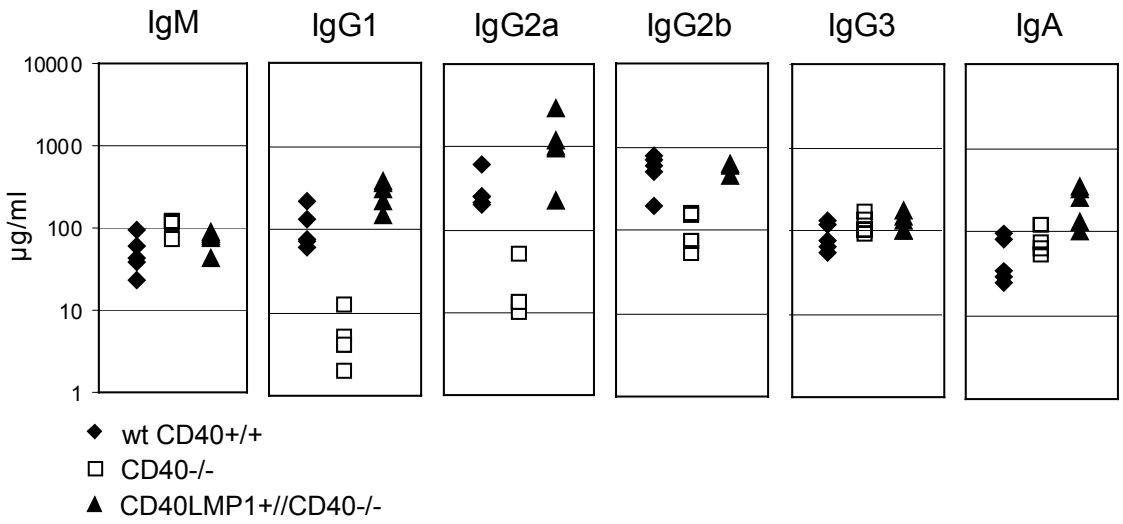

Figure 3.6. CD40/LMP1 can rescue the class switch recombination deficiency in CD40-/- mice
Non-immunized mice were analyzed for total serum immunoglobulin concentrations of the indicated isotypes. Serum of 5 mice per group between the age of 8 and 16 weeks was analyzed.

3.1.5 CD40/LMP1 substitutes CD40 in germinal center formation

To investigate whether CD40/LMP1 could provide the B cells with the essential signals for germinal center (GC) formation, isotype switching and affinity maturation in the absence of CD40, we immunized CD40/LMP1+//CD40-/- and control mice with the hapten nitrophenylacetyl conjugated to chicken-gammaglobulin (NP-CGG). The presence of GC was revealed 0, 7, 14, 21 and 28 days after immunization by flow cytometric and immunohistochemical analyses after staining with peanut agglutinin (PNA), which is specific for germinal center B cells (Rose *et al.*, 1980). PNA stained cells could be observed in

immunized wt CD40+/+ and CD40/LMP1+//CD40-/- mice, but not in immunized CD40-/- mice, nor in un-immunized mice including CD40/LMP1+//CD40-/- mice (Figure 3.7). The immunohistochemical analyses revealed that CD40/LMP1+//CD40-/- mice showed a normal architecture of the follicles with the GC and mantle zone. This result indicates that CD40/LMP1 expression in B cells can rescue optimal GC formation upon TD-immunization in CD40 deficient mice, but does not induce spontaneous GC formation. Compared to wt CD40+/+ controls, CD40/LMP1+//CD40-/- mice showed a higher percentage of GC B as well as plasma cells after immunization (Figure 3.8).

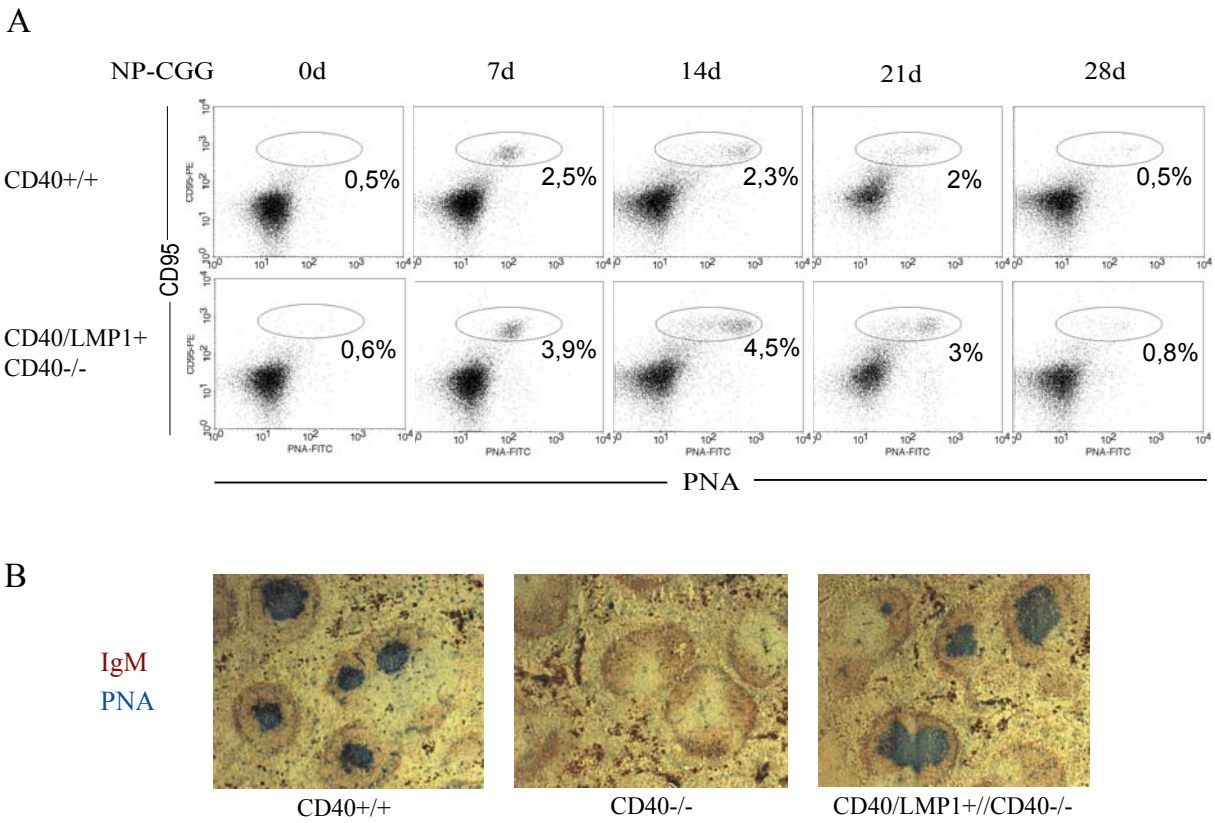

Figure 3.7. CD40/LMP1 is able to substitute for CD40 in germinal center formation
(A) Flow cytometry to identify germinal center B cells ($CD95^{+}PNA^{high}$) in the spleens of CD40+/+ and CD40/LMP1+//CD40-/- mice. Cells were isolated and analyzed at day 0, 7, 14, 21 and 28 after immunization with 100 µg NP-CGG. Cells are gated on $B220^{+}$, numbers indicate mean percentages of B cells displaying a germinal center phenotype of 2 to 5 mice analyzed per group.
(B) Histological analyses of germinal centers in the spleen 14 days after immunization with 100 µg NP-CGG. Cryosections were stained with anti-IgM specific for B cells (red) and PNA specific for germinal center B cells (blue). Original magnification, x 50.

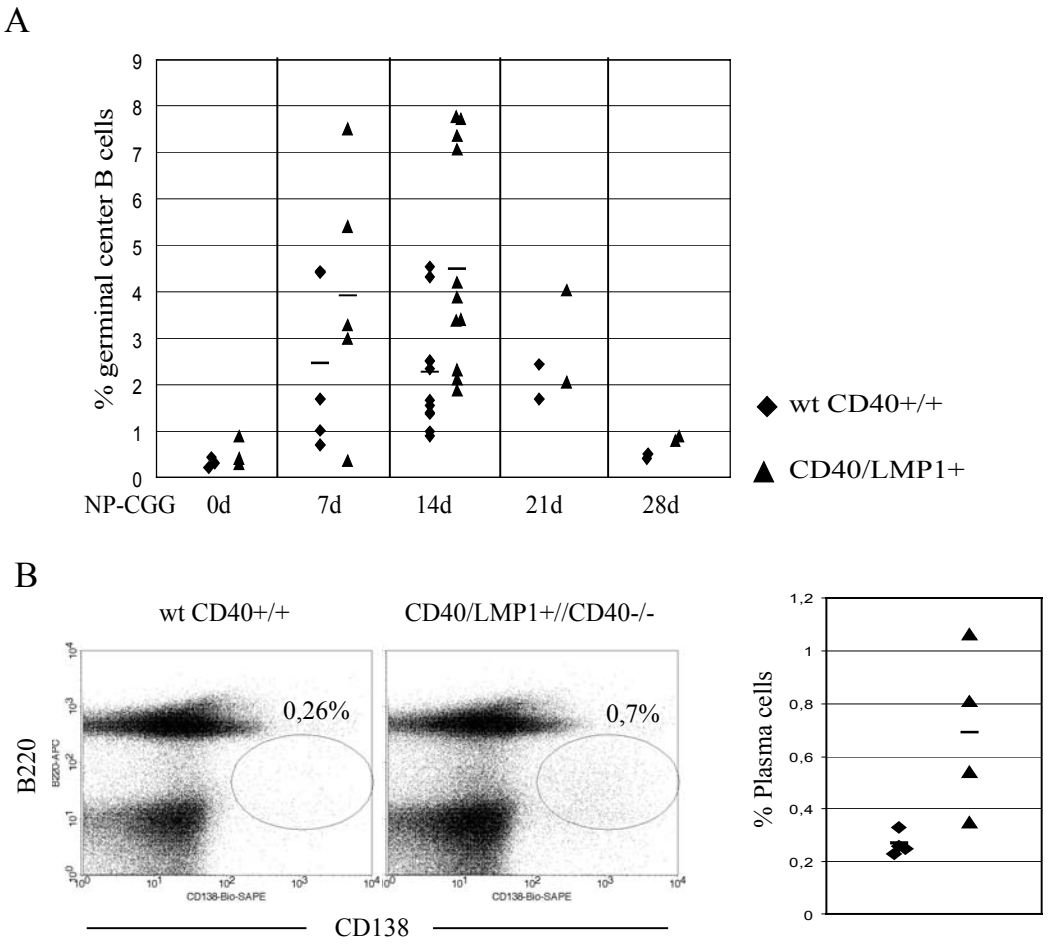

Figure 3.8. CD40/LMP1+//CD40-/- mice show a higher percentage of germinal center B and plasma cells upon immunization
(A) The graph shows the percentages of germinal center B cells in CD40+/+ and CD40/LMP1//CD40-/- mice at day 0, 7, 14, 21 and 28 after immunization with 100 µg NP-CGG. 2 to 11 mice were analyzed per group and time point. At day 14, CD40/LMP1+//CD40-/- mice in average showed a 2 fold increase of germinal center B cells compared to CD40+/+ controls.
(B) Flow cytometry to identify plasma cells ($CD138^{+}B220^{low}$) in the spleens of CD40+/+ and CD40/LMP1//CD40-/- mice 14 days after immunization. Cells are gated on living cells (PI negative). The graph shows the percentages of plasma cells in immunized mice of 4 independent experiments. CD40/LMP1//CD40-/- mice showed a 2 to 4 fold increase in plasma cell percentages compared to CD40+/+ controls.

3.1.6 CD40/LMP1 substitutes CD40 in the production of high affinity antibodies

The ability to produce class switched NP specific antibodies was analyzed by ELISA. CD40/LMP1+//CD40-/- mice were able to produce NP specific antibodies of all isotypes analysed (Figure 3.9A). In comparison to CD40+/+ controls, CD40/LMP1+//CD40-/- mice showed an increase of all class switched NP-specific antibodies. Most obvious was the increase of IgG2b and IgA antibodies.

An important feature of a proper germinal center reaction is the development of high affinity antibodies. By ELISA, NP specific antibodies can be tested to show high or low affinity for NP by binding to low-density hapten and high-density hapten, respectively. To analyze Ig affinity maturation in immunized CD40/LMP1+//CD40-/- and CD40+/+ mice, the concentrations of NP-specific IgG1 antibodies were analyzed 7, 14, 21 and 28 days after immunization. The ratio of anti-NP3/anti-NP17 binding of the antibodies increased with time, showing that CD40/LMP1 can substitute for CD40 in the generation of high affinity antibodies (Figure 3.9B).

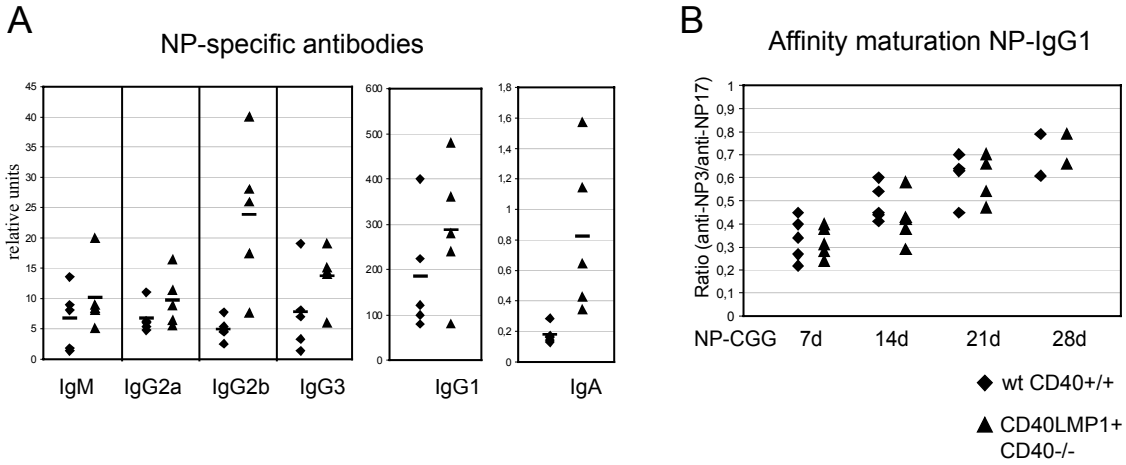

Figure 3.9. CD40/LMP1 substitutes CD40 in the production of class switched and high affinity antibodies

(A) NP-specific antibody response 14 days after immunization with 100µg NP-CGG. NP-specific immunoglobulin concentrations for the indicated isotypes are shown for 5 mice from each group. CD40/LMP1+//CD40-/- mice showed a relative increase of all class switched isotypes analyzed compared to CD40+/+ controls.
(B) Affinity maturation of NP specific IgG1 antibodies at day 7, 14, 21 and 28. The ratio of antibody binding to low-density hapten (NP3-BSA) versus high-density hapten (NP17-BSA) is plotted for 2 to 5 mice per group.

3.1.7 CD40/LMP1+//CD40-/- mice somatically mutate their Ig genes comparable to wild type

Since CD40/LMP1//CD40-/- mice showed affinity maturation of antibodies, we expected the Ig genes to be somatically mutated. To determine adequately if CD40/LMP1 could induce somatic hypermutation to the same extend as CD40, we analyzed the sequences of the J_H4 region of the Ig genes of GC and non-GC B cells of 2 sets of immunized CD40/LMP1+//CD40-/- and CD40+/+ control mice. Whereas non-GC B cells of neither CD40/LMP1+ nor CD40+/+ mice showed SHM, GC B cells of both groups showed approximately the same frequencies of SHM (0,53% versus 0,52%, respectively) (Table 3.1).

This indicates that the LMP1 signaling domain is as efficient as CD40 to induce factors necessary for SHM, and does not lead to uncontrolled mutational activity.

Table 3.1. Frequency and range of mutations in a 500 bp-long region in the intron downstream of the rearranged $V_HD_HJ_H4$ joints of splenic GC B cells of two independent experiments

Genotype	No. clones	No. mutated clones	Range, mutations/clone	No. mutations/no. bp (%)
CD40/LMP1+//CD40-/-	44	38	1-11	130/22000 (0,59%)
CD40+/+	43	37	1-11	146/21500 (0,68%)
CD40/LMP1+//CD40-/-	41	35	1-6	97/20500 (0,47%)
CD40+/+	41	29	1-6	74/20500 (0,36%)

Splenic GC and naïve B cells were isolated 14 days after immunization with NP-CGG. PCR was performed from 40000 cell equivalents with primers annealing in the framework 3 region of most J558 V genes and in the intron downstream of the J_H4 gene segment.
Naïve CD40/LMP1+//CD40-/- B cells had a 0,05% and 0,09% mutation frequency; and naïve CD40+/+ B cells a 0,06% and 0,07% mutation frequency, respectively.

3.1.8 The higher percentage of germinal center B cells in immunized CD40/LMP1+//CD40-/- mice is not a result of a higher proliferation rate

The higher percentages of GC B cells in CD40/LMP1+//CD40-/- mice could be either due to a higher proliferation rate, due to a better survival of CD40/LMP1 expressing GC B cells, or due to a higher recruitment of B cells into the GC.

To analyze the proliferation capacity of GC B cells in CD40/LMP1+//CD40-/- and control mice, we performed BrdU assays in vivo. BrdU, an analogue of thymidine, is incorporated into the DNA of proliferating cells and can be visualized with an anti-BrdU antibody by intracellular staining. Mice were injected intraperitoneally with 150 µl BrdU solution (10 mg/ml) at the peak of GC formation 14 days post-immunization, and sacrificed 2 or 6 hours later. Splenic cells were stained with PNA, anti-CD95 and anti-BrdU and analyzed by flow cytometry. CD40/LMP1+//CD40-/- mice did not show more BrdU positive GC B cells than CD40+/+ mice, indicating that their GC B cells did not proliferate faster than wild type B cells (Figure 3.10).

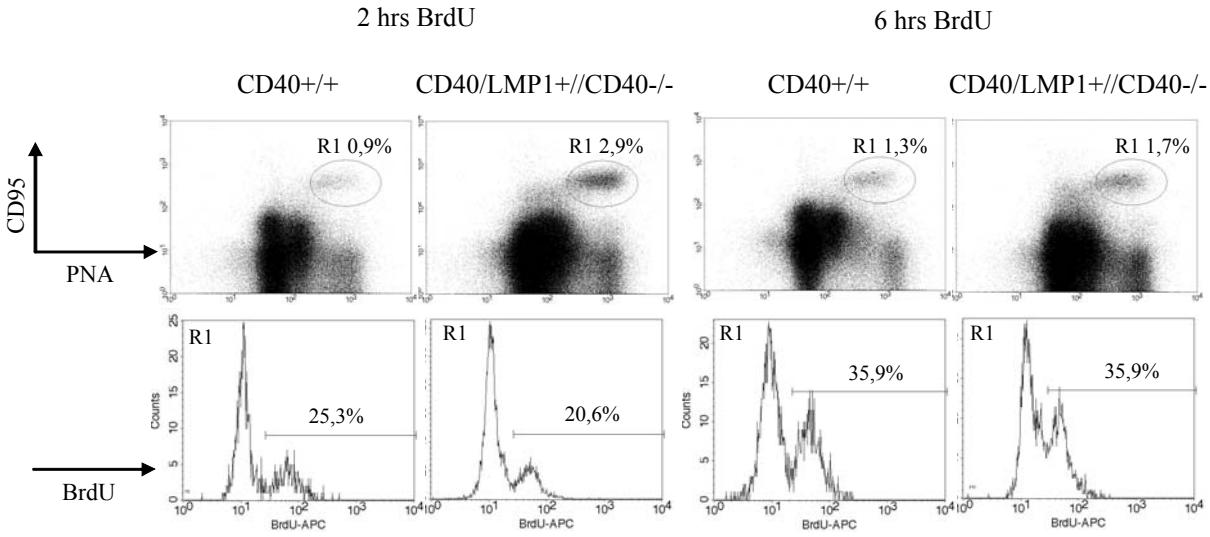

Figure 3.10. CD40/LMP1+//CD40-/- B cells do not show a higher proliferation rate in the germinal center
14 days after immunization with NP-CGG, mice were injected with 150 µl BrdU solution (10 mg/ml) and sacrificed 2 or 6 hours later. The incorporation of BrdU into the DNA was analyzed in germinal center B cells. Cells were stained for CD95 and PNA, fixated and permeabilized to stain intracellular for BrdU incorporation.
The germinal center B cell staining ($CD95^+PNA^+$) and histograms of the BrdU staining of these cells gated on R1 are shown. Numbers indicate the percentages of germinal center B cells and BrdU positive germinal center B cells, respectively.

3.1.9 CD40/LMP1+//CD40-/- mice do not show signs of autoimmunity

It was previously reported that transgenic mice expressing CD40/LMP1 under control of the MHC class II promoter show signs of autoimmune reactivity, as chronic lymphocyte activation, splenomegaly, lymphadenopathy, elevated serum IL-6, spontaneous GC, and anti-ds DNA and anti-phospholipid antibodies in the serum (Stunz *et al.*, 2004). CD40/LMP1+//CD40-/- mice did not show any evidence for autoimmune disease, nevertheless we tested 2-12 months old mice for the presence of anti-phospholipid antibodies by an anti-cardiolipin ELISA. All samples of CD40/LMP1+//CD40-/- and control mice were clearly negative for anti-cardiolipin antibodies (Figure 3.11).

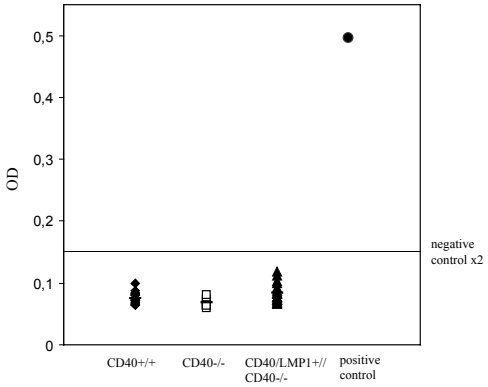

Figure 3.11. CD40/LMP1+//CD40-/- mice do not have anti-cardiolipin antibodies
Serum from un-immunized 2-12 months old mice (7-15 per group) were screened for anti-cardiolipin antibodies by ELISA. Data are means of duplicate wells; all values shown are sera tested together in one assay. The OD of the negative control serum was set two times to define positive and negative tests, according to manufacturer's protocol.

3.1.10 LMP1 signaling induces cytokine-independent class switch recombination

Since we observed an increase of class switched antibodies in CD40/LMP1//CD40-/- compared to CD40+/+ mice, we wished to determine whether this could be due to signaling outcome differences of LMP1 and CD40. It has been reported previously that LMP1 is able to induce class switch recombination in a Burkitt-Lymphoma cell line *in vitro* (He *et al.*, 2003).
To test whether the LMP1 signaling domain is able to induce class switch recombination independent of cytokines in primary B cells, isolated splenic B cells were labeled with CFSE and cultured in the presence of agonistic CD40 antibody (anti-CD40), IL-4, anti-CD40 plus IL-4, or without any stimuli, and stained for surface IgG1 at day 5 (Figure 3.12A). In cultures of CD40/LMP1+//CD40-/- B cells, a distinct fraction of IgG1 class-switched cells could already be detected upon stimulation with anti-CD40 only. The latter subset was absent in cultures of anti-CD40 stimulated wild type B cells and appeared only after co-stimulation with anti-CD40 and IL-4. The CFSE labeling showed that CSR in CD40/LMP1+//CD40-/- B cells did not correlate with a higher proliferation rate of these cells. Staining with anti-CD40 antibody indicated that the endogenous CD40 in wt B cells was expressed at similar levels as CD40/LMP1 upon activation (Figure 3.12B). The ability to induce cytokine-independet CSR was restricted to IgG1, since no IgA, IgG2a and IgG2b positive cells could be detected after anti-CD40 stimulation (data not shown).
By mixed B cell culture experiments we further elucidated whether the CSR of anti-CD40 stimulated CD40/LMP1+ B cells was mediated by an intrinsic effect of LMP1 signaling or by an autocrine mechanism of elevated cytokine release by these cells. Wild type CD40+/+ B cells expressing the Ly5.1 leukocyte marker were cultured together with CD40/LMP1+//CD40-/- B cells expressing Ly5.2 instead of Ly5.1, therefore distinguishable by a specific anti-Ly5.2 antibody. Stimulation with anti-CD40 antibody induced CSR in CD40/LMP1+//CD40-/- but not in CD40+/+ B cells, indicating a unique feature of the LMP1 signaling domain to induce cytokine-independent CSR.

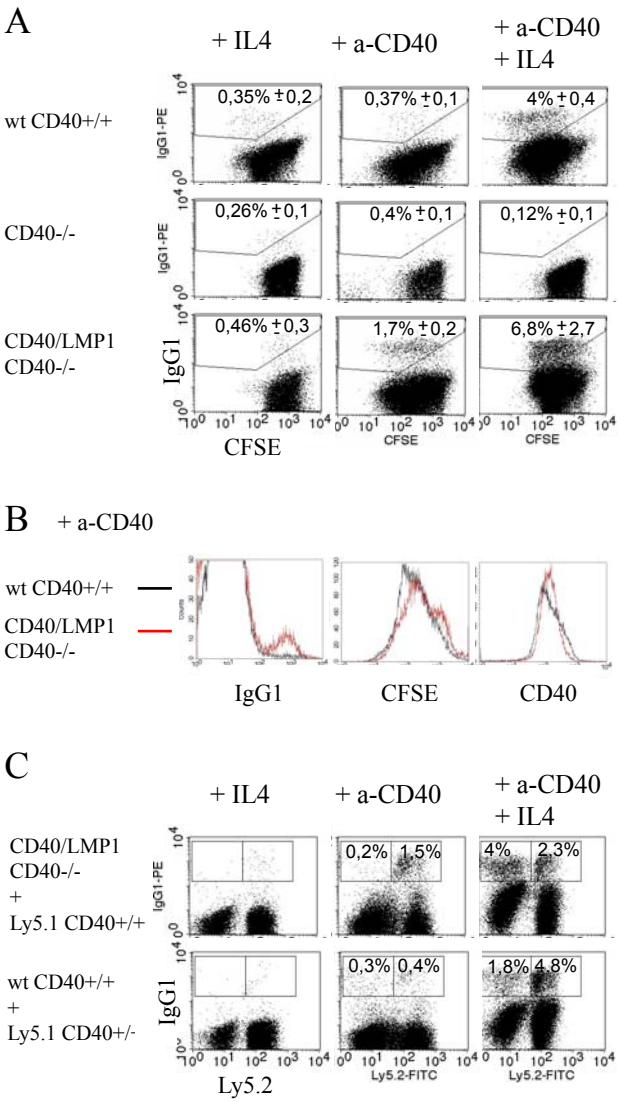

Figure 3.12. LMP1 signaling induces cytokine-independent class switch recombination to IgG1
(A) CD43- and IgG1-depleted splenic B cells were labeled with CFSE and cultured in the presence of the indicated stimuli for five days. CD40/LMP1+//CD40-/- B cells showed CSR to IgG1 after stimulation with agonistic anti-CD40 antibody (a-CD40) only, whereas in wt CD40+/+ B cells CSR was dependent on CD40 and IL4. CD40-/- B cells were added in the study as a negative control. Cells are gated on living (PI^-) cells. Numbers indicate means of percentages of $IgG1^+$ cells of three independent experiments.
(B) After 5 days of culture in the presence of anti-CD40 antibody (a-CD40), CD40/LMP1+//CD40-/- (red line) but not wt CD40+/+ B cells (black line) showed a certain fraction of IgG1 positive cells, although they had comparable division rates (visualized by CFSE labeling), and CD40 or CD40/LMP1 expression levels, respectively.
(C) Mixed B cell cultures of CD40+/+ B cells expressing the leukocyte marker Ly5.1 with Ly5.2-positive CD40/LMP1+//CD40-/- and CD40+/+ B cells, respectively. After 5 days of culture with anti-CD40 antibody, $Ly5.2^+$ CD40/LMP1+//CD40-/- , but not $Ly5.1^+$ CD40+/+ B cells showed CSR to IgG1. This indicates that CSR of CD40/LMP1+//CD40-/- B cells reflects an intrinsic effect of the LMP1 signaling domain and is not mediated by an increased release of cytokines. Numbers indicate means of percentages of $IgG1^+$ cells of two independent experiments.

3.2 The influence of constitutive active CD40 signaling on germinal center B cells *in vivo*

We could show that CD40L regulated LMP1 signaling could substitute for CD40 in GC formation, whereas constitutive CD40 signaling triggered by the transmembrane domain of LMP1 (LMP1/CD40) blocked germinal center formation in mice as it has been shown for LMP1 (Panagopoulos *et al.*, 2004; Rose *et al.*, 1980; Uchida *et al.*, 1999) (Hömig, 2005). However, LMP1 expression could be detected in GC B cells of healthy human EBV carriers (Babcock *et al.*, 2000). Therefore, it was tempting to speculate that the constitutive activity of either LMP1 or CD40 inhibited the cells to enter the GC, but that it did not interfere with already established GC and even help the cells to survive there. To test this hypothesis, we crossed LMP1/CD40^{flSTOP} mice to Cγ1-cre mice to induce LMP1/CD40 expression in GC B cells (Figure 3.13). The LMP1/CD40^{flSTOP} mouse strain was generated with the same targeting strategy like the CD40/LMP1^{flSTOP} mouse strain and described elsewhere (see Figure 1; Hömig, 2005).

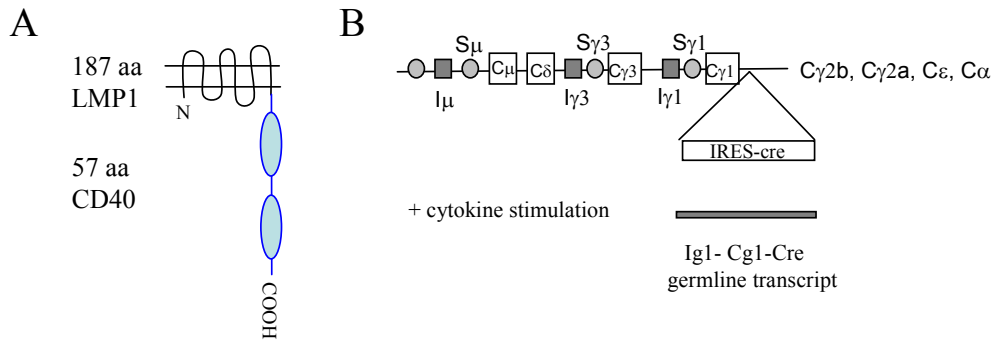

Figure 3.13. LMP1/CD40//Cγ1-Cre mice
(A) Schematic representation of the chimeric protein LMP1/CD40. The N-terminal 187 amino acids (aa) of LMP1 (transmembrane domain) were fused to the C-terminal 57 aa of CD40 (cytoplasmic domain).
(B) Insertion of the cre-gene with a downstream internal ribosome entry site (IRES) into the Cγ1 locus. The figure shows the wild type immunoglobulin (Ig) heavy chain locus with the I exons and its promoters (I), the switch sequences (S) and the constant regions (C) of the indicated Ig isotypes. The IRES-cre DNA cassette was inserted into the 3' region of the Cγ1 locus between the last membrane-coding exon and its polyadenylation site. Upon cytokine stimulation in vitro (LPS+IL4) or immunization in vivo, the bicistronic mRNA consisting of the Cγ1 and the cre transcript is expressed from the Cγ1 locus under control of the Iγ1 promoter.

Cγ1-cre mice were generated by inserting the cre-gene with a downstream internal ribosome entry site (IRES) into the Cγ1 locus (Casola *et al.*, 2006). Thus, expression of the Cre recombinase is induced by onset of germline Cγ1 transcription. Germline Cγ1 transcription

precedes class switch recombination, which is induced in activated B cells in response to cytokine stimulation and cross-linking of the CD40 receptor. Since this process mainly takes place in the GC, Cγ1-Cre mice predominantly show Cre-recombinase activity in GC B cells.

3.2.1 Constitutive CD40 signaling is not compatible with the GC reaction

To induce LMP1/CD40 expression in GC B cells we immunized LMP1/CD40//Cγ1-Cre mice with NP-CGG. Neither 7 nor 14 days after immunization GC could be detected in LMP1/CD40//Cγ1-Cre mice, whereas the wild type controls with only the Cγ1-Cre allele inserted (wt//Cγ1-Cre) showed GC formation (data not shown).

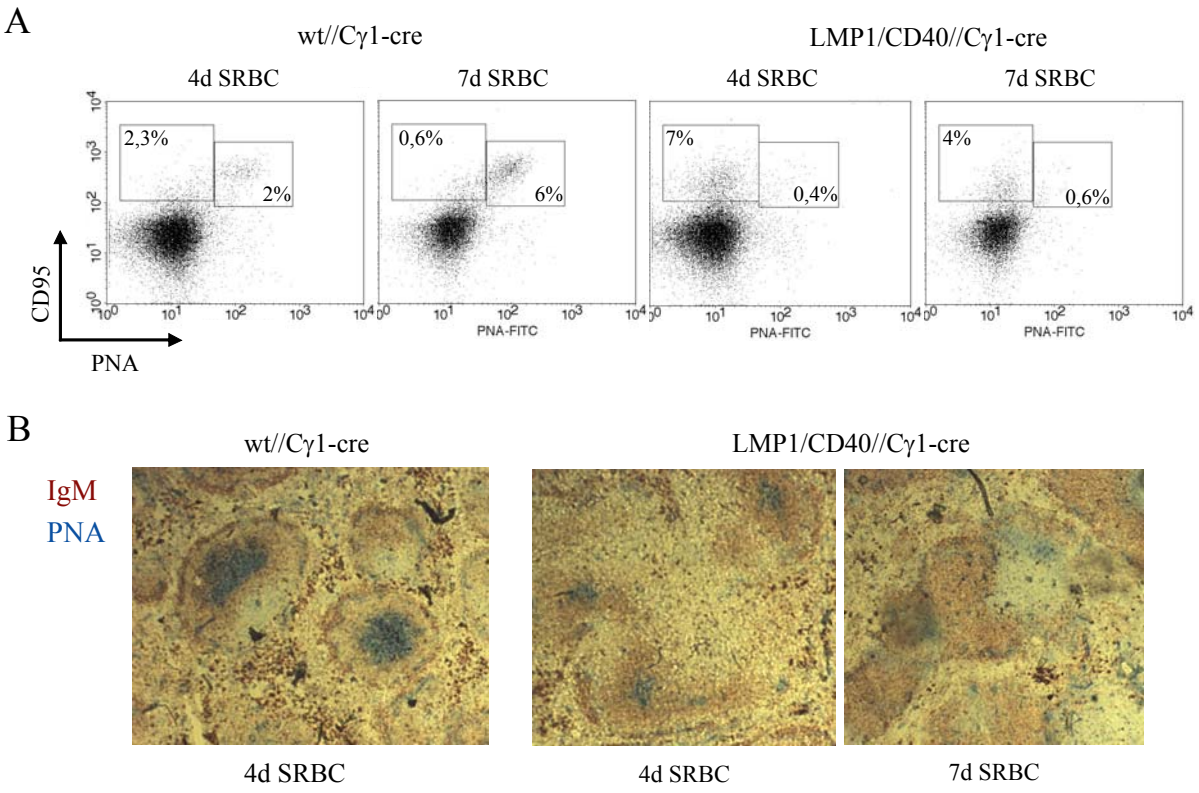

Figure 3.14. Constitutive CD40 signaling is not compatible with the GC reaction
(A) Flow cytometry to identify germinal center B cells ($CD95^+PNA^{high}$) in the spleens of wildtype (wt//Cγ1-cre) and LMP1/CD40//Cγ1-cre mice. Splenic cells were isolated and analyzed at day 4 and 7 after immunization with $2x10^8$ sheep red blood cells (SRBC). Cells are gated on $B220^+$. Results are representative for 2 mice analyzed per group. LMP1/CD40//Cγ1-cre mice did not show $CD95^+PNA^+$ GC B cells, but a certain fraction of $CD95^+PNA^-$ B cells, most likely reflecting the LMP1/CD40 expressing B cells.
(B) Histological analysis of germinal centers in the spleen after immunization with $2x10^8$ sheep red blood cells (SRBC). Cryosections were stained with anti-IgM specific for B cells (red) and PNA specific for germinal center B cells (blue). Original magnification, x 50.

Next we immunized the mice with sheep red blood cells (SRBC), which are known to induce a stronger and faster response than NP-CGG. Mice were injected i.p. with 2 x 10^8 SRBC and analyzed for GC formation after 4 and 7 days (Figure 3.14). Unlike in controls, the fraction of PNA-binding GC B cells detected by FACS was very low in LMP1/CD40//Cγ1-cre mice. However, by immunohistochemistry some lightly stained PNA positive areas could be visualized in the spleen, suggesting that GC had been formed in LMP1/CD40//Cγ1-cre mice by undeleted cells, but were disrupted as soon as LMP1/CD40 was expressed. By FACS, an aberrant population of $CD95^+$ B cells could be detected in the immunized LMP1/CD40//Cγ1-cre mice, which are very likely the LMP1/ CD40 expressing cells, since CD95 expression has been shown to be a hallmark of LMP1/CD40 expressing B cells in LMP1/CD40//CD19-cre mice (Hömig, 2005).

These data indicate that constitutive CD40 signaling is not compatible with the GC reaction. However, since in Cγ1-Cre mice the Cre-recombinase is induced very early during the GC reaction or even already in activated B blasts, we cannot exclude that LMP1/CD40 expression has opposite effects if activated later during the GC reaction.

3.2.2 LMP1/CD40//Cγ1-cre mice show an age dependent increase of splenic weight and accumulation of aberrant B cells

Since LMP1/CD40//CD19-cre mice in which LMP1/CD40 is expressed in all B cells from a pre/pro B cell stage on showed a splenomegaly and an increase of B and T cells at an age of 8 weeks, we wanted to investigate whether LMP1/CD40//Cγ1-cre mice also accumulate lymphocytes within time. Therefore, we immunized 8 weeks old LMP1/CD40//Cγ1-cre and wt//Cγ1-Cre mice with NP-CGG and analyzed these and un-immunized control mice of both groups after 8 and 16 weeks. 16 weeks old LMP1/CD40//Cγ1-cre mice had in average the same splenic weight like the wt//Cγ1-Cre controls, whereas 24 weeks old LMP1/CD40//Cγ1-cre mice showed already a 2-4 fold increase in splenic weight compared to the wt//Cγ1-Cre controls. 10 and 12 months old LMP1/CD40//Cγ1-cre mice showed a further increase of splenic weight (Figure 3.15A). FACS analyses revealed the accumulation of aberrant B cells in the spleens of LMP1/CD40//Cγ1-cre mice. Although shortly after immunization a fraction of $CD95^+$ B cells appeared in LMP1/CD40//Cγ1-cre mice, the aberrant B cell population in older mice did not express CD95 (data not shown), but showed a distinct surface expression pattern of $B220^{low}$ $CD5^+$ $CD43^+$ $CD23^-$ $CD21^{low}$ IgD^{low} and IgM^+ (Figure 3.15B and data not shown). This expression pattern is reminiscent of B1 cells. However, we cannot distinguish

whether these accumulated B cells are really B1 cells, or activated B2 cells which have changed their phenotype upon LMP1/CD40 expression.

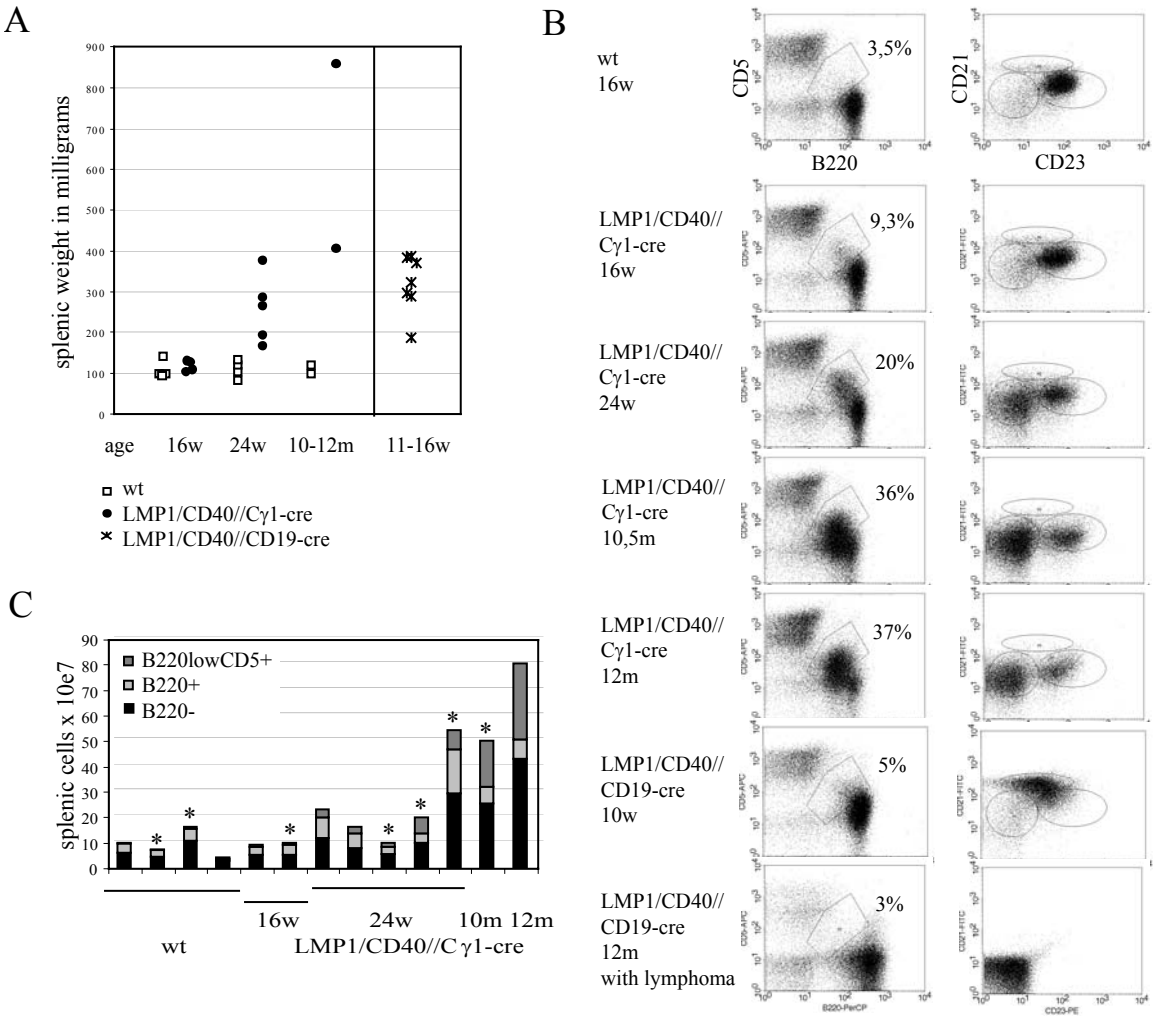

Figure 3.15. LMP1/CD40//Cγ1-cre mice show an increase of splenic weight and an accumulation of an aberrant B cell population with age

(A) Spleen weights in milligrams of wild type (wt) and LMP1/CD40//Cγ1-cre mice of the indicated ages, and of 11-16 weeks old LMP1/CD40//CD19-cre mice are plotted in the graph. LMP1/CD40//Cγ1-cre mice developed a splenomegaly, which increased with age. Compared to wild type mice, 24 weeks old LMP1/CD40//Cγ1-cre mice showed the same 2-4 fold increase of splenic weight as 11-16 weeks old LMP1/CD40//CD19-cre mice, which express the transgene from an early B cell developmental stage on.

(B) Flow cytometric analyses for CD5 and B220 expression on lymphocyte gated splenic cells, and CD21 and CD23 expression on $B220^+$ gated splenic B cells. Numbers indicate the percentages of $CD5^+B220^{low}$ cells. Representative blots of wild type (wt), LMP1/CD40//Cγ1-cre mice of indicated ages and LMP1/CD40//CD19-cre mice, 10 weeks and 12 months old, the latter one bearing lymphoma, are shown.

(C) Absolute numbers of $B220^{low}CD5^+$ and $B220^+CD5^-$ B cells and the residual $B220^-$ cells in the spleens of LMP1/CD40//Cγ1-cre mice and age matched wild type controls (wt). Asterisks indicate preceding immunization at an age of 8 weeks.

Strikingly, the B cell expansion in LMP1/CD40//Cγ1-cre mice was independent of preceding immunization. Since the mice were not kept in totally pathogen-free conditions, they were most likely exposed to natural pathogens, leading to the induction of LMP1/CD40 expression upon immune responses.

3.2.3 The B cell accumulation in the spleens of LMP1/CD40//Cγ1-cre mice can be classified as B cell lymphoma by histology

LMP1/CD40//CD19-cre mice between an age of 12 and 19 months developed lymphoma with a very high incidence. Interestingly, the malignant B cells in those mice showed a different cell surface expression phenotype than the accumulated population in the LMP1/CD40//Cγ1-cre mice. Thus, lymphoma cells in LMP1/CD40//CD19-cre mice were $CD5^-$, showed either high or no B220 expression, and a total loss of CD21 and CD23 expression (Figure 3.15B).

To investigate whether the expansion of the $B220^{low}CD5^+$ B cell population in LMP1/CD40//Cγ1-cre mice could be classified as B cell lymphoma, histopathological analyses were performed by the pathologist Dr. Quintanilla-Martinez (Institute of Pathology, GSF Neuherberg). Parts of the spleens were fixed in formalin and embedded in paraffin to perform hematoxylin and eosin (H/E) and immunohistochemical staining. The spleens of LMP1/CD40//Cγ1-cre mice showed a subtle nodular infiltrate, where the follicles were almost back to back with reduced red pulp in between (Figure 3.16A). The cellular component was relatively homogenous and composed by small mature-looking lymphoid cells. The cells within the nodules were B220 positive (Figure 3.16B). As already observed by FACS analysis, the vast majority of the cells was weak B220 positive. Moreover, cells of this population were larger than normal reactive B cells and seemed to have abundant cytoplasm. The second population was composed of small lymphocytes with a strong, crisp staining for B220, which seemed to correspond to the normal residual B cell population. Immunohistochemistry with CD3 showed that the normal T cell zone was disrupted (Figure 3.16C).

Splenic sections of seven LMP1/CD40//Cγ1-cre mice (five of them 24 weeks, one 10 and one 12 months old) were analyzed and all cases showed the same signs of neoplastic disease in morphology and immunophenotype, although the older mice showed a more advanced stage. Based on these histological data it is very likely that these mice developed lymphomas.

The surface expression phenotype of the aberrant B cell population in the LMP1/CD40//Cγ1-cre mice corresponded to the phenotype described for mantle cell lymphoma in humans

(Bertoni *et al.*, 2004). A hallmark of mantle cell lymphoma is the t(11;14)(q13;q32) translocation, which juxtaposes the cyclin D1 gene to the immunoglobulin heavy chain junctional region, resulting in the overexpression of cyclin D1. Therefore, we analyzed whether LMP1/CD40 expression induced an upregulation of cyclin D1 in these B cells. However, an anti-cyclin D1 antibody did not stain B cells in splenic sections of LMP1/CD40//Cγ1-cre mice (Figure 3.16D).

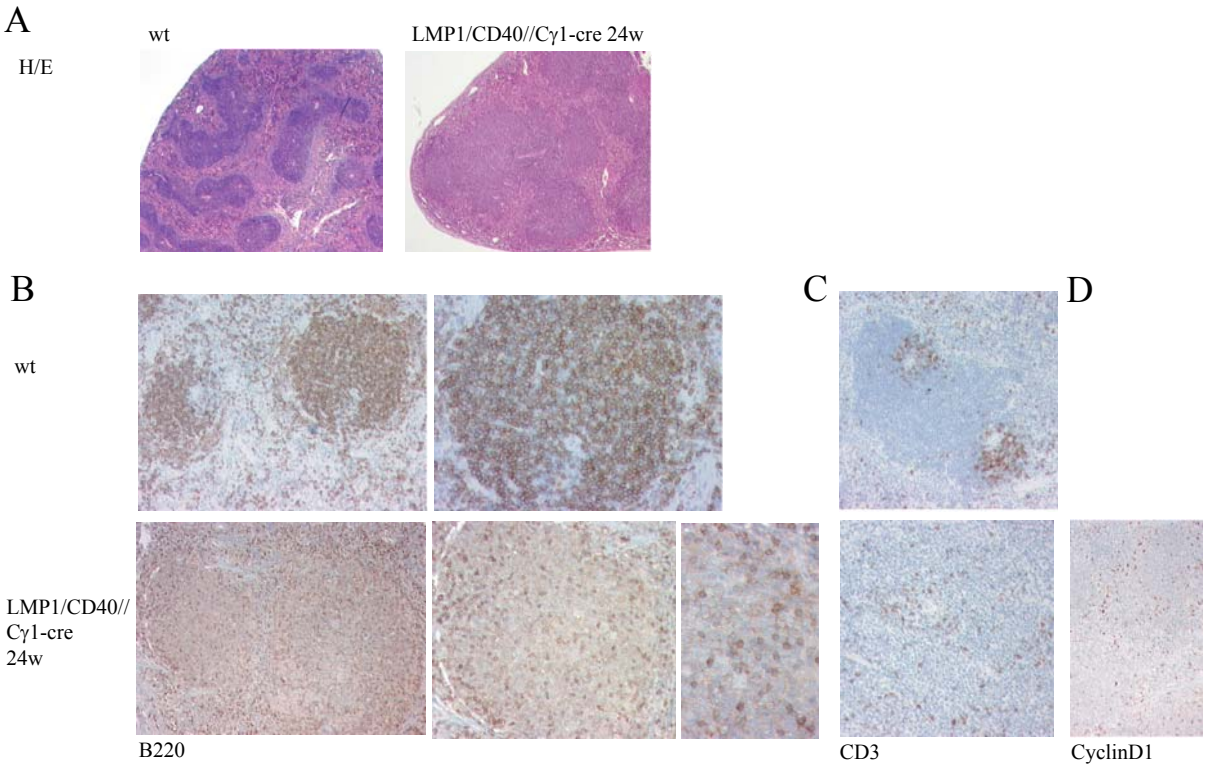

Figure 3.16. Representative histological analyses of one 24 weeks old LMP1/CD40//Cγ1-cre and one wild type control mouse
(A) Low magnification of hematoxylin and eosin (H/E) stained spleens. The LMP1/CD40//Cγ1-cre spleen shows nodular infiltrates, with the follicles almost back to back replacing the red pulp in between.
(B) Immunohistochemistry using an antibody specific for B220 (brown) to detect B cells. In the wildtype, the B cell follicles are brightly stained for B220 and separated by the red pulp, whereas in the LMP1/CD40//Cγ1-cre, the red pulp is infiltrated by $B220^{+}$ cells. The higher magnifications show the characteristic double positivity for B220 in the LMP1/CD40//Cγ1-cre spleen, with the vast majority of cells showing a weak staining for B220.
(C) Immunohistochemistry using an antibody specific for CD3 (brown) to detect T cells. In the wild type, organized T cell areas surround the central arterioles of follicles, whereas in the LMP1/CD40//Cγ1-cre, T cells are dispersed within the infiltrate.
(D) Immunohistochemistry using an antibody specific for cyclin D1 (brown). B cells in the LMP1/CD40//Cγ1-cre mice are negative for cyclin D1; the positively stained cells are endothelial cells and histiocytes.

3.2.4 The Ig genes in the aberrant B cell population are not somatically mutated

To analyze whether the aberrant B cell population originated from GC B cells, the $CD5^{+}B220^{low}$ and the $CD5^{-}B220^{+}$ populations of one LMP1/CD40//Cγ1-cre mouse showing signs of disease (12 months old) were analyzed for SHM in their Ig genes. Cells were sorted by FACS to isolate DNA and to amplify the JH4 region of the Ig genes by PCR. 23 clones of each population were sequenced and analyzed for SHM, but none of them showed mutated sequences in the JH4 genes (data not shown). This indicates that the cells did not originate from GC B cells, and is in line with our data showing impaired GC formation in these mice. However, since SHM starts not earlier than 8 days after immunization (Jacob *et al.*, 1993), it is still possible that the aberrant B cell population in LMP1/CD40//Cγ1-cre mice originated from early GC B cells.

Analyzes of the Ig rearrangements revealed that four clones were preferentially amplified (up to five times in 23 sequences), indicating an oligoclonal origin of the expanded B cell population.

3.3 Modeling of EBV and the germinal center reaction *in vitro*

The observation that LMP1 blocks GC formation in transgenic mice is in contrast to the proposed model of Thorley-Lawson and colleagues that EBV infected B cells have to pass the GC reaction to establish latency in memory B cells. Since we could show that LMP1 signaling not only has properties to activate B cells, but is able to initiate differentiation processes like CSR, we wanted to test whether EBV by itself is able to convert a naïve B cell into a B cell with a memory B cell like phenotype. Moreover, we analyzed the influence of LMP1, LMP2A and EBNA1 independent of EBNA2 on a human B cell line, recapitulating the proposed EBV expression program in GC B cells.

We made use of the EREB2-5 cell line, which is a conditional LCL, where the EBV program can be switched on and off via an estrogen regulated EBNA2 (Kempkes *et al.*, 1995b). Upon estrogen withdrawal, EBNA2 is retained in the cytoplasm and not able to transactivate the other EBV genes, leading to cell cycle arrest and death of the cells within six days.

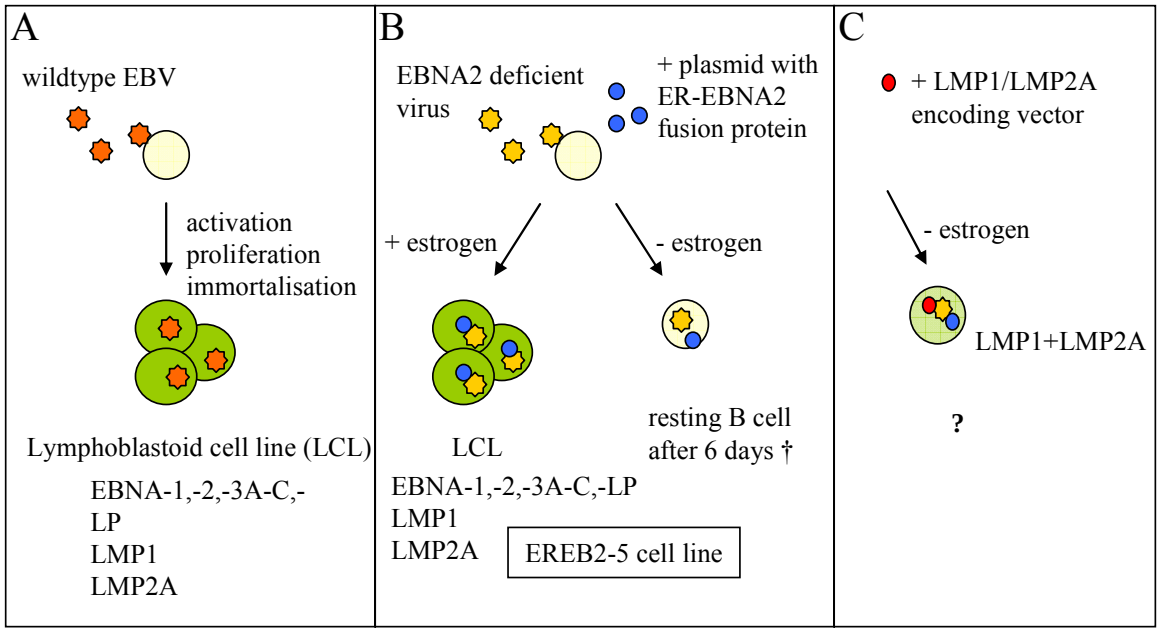

Figure 3.17. Schematic representation of the generation of lymphoblastoid cell lines (LCLs) and the EREB2-5 cell system
(A) The transfection with wildtype EBV leads to the transformation and immortalisation of primary B cells. These lymphoblastoid cell lines (LCLs) express the so called latency III program, where all nine latent EBV proteins EBNA1, EBNA2, EBNA3A-C, EBNA-LP, LMP1, LMP2A and B are expressed.
(B) The conditionally transformed cell line EREB2-5 was established by co-infection with two virions, the so-called P3HR1 virus genome, which has a deletion removing the EBNA2 gene and a mini-EBV plasmid carrying a EBNA2 gene fused to the hormone binding domain of the estrogen receptor gene (ER-EBNA2). In the presence of estrogen the chimeric ER-EBNA2 protein substitutes for the wild-type EBNA2, leading to the transactivation of the latent EBV genes and to the immortalization of B cells. Upon withdrawal of estrogen, EBNA2 function is inactivated and consequently immortalized B cells stop to proliferate and adopt a phenotype similar to EBV-negative, resting B cells, and die within 6 days.
(C) The EREB2-5 cell line was transfected with vectors coding for EBNA1, LMP1 and LMP2A to express these proteins independent of the EBV latency III program.

3.3.1 EREB2-5 cells gain a memory B cell phenotype upon EBNA2 inactivation

The EREB2-5 cell line was established by infecting B cells isolated from umbilical cord blood cells, therefore originating from a naïve B cell (Kempkes *et al.*, 1995b). In line with this, EREB2-5 cells appeared to be negative for the memory B cell surface marker CD27 and positive for IgM. However, upon estrogen withdrawal, EREB2-5 cells up-regulated CD27 (Figure 3.18). To rule out that the EREB2-5 cell line did not originate from naïve but from memory B cells, the VH region of the immunoglobulin locus was amplified and sequenced. The sequence clearly showed a clonal origin without any sign of somatic hypermutation, indicating that the EREB2-5 cell line originated from a naïve, but not a memory B cell (data not shown).

Next, the cells were tested for their ability to gain other memory B cell specific fates upon EBNA2 inactivation. Since memory B cells mainly show isotype switched immunoglobulin, the cells were tested for CSR by RT-PCR for the mature Ig transcripts. EREB2-5 cells without EBNA2 activity showed an increase of mature IgA transcripts (Figure 3.22).

These data suggest that the EBV latency III program keeps the cells in an activated status, and as soon as EBNA2 is down-regulated by a mechanism still unknown, the cells gain the phenotype of resting memory B cells ($CD27^+$). Since EBNA2 transactivates all the other latent EBV genes, its inactivation should consequently lead to a down-regulation of the other viral proteins. Nevertheless, some viral proteins may stay stable for a certain time and act EBNA2 independent on a cell. To study the influence of LMP1, LMP2A and EBNA1 independent from EBNA2 and other EBV proteins, we transfected EREB2-5 cells with expression vectors coding for EBNA1, LMP1 and/or LMP2A (Figure 3.17C).

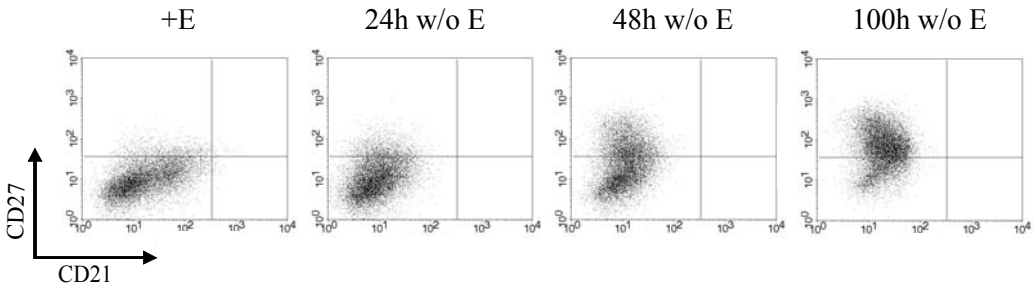

Figure 3.18. EREB2-5 cells up-regulate the memory B cell marker CD27 upon EBNA2 inactivation
Cells were washed three times with estrogen free medium and then cultured in the absence of estrogen for several hours and analyzed for the expression of CD27 by flow cytometry. The cells were gated on PI- living cells and co-stained for CD21 as a marker for EBNA2 activity. Cells cultured in the presence of estrogen (+E) showed CD21, but no CD27 surface expression. Upon estrogen withdrawal, cells became $CD21^{low}$ and positive for the memory B cell marker CD27.

3.3.2 Generation of the expression vectors coding for LMP1, LMP2A and EBNA1

The coding sequences from LMP1 and LMP2A were cloned into the expression vector pRT-1 at each site of a tetracycline dependent bidirectional promoter (P_{tet}bi-1), which allows simultaneous expression of both genes (Bornkamm *et al.*, 2005). The pRT-1-vector contains the EBNA1 coding sequence and the EBV origin of replication (oriP) for episomal maintenance of the plasmid in the cell.

Beside the vector coding for LMP1 and LMP2A, three other expression vectors were generated as controls, coding for: (a) LMP1 and the enhanced green fluorescent protein (EGFP) (Figure 3.19B); (b) LMP2A and the truncated form of the nerval growth factor

receptor without cytoplasmic tail (NGFR) (Figure 3.19C); and (c) NGFR and the luciferase gene (luc) (Figure 3.19D). EGFP and NGFR could be used as markers to detect LMP1 and LMP2A expression, respectively.

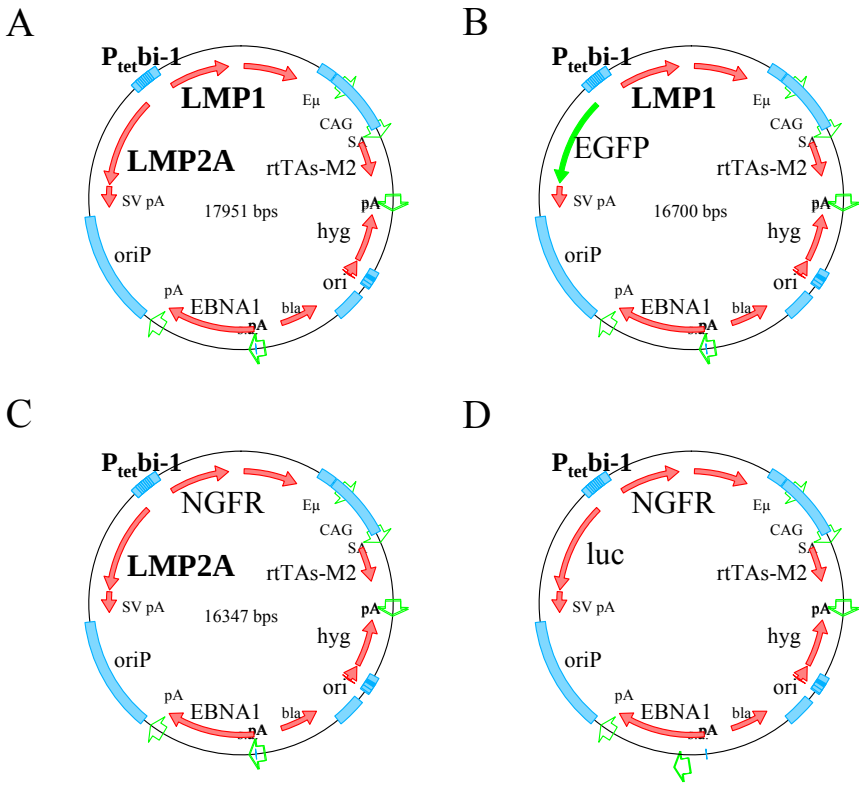

Figure 3.19. pRT-1 expression vectors
(A) The coding sequences from LMP1 and LMP2A were cloned at each site of the tetracycline dependent bidirectional promoter (P_{tet}bi-1) of the pRT-1 vector. EREB2-5 cells were transfected with this vector to generate the EREB-LMP1/2A cell line, which is able to express LMP1 and LMP2A independent from EBNA2.
(B) LMP1 and the EGFP coding sequence were cloned at each site of the P_{tet}bi-1 promoter. EREB2-5 cells were transfected with this vector to generate the EREB-LMP1 cell line, which is able to express LMP1 independent from EBNA2.
(C) LMP2A and the truncated form of the nerval growth factor receptor without cytoplasmic tail (NGFR) were cloned at each site of the P_{tet}bi-1 promoter. EREB2-5 cells were transfected with this vector to generate the EREB-LMP2A cell line, which is able to express LMP2A independent from EBNA2.
(D) As a control without LMP1 and LMP2A, the sequences for the truncated NGFR and the luciferase gene (luc) were cloned at each site of the P_{tet}bi-1 promoter. EREB2-5 cells were transfected with this vector to generate the EREB-control cell line.
P_{tet}bi-1, tetracycline dependent bidirectional promoter; rtTA2^{s}-M2, reverse tetracycline controlled transcriptional activator (doxycycline sensitive), transcribed from a promoter/enhancer consisting of the mouse heavy chain intron enhancer (Eμ) and the chicken β-actin promoter (CAGp); hyg, the hygromycin phosphotransferase gene; SVp, the SV40 early promoter; ori, the bacterial origin of replication; bla, β-lactamase; oriP, EBV-episomal origin of replication; pA, polyadenylation site; bps, base pairs.

All vectors were transfected into EREB2-5 cells to generate four different cell lines, expressing either EBNA1 alone or in combination with LMP1, LMP2A or both LMP1 and LMP2A, called EREB-control, EREB-LMP1, EREB-LMP2A and EREB-LMP1/2A, respectively.

3.3.3 EBNA2 independent expression of LMP1 and LMP2A in a human B cell line

After stable transfection of the expression plasmids into EREB2-5 cells, they were examined for the expression of LMP1 and LMP2A. For the EREB-LMP1/2A cell line a Northern blot analysis was performed (Figure 3.20A). The cells were washed three times in estrogen free medium and then cultured for three days in the presence and absence of estrogen and doxycycline (a derivate of tetracycline to induce expression of LMP1 and LMP2A). The treatment with doxycycline induced a very strong expression of LMP1 and LMP2A mRNA expression in EREB-LMP1/2A cells, independent of the presence of estrogen. Cells cultured with neither estrogen nor doxycycline showed LMP1 and LMP2A mRNA expression as well. This was not surprising since the expression vector did not contain the silencer of the tetracycline dependent promoter, and a certain leakiness of the promoter in the absence of tetracycline or doxycycline was already known (Bornkamm *et al.*, 2005). Western blot analyses approved the estrogen and doxycycline independent expression of LMP1 in EREB-LMP1/2A cells (Figure 3.20B). Whereas in the EREB-control cell line 5 days after estrogen withdrawal no LMP1 protein was detectable, it was clearly detectable in EREB-LMP1/2A cells grown without estrogen and even without doxycycline. EREB-LMP1/2A and EREB-control cells grown in the presence of estrogen showed approximately the same LMP1 protein levels. Since we observed that EREB-LMP1/2A cells survived better without than in the presence of doxycycline (Figure 3.24A), we concluded that the induced levels of LMP1 and LMP2A were too high and therefore toxic for the cells. Hence, we decided to continue the studies without adding doxycycline to the culture medium.

In the EREB-LMP1 and EREB-LMP2A cell lines the expression of LMP1 and LMP2A was examined by Western blot and light cycler analyses, respectively (Figure 3.20C and D).

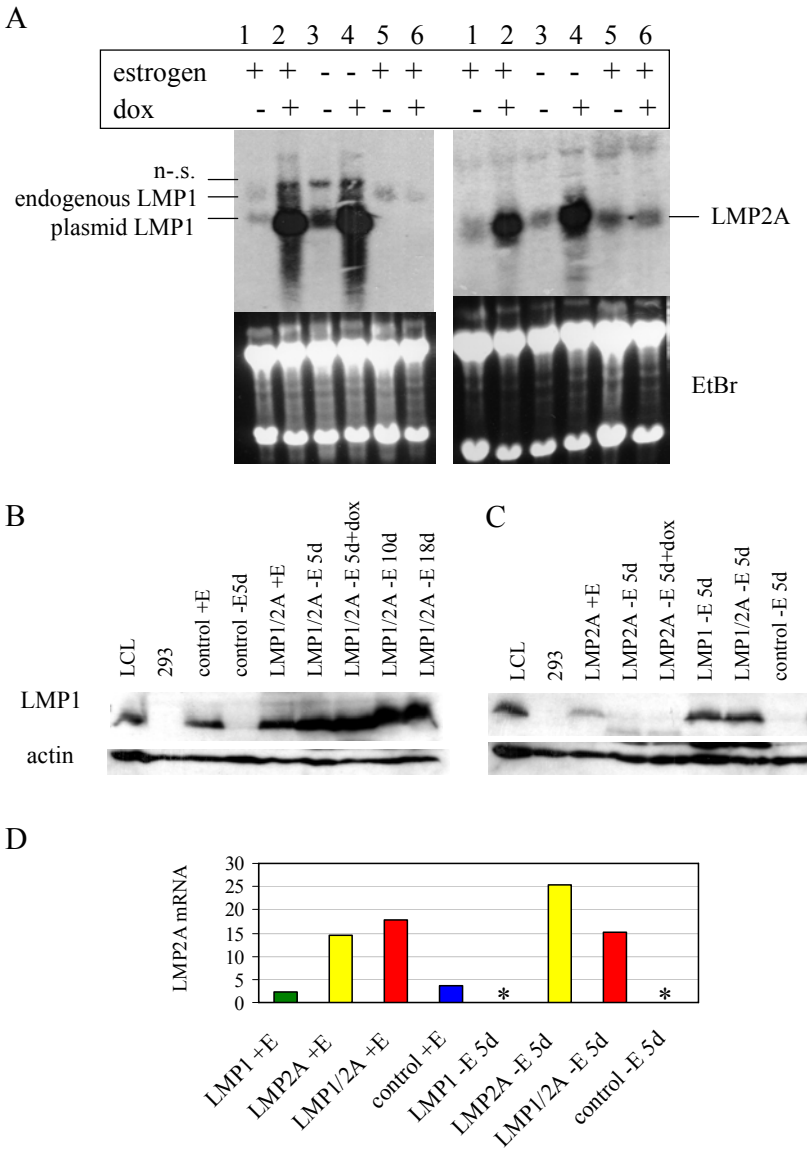

Figure 3.20. Estrogen independent expression of LMP1 and LMP2A
(A) Northern blot analysis to examine the mRNA expression of LMP1 and LMP2A in the EREB-LMP1/2A cell line. Lanes 1-4, EREB-LMP1/2A cell line cultured for three days in the presence and absence of estrogen and doxycycline (dox) as indicated; lanes 5-6, EREB-control cell line in the presence of estrogen with and without doxycycline as a negative control for the exogenous LMP1 and LMP2A expression from the vector. The mRNA of vector and endogenous LMP1 can be distinguished by size. n.s., non specific band. As a loading control, the ethidium bromide (EtBr) stained agarose gel is shown.
(B) Western blot analysis for LMP1 protein expression in the EREB-LMP1/2A and EREB-control cell lines. Cells were cultured in the presence of estrogen (+E) or without estrogen (-E) for 5, 10 and 18 days. A conventional LCL served as a positive and the 293 cell line as a negative control. To detect the 63 kDa LMP1 protein, the anti-LMP1 antibody CS1-4 was used. Equal protein loading was verified with an anti-actin antibody.
(C) Western blot analysis to compare the LMP1 protein expression levels in the different EREB-cell lines 5 days after estrogen withdrawal.
(D) Light Cycler analysis for LMP2A expression in the different EREB cell lines. The relative copy numbers of LMP2A mRNA normalized to c-abl are plotted. The graph shows the LMP2A mRNA expression in the presence of estrogen (+E) and 5 days after withdrawal of estrogen (-E 5d). ∗ no product detected.

3.3.4 EBNA2 independent LMP1 and LMP2A expression induces a GC B cell phenotype *in vitro*

The model of Thorley-Lawson, which states that EBV positive cells have to pass the germinal center to get access to the memory B cell compartment, is based on EBV gene expression studies in tonsillar B cells of healthy persons (Babcock *et al.*, 2000). They showed that only B cells with a naïve phenotype (IgD^+CD10^-) express the genes of the latency III program, whereas B cells with a GC phenotype of centroblasts ($CD10^+CD77^+$) and centrocytes ($CD10^+CD77^-$) express the more restricted latency II program, where only EBNA1 and the two membrane proteins LMP1 and LMP2A can be detected. We wanted to elucidate whether it could also be the vice versa effect, that not GC B cells express LMP1 and LMP2A, but that LMP1 and LMP2A expression in any B cell induces a GC B cell like phenotype. To this end we analyzed the EREB-LMP1/2A cell line cultured without estrogen for the expression of specific GC surface marker by flow cytometry. Five days after estrogen withdrawal, neither EREB-control nor EREB-LMP1/2A cells showed any GC surface marker expression. However, 15 days after EBNA2 inactivation, the GC B cell surface marker CD10, CD77 and CD38 were all up-regulated in comparison to EREB-control and EREB-LMP1/2A cells cultured in the presence of estrogen (Figure 3.21). EREB-LMP1/2A cells also showed an increase of cell size, reminiscent of blasts in the GC. Gating on large cells in the forward scatter histogram visualized an even pronounced up-regulation of GC B cell surface marker on these cells. EREB-control cells cultured without estrogen could not be included in this analysis since these cells did not survive more than six days, but did not show an increase of GC markers at any time point after estrogen withdrawal (data not shown).This indicates that LMP1 and LMP2A expression is able to induce a GC B cell surface marker phenotype *in vitro*.

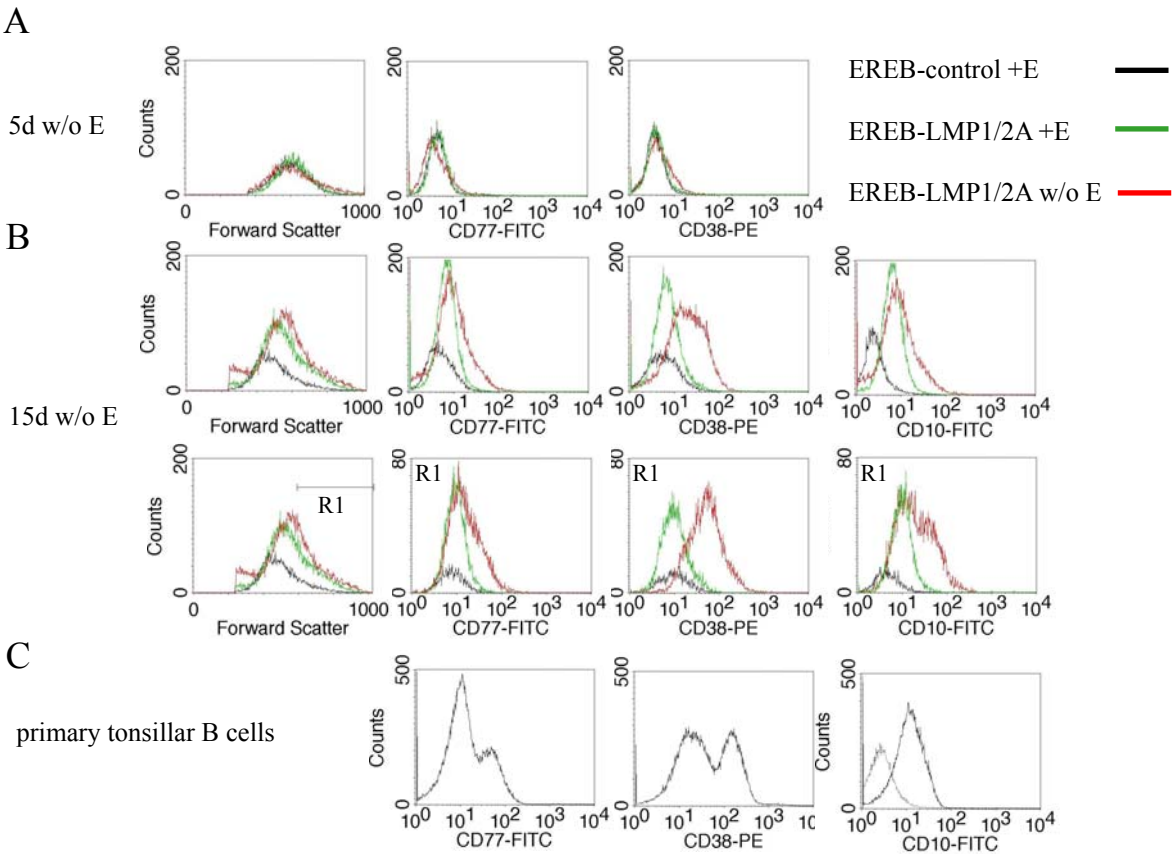

Figure 3.21. CD10, CD77 and CD38 expression in EREB-LMP1/2A cells
(A) and (B) EREB-LMP1/2A cells were washed three times with estrogen free medium and then cultured in the absence of estrogen for (A) 5 and (B) 15 days to perform flow cytometry. Cells size (forward scatter), CD77, CD38 and CD10 surface expression was analyzed and compared to EREB-control and EREB-LMP1/2A cells cultured in the presence of estrogen. 5 days after estrogen withdrawal, EREB-LMP1/2A cells did not show any change in cell size or CD77 and CD38 expression levels. After 15 days, an increase in size as well as in CD10, CD77 and CD38 expression could be observed. Cells gated on R1 to include only large cells showed a pronounced increase of GC marker. All cells were gated on PI^- living cells. EREB-control cells cultured without estrogen where not included since they did not survive more than 6 days.
(C) Human tonsils were prepared to analyze primary GC B cells for CD77, CD38 and CD10 expression. Cells are gated on $CD19^+$.

3.3.5 LMP1 induces expression of mature IgG transcripts, which is abrogated by co-expression of LMP2A

Since we could show that primary CD40/LMP1 expressing murine B cells induced cytokine-independent CSR, we assessed the ability of EREB-LMP1, EREB-LMP1/2A, EREB-LMP2A and EREB-control cell lines to induce class switching. The EREB cell lines in the presence of estrogen did not show any detectable mature IgG transcripts. Therefore, the cells were washed three times with estrogen free medium and cultured in the absence of estrogen to shut off the EBV program. On several days after estrogen withdrawal, cells were harvested, their RNA isolated for cDNA synthesis and RT-PCR for the mature Ig transcripts performed. In contrast

to EREB-control and EREB-LMP2A cells, EREB-LMP1 cells showed mature transcripts of all IgG and IgA isotypes (Figure 3.22 and Table 3.2). Strikingly, the transcription of mature class switched Ig declined again in EREB-cells co-expressing LMP1 and LMP2A, indicating that LMP2A counter-regulates the unique ability of LMP1 to induce CSR.

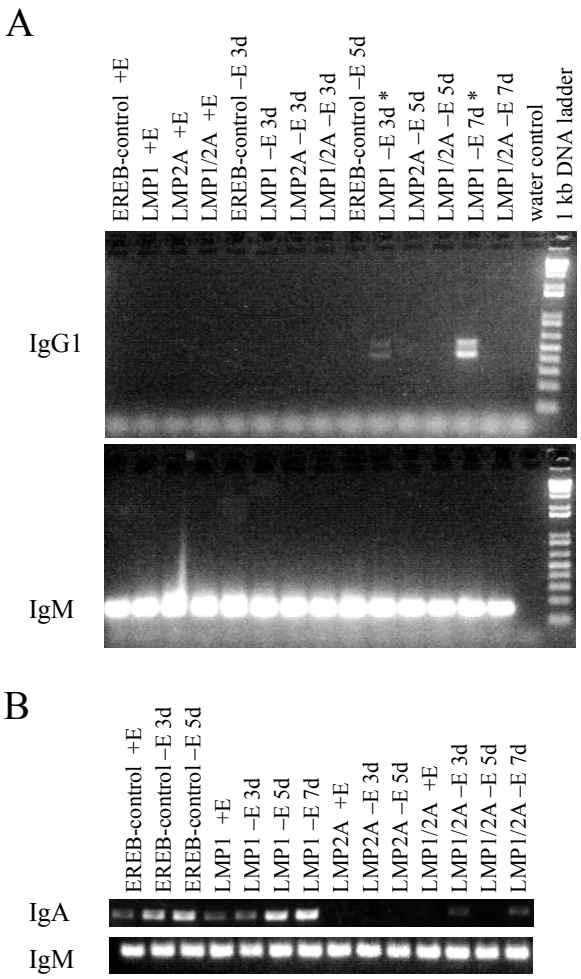

Figure 3.22. RT-PCR for mature IgG1 and IgA1 transcripts in EREB cells
EREB cell lines were cultured in the presence or absence of estrogen and examined for expression of the mature (A) IgG1 and (B) IgA transcripts using a 5' primer in the FR3 region of the variable domain and a 3' primer in the constant domain of the respective Ig isotype. The RT-PCR for mature IgM transcripts was plotted as a positive control.
(A) RT-PCR with 30 amplification cycles for the mature IgG1 transcript. Only EREB-LMP1 cells cultured without estrogen showed a product, indicated by asterisks.
(B) RT-PCR with 40 amplification cycles for the mature IgA1 transcript. Products could be detected in EREB-control and EREB-LMP1 cells, which amount increased upon estrogen withdrawal, but not in EREB-LMP2A and only slightly in EREB-LMP1/2A cells.

Table 3.2. RT-PCR for mature Ig transcripts

	IgA	IgG1	IgG2	IgG3
EREB2-5 +E	+/-	-	-	-
EREB2-5 w/o E	++	-	-	-
pEBNA-LMP1 +E	+/-	+/-	-	-
pEBNA-LMP1 w/o E	++	++	+	++
pEBNA-LMP2A +E	-	-	-	-
pEBNA-LMP2A w/o E	-	-	-	-
pEBNA-LMP1+2A +E	-	-	-	-
pEBNA-LMP1+2A w/o E	+/-	-	-	-

EREB cell lines were cultured in the presence or absence of estrogen (5 days) and examined for expression of the mature IgA, IgG1, IgG2 and IgG3 transcripts by RT-PCR with 30 amplification cycles using a 5' primer in the FR3 region of the variable domain and a 3' primer in the constant domain of the respective Ig isotype. -, no PCR product detected; +/-, PCR product as a slight band detectable; +/+, PCR product as a strong band detectable;

3.3.6 LMP2A down modulates AID protein expression

AID is the key player in the processes of SHM and CSR. Therefore, we analyzed the EREB-cell lines for AID protein expression by Western blotting. In the presence of estrogen, all cell lines showed the same levels of AID expression (Figure 3.23A). Five days after estrogen withdrawal, the EREB-LMP1 cell line showed an increase of AID expression, reflecting the ability of LMP1 to induce AID expression (He *et al.*, 2003). Strikingly, this effect was abrogated by co-expression of LMP2A in the EREB-LMP1/2A cell line. It has been shown previously that EBNA2 mediates AID down-regulation, thus leading to an AID up-regulation in EREB2-5 cells upon estrogen withdrawal (Tobollik *et al.*, 2006). We show now that not only EBNA2 but also LMP2A down-modulates AID expression (Figure 3.23B), suggesting that LMP2A counter-regulates AID expression induced by LMP1. Thus, the abrogation of LMP1 induced mature IgG transcription by LMP2A co-expression could be the result of the AID down regulation by LMP2A.

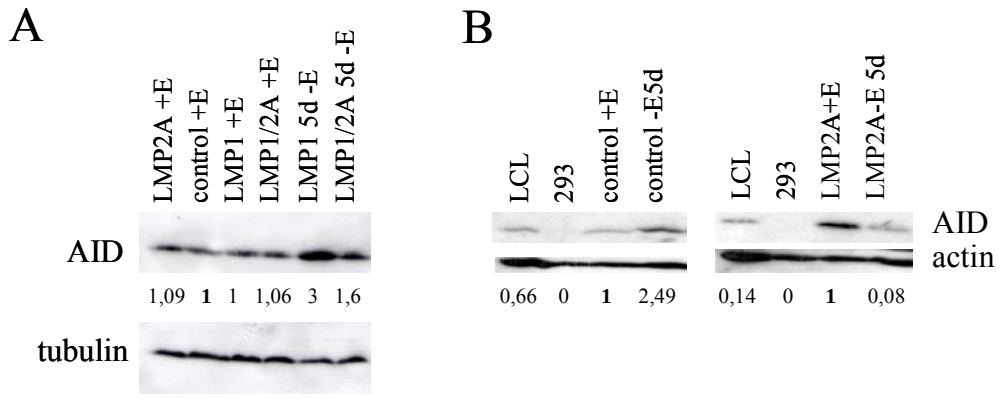

Figure 3.23. AID protein expression
(A) Western blot analyses to examine AID protein levels in EREB cell lines cultured in the presence and absence of estrogen. AID protein levels were standardized to tubulin, numbers indicate x-fold induction of AID protein level in EREB-control cells +E, which was set to 1. In the absence of estrogen, EREB-LMP1 cells show an up-regulation of AID; this is not observed in the EREB-LMP1/2A cell line.
(B) EREB-control cells show an up-regulation of AID protein expression upon estrogen withdrawal, reflecting the EBNA2 inhibitory effect on AID expression in the presence of estrogen. EREB-LMP2A cells do not up-regulate AID upon estrogen withdrawal, indicating an inhibitory effect of LMP2A as well. A classical LCL serves as a positive control, the human 293 kidney fibroblast cell line as a negative control. AID protein levels were standardized to actin, numbers indicate x-fold induction of AID protein level in EREB-control or EREB-LMP2A cells +E, respectively, which was set to 1.

3.4 Does EBNA2 independent expression of LMP1, LMP2A and EBNA1 elicit transforming capacity in human B cells?

EBV-positive Hodgkin's lymphoma (HL) cells express the EBV proteins LMP1, LMP2A and EBNA1, which are suspected to play a role in tumorigenesis. We made use of the different EREB cell lines to test the ability of these EBV proteins expressed either alone or in combination to induce proliferation and survival.

3.4.1 Co-expression of LMP1 and LMP2A prolongs B cell survival in vitro

The survival ability of the cell lines EREB-control, EREB-LMP1, EREB-LMP2A and EREB-LMP1/2A was tested after estrogen withdrawal from the culture medium. Cells were washed three times with estrogen free medium and cultured without estrogen for several days. Cell numbers were determined by counting every day and the percentages of living cells were analyzed by staining with propidium iodide (PI) and flow cytometry (Figure 20). As expected, numbers of living EREB-control cells dropped dramatically in between 5 days. EREB-LMP2A cells did not survive for more than 6 days either. In contrast, EREB-LMP1 and EREB-LMP1/2A cells survived significantly better, showing 8 days after estrogen withdrawal around 10 and 30% living cells, respectively.

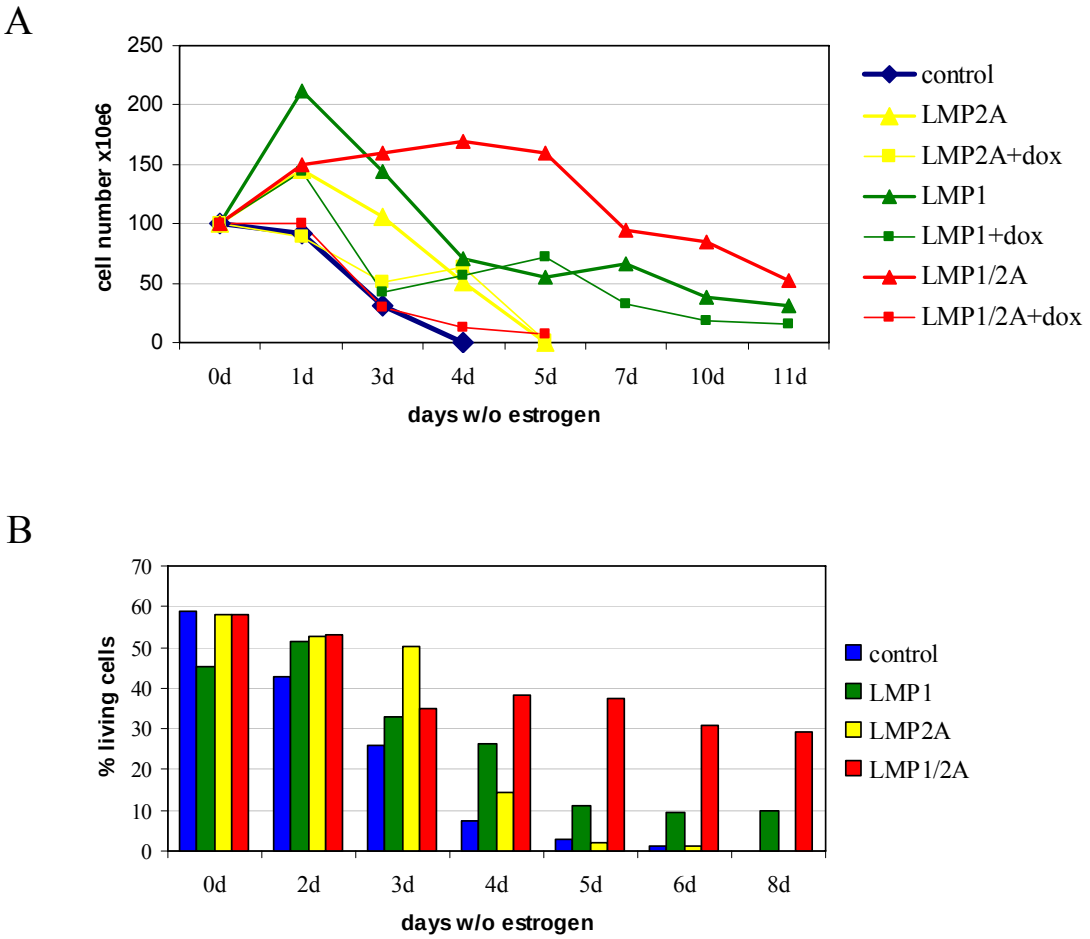

Figure 3.24. Co-expression of LMP1 and LMP2A prolongs B cell survival after EBNA2 inactivation
The cells were washed three times with estrogen free medium and then cultured in the absence of estrogen with or without doxycycline (dox) as indicated.
(A) Numbers of viable cells examined daily by counting. EREB-LMP1/2A cells cultured without doxycycline showed a better survival, indicating that the doxycyline induced LMP1 and LMP2A levels are too high and toxic for the cells.
(B) The percentages of living cells (PI negative) determined on the indicated days after estrogen withdrawal by flow cytometry.

3.4.2 Co-expression of LMP1 and LMP2A in EREB cells cultured without estrogen prolongs proliferation in vitro

To test the ability of the EREB cell lines to proliferate in the absence of estrogen, cell cycle analyses were performed. At day 2, 3, 5 and 9 after estrogen withdrawal the cells were treated with BrdU for 4 hours and then harvested for intracellular staining of the incorporated BrdU. In parallel, the DNA content was determined by a 7-AAD staining to distinguish cells in G0/1, S and G2 phase of the cell cycle (Figure 3.25). In the presence of estrogen, all cell lines

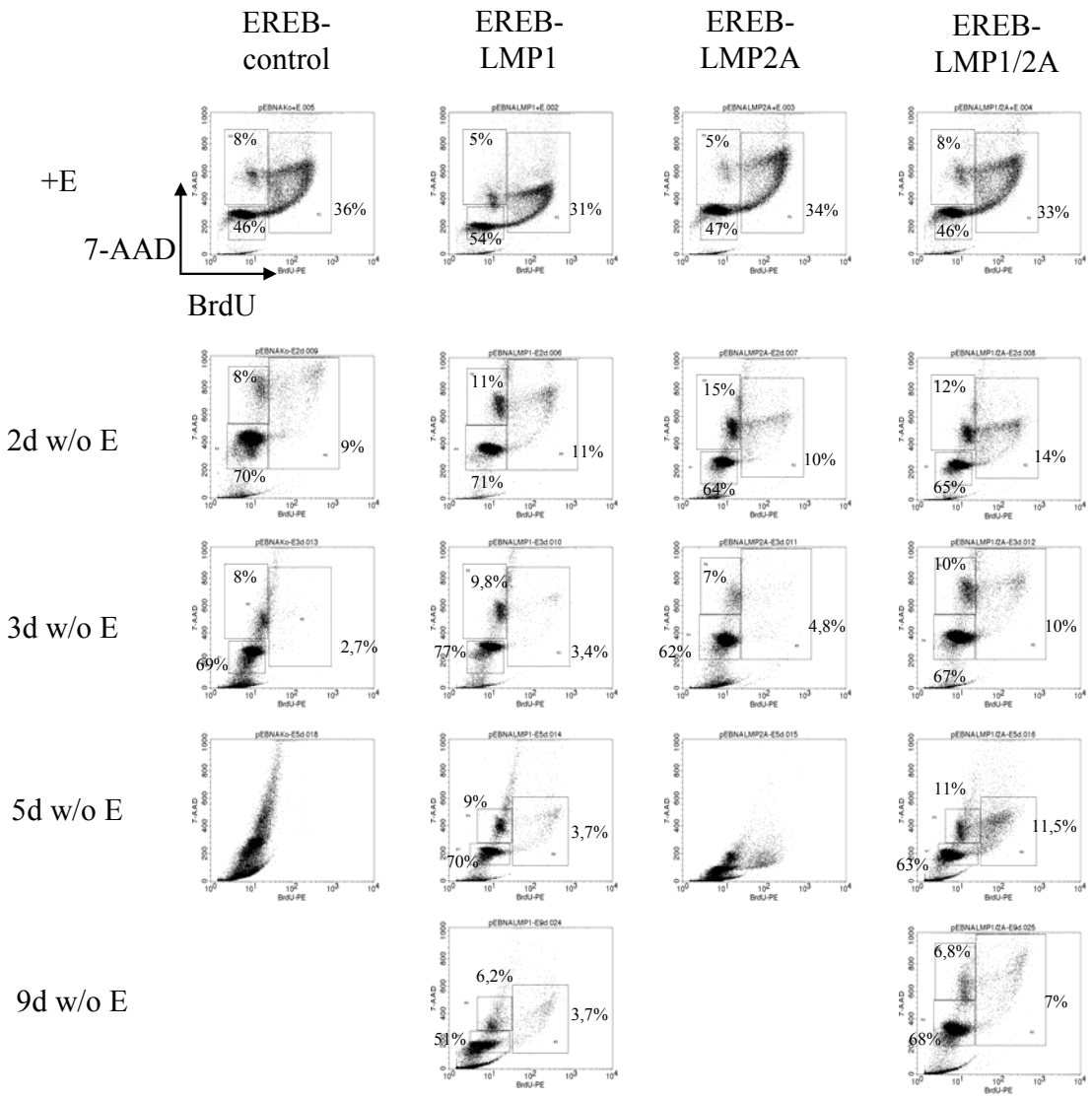

Figure 3.25. Co-expression of LMP1 and LMP2A sustains proliferation upon EBNA2 inactivation
Cell cycle analyses of the EREB-control, EREB-LMP1, EREB-LMP2A and EREB-LMP1/2A cell lines in the presence and absence of estrogen (E).
At the indicated days after estrogen withdrawal, 10^6 cells were cultured in the presence of BrdU for 4 hours and then harvested for intracellular staining of BrdU and the DNA content (7-AAD).
Resting cells in G0/G1-phase ($BrdU^-$, $7\text{-}AAD^-$), replicating cells in S-phase ($BrdU^+$) and cells in G2-phase with a duplicated DNA content ($7\text{-}AAD^+$) are shown. Numbers indicate the percentages of cells in G0/G1, S and G2 phase.

showed approximately the same percentages of cells in the different cell cycle phases, with around 30% in the S phase. The percentages of cells in the S phase dropped in all four cell lines to approximately 10% at day 2 of estrogen withdrawal. However, whereas EREB-control cells hardly showed any proliferation at day 3, the percentages of EREB-LMP1/2A cells in S phase stayed more or less stable up to 9 days. EREB-LMP1 cells also showed a certain percentage of cells in S phase 9 days after estrogen withdrawal, but it was less than in

EREB-LMP1/2A cells. None of the cell lines showed a significant arrest in the G0/1 or G2 phase.

The survival and proliferation data indicate that LMP1 expression alone helps B cells to survive and proliferate, but that co-expression of LMP1 and LMP2A is more potent to sustain proliferation independent from the EBV latency III program.

3.4.3 LMP1 and LMP2A co-expression is not sufficient to maintain immortalization of B cells *in vitro*

In contrast to EREB-control cells, EREB-LMP1 and EREB-LMP1/2A cells cultured without estrogen continued to form LCL-typical tight clumps and did not change their morphology and cell surface marker protein expression ($CD23^{+}CD21^{+}IgM^{low}$) for several days (data not shown). During this time, particularly the EREB-LMP1/2A cells showed a high metabolism rate indicated by the yellow discoloration of the culturing medium. After approximately 15 days, cells started to change their morphology, became big and round and showed a reduced metabolism (Figure 3.26). After approximately 30 days, cells were all dead. This shows that co-expression of LMP1 and LMP2A improves the survival and proliferation capacity of B cells, but is not able to transform and immortalize B cells *in vitro*.

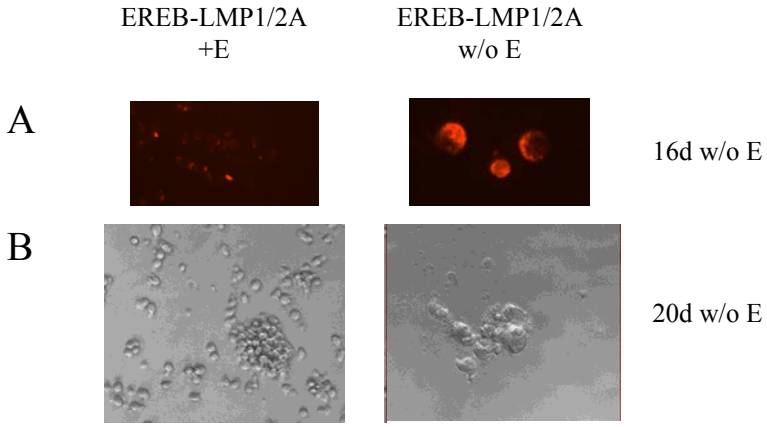

Figure 3.26. Morphology of EREB-LMP1/2A cells in the absence of estrogen
(A) Immunofluorescent staining for LMP1 (in red) on EREB-LMP1/2A cells cultured with and without estrogen for 16 days, shown with the same magnification.
(B) Light microscopy of EREB-LMP1/2A cells cultured with and without estrogen for 20 days, shown with the same magnification.

4 Discussion

So far many properties of EBV have been studied extensively thanks to its ability to immortalize primary B cells in culture. Nevertheless, it is still elusive how EBV establishes persistence *in vivo* and how it contributes to tumorigenesis - two features which might be linked. It has been proposed that EBV uses the normal germinal center (GC) differentiation pathway during its latent infection cycle to gain access to the memory B cell compartment, and indeed, many of the EBV associated lymphomas seem to originate from GC B cells. However, many of the data regarding EBV and the GC reaction are contradictory, querying whether EBV really has to pass the GC to establish viral persistence.

Studying EBV persistence is restricted to the human being, since EBV does not infect rodents. However, one approach to study immuno-modulatory functions of EBV genes is to use transgenic mouse model systems where single EBV gene products are introduced into the murine genome. In the present work, *in vivo* studies in transgenic mice and *in vitro* studies using a conditional EBV immortalized human cell line were combined to examine the interplay of EBV and GC B cell differentiation, with a special focus on the EBV proteins LMP1 and LMP2A.

4.1 The influence of a CD40 ligand regulated LMP1 signaling on B cells in vivo

The Epstein-Barr viral protein LMP1 and the cellular receptor CD40 are considered to be functional homologues. Evidence for similar effects on B cell biology was mainly based on cell culture experiments, although recently several transgenic mice expressing LMP1 and fusion proteins of LMP1 and CD40 were reported (Panagopoulos *et al.*, 2004; Stunz *et al.*, 2004; Uchida *et al.*, 1999) (Hömig, 2005). However, the present report is the first to show that LMP1 signaling in B cells perfectly mimics CD40 function *in vivo*. In normal conditions, EBV latent infection is restricted to B cells, thus our goal was to investigate the effects of the chimeric CD40/LMP1 protein expression exclusively in B cells. Expression of CD40/LMP1 was induced upon deletion of the upstream stop-cassette by Cre-recombinase, which was expressed under control of the CD19 promoter, which is specifically active in B cells from the pro-B cell stage on. We could show that more than 95% of B cells in the periphery expressed CD40/LMP1, whereas only 1% of $B220^-$ cells expressed the transgene, which could reflect rare myeloid populations which either express CD19 or originate from $CD19^+$ progenitor cells (Munn *et al.*, 2004; Montecino-Rodriguez *et al.*, 2001). We could not detect CD40/LMP1 expression in bone marrow derived dendritic cells.

The lymphoid compartment of CD40/LMP1 expressing mice was absolutely normal showing comparable B and T cell numbers and ratios, B cell subset percentages and lymphoid organ architecture to wt mice. Un-immunized, they did not show any spontaneous GC formation, nor did they show signs of autoimmunity, since there was no expansion or hyper-activation of immune cells and no auto-antibodies in the serum detectable. These data indicate that most of the abnormalities of the previously reported CD40/LMP1 expressing mice are due to CD40/LMP1-expression in non-B cells (Stunz *et al.*, 2004). In that study, the transgene was set under control of the MHC class II Eα promoter, allowing expression of CD40/LMP1 not only in B lymphocytes but in all antigen presenting cells, including macrophages and bone-marrow derived dendritic cells. Those mice showed a splenomegaly and lymphadenopathy, spontaneous germinal center formation, disordered lymphoid architecture and elevated IL6 and auto-antibodies in the serum. In contrast, a CD40L regulated LMP1 signal restricted to the B cell compartment did not induce any abnormality of the murine immune system.

Unlike CD40/LMP1, B cell specific LMP1 and LMP1/CD40 expression have been shown to induce pathologic phenotypes in mice, suggesting that the constitutive activity of LMP1 harbors a certain risk for disease development, and that in the normal infection cycle of EBV LMP1 has to be tightly regulated not to cause any malignancies in the host. Nevertheless, the rate of EBV-associated tumors expressing LMP1 is rather low taking into account that more than 95% of the world population is infected by EBV. Many so-called tumor viruses encode products that are homologous to cellular proteins, which allow them to interfere in cellular pathways, and eventually promote tumorigenic processes. Our data indicate a striking functional homology of the LMP1 and CD40 signaling domains, since upon TD immunization LMP1 signaling could provide the B cells with all essential signals for GC formation, isotype switching, SHM and affinity maturation in the absence of CD40, and showed an optimal organization of the follicles with GC and mantle zone. The only slight difference we could observe in CD40/LMP1+//CD40-/- mice was an approximately two fold increase in the percentages of GC B and plasma cells and elevated Ig titers after TD immunization compared to CD40+/+ wt controls. We could exclude that CD40/LMP1 expressing GC B cells proliferate faster or show a better survival rate by in vivo BrdU assays and SHM analyses, respectively. We expected GC B cells with a survival advantage to undergo more division rounds and therefore showing higher rates of SHM in their Ig genes, which was not the case for CD40/LMP1 expressing GC B cells. Additionally, CD40/LMP1 expressing B cells did not show a better survival than wt B cells ex vivo. Therefore, the increase of GC B cell percentages in CD40/LMP1+//CD40-/- mice is most likely due to an

increased recruitment of B cells into the GC. The reason for this could be either a specific effect of the LMP1 signaling domain, or the higher expression level of CD40/LMP1 compared to the endogenous CD40 in wt, which could facilitate the entry into the GC. The high CD40/LMP1 expression however did not seem to have any other influence on B cells, since GC did not form spontaneously nor was the GC reaction prolonged, and the CD40/LMP1 receptor molecule did not induce spontaneous signaling by self aggregation, since un-stimulated CD40/LMP1 expressing B cells showed the same properties as CD40+/+ B cells in regard to NFκB activity, survival, proliferation and class switch recombination after ex vivo isolation (data not shown).

Beside its ability to perfectly mimic CD40 *in vivo*, the LMP1 signaling domain seems to contain unique features that stimulate cytokine independent class switch recombination (CSR). Thus, a fraction of CD40/LMP1 expressing B cells stimulated *in vitro* with agonistic anti-CD40 antibody mediated CSR to IgG1, whereas CD40+/+ control B cells were dependent on co-stimulation with IL4. Co-culture experiments of CD40/LMP1+ and wt CD40+/+ B cells indicated that this was a LMP1-intrinsic effect, since a release of cytokines or other CSR-promoting factors of CD40/LMP1+ B cells would have induced CSR in both cell types and not only in CD40/LMP1+ B cells. It was previously reported that EBV infected primary B cells and Burkitt's Lymphoma (BL) cell lines transfected with LMP1 switch to several isotypes (He *et al.*, 2003). Since EBV infected B cells and BL cell lines are known to secrete several cytokines (Klein *et al.*, 1996), it could not be excluded that LMP1 acts in cooperation with these cytokines in those systems. We could show now that the LMP1 signaling domain on its own is able to induce CSR in primary B cells. Thus, LMP1 not only mimics CD40 receptor stimulation by CD40L in B cells, but additional effects physiologically mediated by T helper cells. It will be interesting to elucidate the unique mechanism of LMP1 to induce CSR, since we could exclude that LMP1 signaling induces an increase of STAT6 phosphorylation (data not shown), which has been shown to be essential for IL4 mediated CSR to IgG1 (Linehan *et al.*, 1998).

The superior ability of LMP1 in contrast to CD40 to induce cytokine-independent class switch recombination is in line with the observation that in CD40-deficient mice LMP1 can rescue CSR to IgG1 (Uchida *et al.*, 1999), whereas the LMP1/CD40 chimeric protein mediating constitutive active CD40 signals can not (Hömig, 2005). This suggests that the constitutive activity of both molecules not only blocks the GC but also the extrafollicular differentiation. Thus, the CSR observed in LMP1 expressing mice most likely reflects the ability of LMP1 to

induce cytokine- and T cell independent CSR rather than the rescue of a normal extrafollicular differentiation process.

4.2 The influence of constitutive active CD40 signaling on germinal center B cells *in vivo*

Constitutive CD40 signaling blocks GC formation as it has been shown for LMP1. Thus, it is evident that not the LMP1 signaling per se but its constitutive activity interferes with GC formation. In the present work we intended to investigate the influence of constitutive CD40 signaling on already established GC. To this end, the conditional LMP1/CD40 transgenic mouse line was crossed to Cγ1-cre mice to induce LMP1/CD40 expression mainly in GC B cells. Immunized LMP1/CD40/Cγ1-cre mice did not show GC formation. However, by immunohistochemical analyses of spleen sections some lightly PNA-stained areas could be detected, suggesting that GC had been formed by undeleted B cells, but were dissolved as soon as LMP1/CD40 was expressed. In Cγ1-cre mice Cre expression is induced upon induction of Cγ1 transcription, implying that the stop cassette is deleted very early during the GC reaction or even in activated B cells. Since LMP1/CD40 interfered with the progression of the GC reaction, we were not able to analyze the effects of constitutive CD40 signaling in later stages of the GC reaction. CD40 has been shown to be involved in selection processes of late GC B cells, the so-called centrocytes. In addition, LMP1 has been found to be expressed in these cells, where it may contribute to the pathogenesis of Hodgkin's lymphoma by enhancing proliferation and survival. Thus, it will be interesting to study the effect of a constitutive CD40 signaling in these cells. However, this requires another Cre-mouse strain which induces Cre recombinase activity in later stages of GC B cell differentiation, which unfortunately is not available yet.

LMP1/CD40//Cγ1-cre mice showed an age-dependent accumulation of an aberrant B cell population with a B1 cell phenotype. This was striking, since we expected to induce LMP1/CD40 expression mainly in activated B2 cells undergoing class switch recombination. B1 cells are the main producers of IgM antibodies and usually do not participate in GC reactions. It remains elusive whether the aberrant B cell population originates from real B1 cells or from B2 cells acquiring a B1-like phenotype upon transformation. LMP1/CD40 expression in B1 cells could be theoretically possible, since by using a GFP-reporter it was shown that in Cγ1-cre mice Cre-recombinase is active in around 1% of peritoneal cavity B1

cells (Casola *et al.*, 2006). This would however assume that B1 cells selectively expand and that B2 cells are negatively selected upon LMP1/CD40 expression.

It cannot be excluded that the aberrant population originated from activated B2 and/or early GC B cells, although they neither showed a GC B cell phenotype nor mutations in their Ig genes. LMP1/CD40 has been shown to down-regulate bcl6 – a key molecule in the GC reaction – making it likely that the cells have lost their GC B cell properties upon LMP1/CD40 expression. In addition, early GC B cells are not expected to show signs of SHM, since this process is known to start not earlier than 8 days after immunization (Jacob 1993).

Interestingly, most of mouse B cell malignancies have been reported to be positive for the B1 cell marker CD5 (Morse *et al.*, 2002). The histological analyses indicated that the B cell expansions in LMP1/CD40//Cγ1-cre mice are malignant lymphomas, showing a disruption of the overall follicular structure with nodular infiltrates mainly composed of $B220^{low}$ B cells. Lymphomas are characterized by their mono- or oligoclonal origin, and although we did not perform Southern Blot analyses to test this issue, the sequence analysis of the IgH rearrangements of one case indicated an oligoclonal origin of the expanded B cell population. Thus, although the final proof is still missing, the histological analyses indicated lymphoma development in LMP1/CD40//Cγ1-cre mice with an incidence of 100%. The surface marker expression of the aberrant B cells resemble that of mantle cell lymphoma cells in humans (Bertoni *et al.*, 2006), but however lack the over-expression of Cyclin D1, a hallmark of mantle cell lymphoma. Recently, cases of mantle cell lymphoma were described which do not express Cyclin D1, but Cyclin D2 or Cyclin D3 (Fu *et al.*, 2005). Thus, it will be interesting to analyze the expression pattern of the aberrant B cell population in LMP1/CD40//Cγ1-cre mice in more detail.

LMP1/CD40//CD19-cre mice, expressing the transgene in all B cells from a pro/pre B cell stage on, develop lymphoma as well (Hömig, 2005). Those mice show a pre-malignant expansion of activated B2 cells, which lead to lymphoma development in 60% of the cases. Although the splenic architecture of lymphoma-bearing mice look quite similar to LMP1/CD40//Cγ1-cre mice, the surface marker expression of expanded B cell populations differs strikingly in the two mouse strains. Lymphoma cells in LMP1/CD40//CD19-cre mice express CD43, but are devoid of CD5 or any other B1 cell marker, and either show a strong or no B220 expression, a high CD95 expression and a total loss of CD21 expression. In contrast, the expanded B cells in LMP1/CD40//Cγ1-cre mice always showed a characteristic expression pattern of $B220^{low}CD5^{+}CD43^{+}CD21^{low}CD95^{-}$. The different phenotypes of the

expanded B cell populations in the two mouse strains are most likely due to the different onset of LMP1/CD40 expression. Thus, LMP1/CD40//CD19-cre mice express the transgene in all naïve B cells, which leads to their activation and to a block of further differentiation. The enhanced proliferation and survival of these B cells increase the risk for malignant transformation. The late onset between an age of 12 and 19 months and the incidence of about 60% indicate that LMP1/CD40 expressing B cells have to acquire secondary mutations to develop malignant lymphomas.

In contrast, LMP1/CD40//Cγ1-cre mice show a lymphoma incidence of 100%. In these mice, B cells are activated by antigen and T helper cells before LMP1/CD40 starts to be expressed. This suggests a high risk of pre-activated B cells to become transformed upon acquiring one single oncogenic event, and might reflect the superior number of GC derived B cell malignancies in humans. A role for CD40 signaling in the pathogenesis of human B cell lymphomas and carcinomas has previously been suggested. Co-expression of CD40 and CD40L has been found in several malignancies like Chronic Lymphocytic Leukemia, Mantle cell Lymphoma, Follicular Lymphoma, Burkitt's Lymphoma and breast cancer (Challa *et al.*, 2002; Clodi *et al.*, 1998; Furman *et al.*, 2000; Pham *et al.*, 2002; Baxendale *et al.*, 2005). In Non-Hodgkin Lymphomas, disruption of the receptor-ligand interaction by antibodies against CD40 or CD40L was shown to result in growth arrest (Pham et al., 2002), providing evidence that the auto-activation of the CD40-signaling pathway by co-expression of CD40 and CD40L on tumor cells can lead to a growth advantage of malignant cells. In normal conditions, CD40L expression is tightly regulated and the co-expression with CD40 is only observed in a small subset of germinal center B cells (Grammer et al., 1999). However, disruption of this tight regulation could bear a substantial risk for cellular transformation.

4.3 Modeling of EBV latent infection *in vitro*

It has been suggested that *in vivo* freshly EBV-infected B cells start to express the latency III program, reflecting LCLs in culture. The expression of the latent EBV genes drives the cells to proliferate and enlarges the EBV infected B cell pool. However, the viral key transactivator EBNA2 and its viral target genes have to be shut off to guarantee survival of EBV infected cells. Otherwise, EBV infected B cells expressing the immunogenic viral proteins would be recognized and killed by cytotoxic T cells. The virus finally persists silently without any viral gene expression in resting memory B cells. The mechanisms how the EBV gene expression is shut off and how EBV establishes persistence in memory B cells are still elusive.

We used the conditional EREB cell line, in which the EBV program can be shut on and off by estrogen, to recapitulate the EBNA2 down-regulation of EBV-infected B cells *in vitro*.

Upon EBNA2 inactivation, EREB cells lose their LCL properties and go into a resting state. Strikingly, although the cell line originated from naïve umbilical cord blood cells, we could observe an up-regulation of the memory B cell marker CD27. To rule out any cell culture contamination, the IgH gene of the EREB cell line was sequenced and analyzed. The lack of somatic mutations and the unique rearrangement revealed that it originated from one naïve cell clone. CD27 is the accepted memory B cell marker so far, although its expression is not restricted to memory B cells. Thus, CD27 is expressed on GC B cells, plasma cells and T cells, but not on naïve B cells (Borst *et al.*, 2005). Several B cell malignancies are also positive for CD27 (van Oers *et al.*, 1993), and LCLs are either $CD27^+$ or $CD27^-$, since they can originate from both memory and naïve B cells, which are infected equally by EBV *in vitro* (Ehlin-Henriksson *et al.*, 2003). CD27 belongs to the TNF-R family and binds to its ligand CD70, which is expressed on activated B, T and dendritic cells. The definitive function of CD27, especially on memory B cells, is not known so far. CD27 was speculated to play a role in apoptosis through binding to Siva, a pro-apoptotic protein (Prasad *et al.*, 1997). In our assay we could rule out that the up-regulation of CD27 was a side effect of cells undergoing apoptosis upon EBNA2 inactivation, since the $CD27^+$ cells did not co-stain for AnnexinV (data not shown).

Another, even more unique characteristic of memory B cells is class switched Ig. We could find an increase of mature IgA transcripts in EREB cells after EBNA2 inactivation. However, we cannot rule out that this effect reflects the regulation of the IgH transcription rather than CSR, since EBNA2 has been shown to down-regulate the IgH (Jochner *et al.*, 1996). Nevertheless, it is very likely that LMP1 induces CSR in the EREB cell system. Previously, LMP1 has been shown to induce CSR in a BL cell line (He *et al.*, 2003). In the present work we give further evidence for this, since we could show that in primary murine B cells the LMP1 signaling domain was able to induce cytokine-independent CSR to IgG1. However, in the EREB cell system LMP1 induced mature Ig gene transcription of all IgG and IgA isotypes. This discrepancy most likely reflects the different cytokine availabilty in the various cell culture conditions. Thus, EBV has been shown to induce secretion of several cytokines in B cells (Klein *et al.*, 1996), which might facilitate CSR towards further isotypes in the EREB cell line.

It has been shown that *in vivo* EBV persists in human memory B cells, characterized by surface marker expression and class switched Ig (Babcock *et al.*, 1998). However, it has not

been shown so far whether EBV harboring cells in healthy individuals are real memory B cells carrying somatic mutations in their Ig genes. Here we show now that naïve EBV infected B cells gain a surface marker expression reminiscent of memory B cells as soon as EBNA2 is down-regulated. The additional ability of LMP1 to induce CSR suggests a unique feature of EBV to imitate memory B cell differentiation. These results inquire whether EBV infected B cells really have to pass the GC reaction to enter the memory B cell compartment, since several recent data contradict this model. Thus, LMP1 and LMP1/CD40 expression are not compatible with the GC reaction. Nevertheless, LMP1 and LMP2A expression has been detected in B cells of healthy EBV carriers resembling GC B cells by surface marker expression (Babcock *et al.*, 2000). Since it is known that EBV gene expression modulates the cellular gene expression pattern in B cells, we speculated whether EBNA2-independent LMP1 and LMP2A expression could induce a GC B cell surface marker phenotype in B cells, therefore only imitating GC B cells. To this end we generated the EREB-LMP1/2A cell line from the parental EREB2-5 cell line expressing LMP1, LMP2A and EBNA1 independent from EBNA2. Indeed, the EBV latency II expression pattern induced a GC B cell surface marker phenotype, leading to an up-regulation of CD10, CD77 and CD38. Moreover, these cells showed an increase in size, reflecting the immunoblastic morphology of GC B cells. Thus, different phases of EBV latent infection mimic normal B cell differentiation pathways independent of a classical GC reaction, implying a phase of activation (EBV latency III) and a phase of differentiation including CSR (EBV latency II and LMP1 expression alone) to finally become a resting memory like B cell (EBV latency 0).

Strikingly, the LMP1 induced CSR processes were abrogated upon co-expression of LMP2A. It has been shown that cross-linking of surface Ig delays CD40 ligand- and IL-4-induced B cell Ig class switching (Rush *et al.*, 2002). Thus, it could be that LMP2A, as a functional homologue of the BCR, has the same influence on LMP1 induced CSR. Whereas LMP1 induces up-regulation of AID (He *et al.*, 2003), we now provide evidence that LMP2A mediates the down-modulation of AID protein expression, which could be the mechanism of abrogating CSR.

So far it is not clear how the EBNA2 independent LMP1 and LMP2A expression is mediated *in vivo*. It could be that these proteins either stay stable for a certain time after EBNA2 down-regulation, or are re-induced by cellular stimuli. *In vitro* studies revealed that LMP1 expression can be induced by cytokines, like IL10, and also by an auto-regulatory mechanism (Kis *et al.*, 2006; Goormachtigh *et al.*, 2006). In addition, LMP1 and LMP2A expression have been shown to be induced by the cellular EBNA2 homologue Notch, which however elicits a

higher potential to induce LMP2A (Hofelmayr *et al.*, 1999). So far, gene expression studies of B cells in healthy EBV carriers have been done by RT-PCR, where both LMP1 and LMP2A transcripts could be detected in the bulk of GC marker expressing B cells. However, no co-staining of LMP1 and LMP2A protein expression in tissues were performed so far, remaining it elusive whether in the normal EBV infection cycle latencies exist where only one of the latent membrane proteins is expressed. Since we could show that the LMP1 induced CSR is down-modulated by LMP2A, we propose a certain phase of EBV infection where LMP1 is expressed independent of EBNA2 and LMP2A. Nevertheless, LMP2A could play an essential role in the control of LMP1 signaling outcomes in EBV infected cells, since up-regulation of AID and CSR induction harbor a certain risk for cells to become mutated and negatively selected by apoptosis. Therefore, if LMP1 and LMP2A are ever expressed in real GC B cells, LMP2A might inhibit further SHM and CSR, but improves survival and proliferation of EBV infected B cells, which could be a pre-requisite for lymphomagenesis. It is believed that the viral proteins LMP1 and LMP2A play an important role in the pathogenesis of Hodgkin's lymphoma, but their contribution to the initiation or maintenance of the tumor is not well understood. We therefore analyzed the different EREB cell lines expressing LMP1, LMP2A and EBNA1 either alone or in combination for their potential to maintain the immortalization after inactivation of EBNA2. The EREB-LMP1/2A cell line expressing all three proteins showed the best capacity to maintain proliferation and survival. These cells survived up to 30 days after EBNA2 inactivation, whereas control cells expressing EBNA1 only died within six days. This indicates that the co-expression of LMP1, LMP2A and EBNA1 provides B cells with survival and proliferation inducing signals, which may play a tumor promoting role *in vivo*. However, LMP1, LMP2A and EBNA1 co-expression in our system was not sufficient to maintain the immortalization of B cells *in vitro*. Around day 14 after EBNA2 inactivation, cells changed their morphology, became large and round, most likely entering a senescent stage, which they were not able to overcome by themselves. Interestingly, it is also very difficult to establish stable cell lines from Hodgkin's lymphoma. A characteristic of Hodgkin's lymphoma is that the malignant cells contribute to only 1-2% of the tumor mass, which is infiltrated by T lymphocytes, histiocytes, eosinophil granulocytes and plasma cells. Thus, it is suggested that the tumor cells are highly dependent on this special microenvironment, and not able to survive on their own in normal cell culture conditions. The EREB-LMP1/2A cell line reflecting the EBV expression pattern of Hodgkin lymphoma cells was not able to immortalize B cells *in vitro*, but may exhibit unique properties to survive and initiate tumorigenic processes *in vivo*. This cell line therefore can serve as a tool to study the

transforming capability of LMP1, LMP2A and EBNA1 *in vivo*. The injection of LCLs transformed with wild type EBV in immunodeficient mice of the severe combined immunodeficiency (SCID) genotype induces lymphoma development within four weeks with characteristics of lymphoproliferative disorders in humans (Rowe *et al.*, 1991). It will be interesting to investigate if injection of the human EREB cell line co-expressing EBNA1, LMP1 and LMP2A independent of other EBV genes can lead to tumor development in mice, and whether the tumors resemble Hodgkin's lymphomas in humans.

4.4 A new scenario of EBV latent infection

The data of the LMP1 and LMP1/CD40 transgenic mice indicate that constitutive LMP1 signaling is not compatible with the GC reaction. In contrast, the LMP1 signaling domain regulated by CD40L perfectly mimics CD40 *in vivo* in regards of GC formation, CSR and affinity maturation of antibodies. Beside this, the LMP1 signaling domain seems to contain unique features that stimulate cytokine independent class switch recombination. Thus, LMP1 signaling has not only properties to activate B cells, but is able to initiate differentiation processes like CSR independent of T cell help. In the EREB cell line we could show that naïve B cells, infected by EBV, do not return to a naïve B cell status upon switching off the EBV program, but seem to be converted to a memory like B cell. Moreover, EBNA2 independent expression of LMP1 and LMP2A induced a GC B cell like phenotype.

Taking into account these results, we propose a new scenario to explain how EBV establishes persistent infection (Figure 4.1). We assume that naïve EBV infected B cells do not have to undergo a classical GC reaction to enter the memory B cell pool, but that EBV by itself is able to convert a naïve B cell into a B cell with a memory B cell like phenotype by imitating several cellular signaling pathways involved in the GC reaction. This EBV induced differentiation process is independent from any T cell help, guaranteeing immune surveillance of EBV infected B cells.

However, several EBV associated tumors seem to originate from GC B cells, implicating that EBV infected B cells can be involved in the GC reaction. Indeed, we cannot exclude that B cells co-expressing LMP1 and LMP2A can take part in the GC reaction. Taking into consideration our results that LMP2A down-modulates some LMP1 induced effects, it still could be that it abrogates the GC inhibitory effect of LMP1.

Alternatively, EBV infected B cells may enter a GC reaction after they have undergone the GC independent EBV induced differentiation process. Thus, a resting EBV positive memory like B cell devoid of any EBV gene expression eventually enters a GC upon activation by

cognate antigen. Subsequently, the cytokine milieu and interaction with other immune cells during the GC reaction could re-induce LMP1 and LMP2A expression in this cell. This event may not happen very often, since in healthy EBV-infected individuals only 1 in 10^4 to 1 in 10^6 of peripheral blood cells carries EBV. However, our data showing that LMP1/CD40 expression in pre-activated compared to naïve B cells increases the lymphoma incidence from 60 to 100% provide evidence that LMP1 harbors a dramatic oncogenic potential in activated and GC B cells. This suggests that the risk of malignant transformation increases immense ones an EBV infected B cell enters the GC. This hypothesis could also explain the substantial increase of EBV-associated malignancies in areas prevalent of other infectious diseases, like malaria in regions of the so called "lymphoma belt" in Africa (Magrath *et al.*, 1992).

Taken together our results we propose that EBV establishes persistence in B cells independent of a GC reaction. This mechanism might have evolved to be beneficial for both the virus and the host, since virus-infected B cells not only evade immune surveillance, but also extrinsic activation, which limits the risk of malignant transformation.

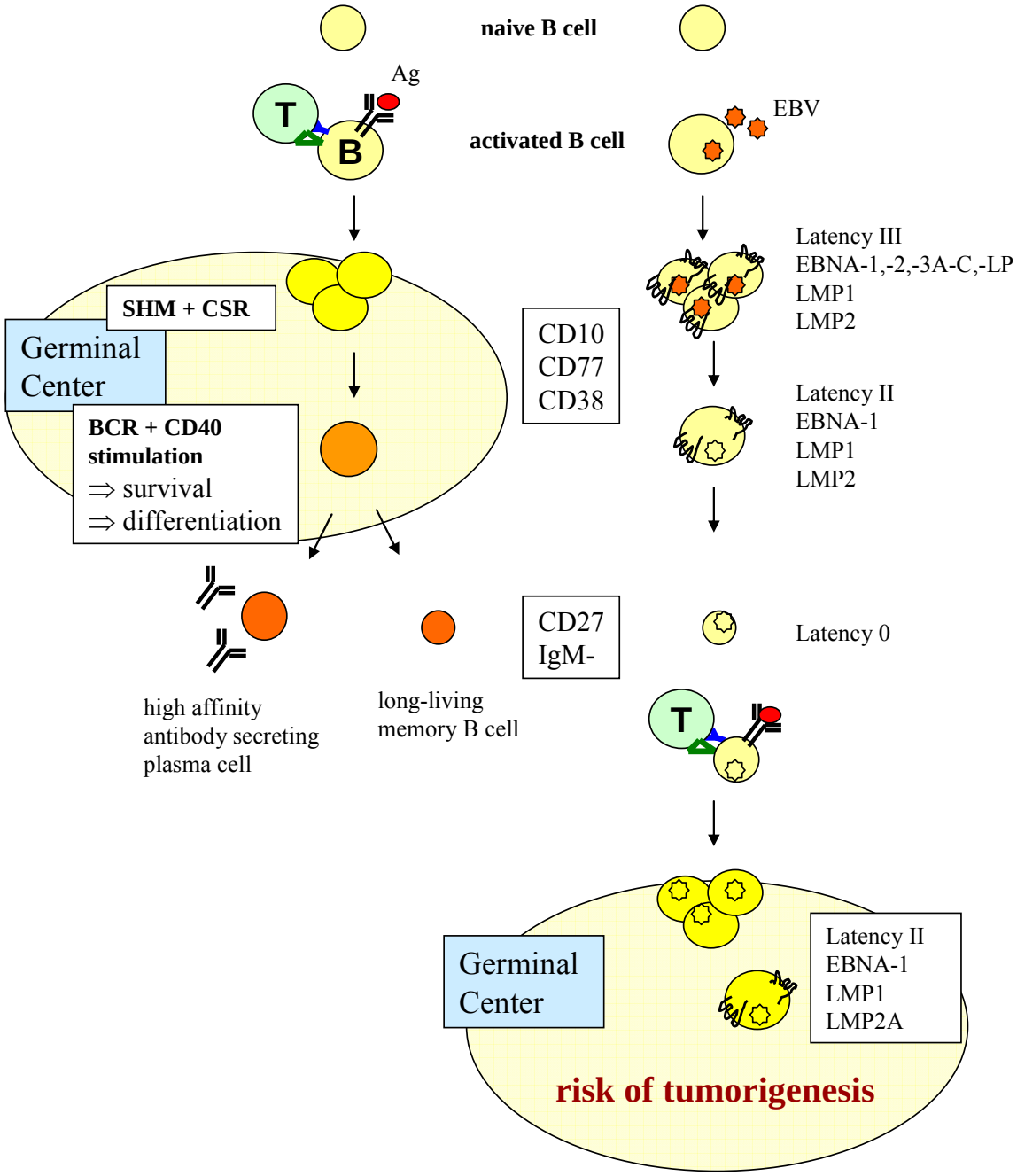

Figure 4.1. A new scenario of EBV latent infection
EBV latent infection mimics normal B cell differentiation processes. EBV infected B cells expressing the EBV latency III program become activated and proliferate like B cells stimulated through cognate antigen (Ag) and T cell help. EBNA2 down-regulation and the subsequent EBV latency II program induces a GC B cell like surface marker phenotype. LMP1 induces T cell independent class switch recombination. Finally, EBV infected B cells shut off EBV gene expression and gain a B cell memory like phenotype, expressing CD27 and class switched Ig on the surface. The activation of these cells by cognate antigen can lead to the recruitment of EBV infected cells into the GC reaction. There, the cytokine milieu and various cell to cell interactions can induce EBNA2 independent LMP1 and LMP2A expression, leading to an increased risk of GC B cell transformation.

5 Summary

Epstein-Barr virus (EBV) is a γ-herpes virus which preferentially infects human B lymphocytes. It is highly adapted to persist in B cells since it encodes for proteins which mimic several cellular proteins playing an important role in B cell biology. Thus, the viral Latent Membrane Proteins (LMP) 1 and 2A are considered to be functional homologues of the CD40 receptor and the B cell receptor, respectively. It has been postulated that EBV uses the normal T cell dependent immune response B cell differentiation pathway (the so-called germinal center reaction) for its infection cycle to gain access to the long living memory B cell compartment. LMP1 and LMP2A are suggested to play an important role during this process, since they are able to provide B cells with survival and proliferation signals, and may help the EBV-infected B cells to evade negative selection during the germinal center reaction. LMP1 and LMP2A are also expressed in germinal center B cell derived EBV-associated malignancies, suggesting a contribution of these viral proteins to tumor development. However, the data concerning the role of the germinal center reaction in the latent EBV infection circle is controversial, remaining it elusive (i) if EBV-infected B cells have to pass the germinal center to establish persistence, and (ii) if LMP1 signaling has any influence on germinal center B cells.

In the present work, the interplay of EBV and the germinal center reaction was examined, focusing on the roles of LMP1 and LMP2A in normal B cell biology and lymphomagenesis.

LMP1 was shown to mimic a constitutive active CD40 receptor *in vitro*, but *in vivo* LMP1 only partially restored the CD40-deficiency in transgenic mice; it even blocked germinal center formation in the presence of CD40. This could be due to differences in the signaling mediated by LMP1 and CD40, or to the constitutive activity of LMP1. To compare CD40 and LMP1 signaling *in vivo*, we generated a transgenic mouse line which conditionally expresses a CD40-ligand regulated LMP1 protein (CD40/LMP1). We show that LMP1 signaling in B cells perfectly mimics CD40 function *in vivo*, leading to normal B cell development, B cell activation, and T cell dependent immune responses in CD40 deficient mice.

Thus, we conclude that not the LMP1 signaling domain but its constitutive activity interferes with the germinal center reaction. This is in accordance with a previous study of our group, showing that ligand-independent constitutive active CD40 signaling (LMP1/CD40) blocks germinal center initiation like LMP1. However, the influence of a constitutive active CD40 signaling directly on germinal center B cells remained open. In the present study, LMP1/CD40 expression was specifically induced in germinal center B cells. We show that a

constitutive active CD40 signaling also interferes with early germinal centers, but leads to lymphoproliferation, which most likely reflects malignant lymphoma. The incidence of 100% suggests a substantial risk of pre-activated B cells to become transformed upon deregulation of CD40 signaling or LMP1 expression.

Since constitutive LMP1 and CD40 signals are not compatible with the germinal center B cell differentiation process, and LMP1 expression however has been detected in B cells resembling a germinal center phenotype of healthy EBV-carriers, we questioned whether these are real germinal center B cells. We studied the influence of EBV protein expression on the phenotype of human B cells *in vitro*, and indeed could show that EBNA2-independent LMP1 and LMP2A expression induces a germinal center cell like phenotype in B cells. Further, the inactivation of EBNA2, the key transactivator of EBV gene expression, does not lead to the original naïve phenotype of a B cell, but induces up-regulation of the memory B cell marker CD27. Beyond, we show that in CD40/LMP1-expressing B cells of the transgenic mice as well as in the human cell lines, LMP1 signaling induces class switch recombination independent from cytokines. This implies a unique feature of the LMP1 signaling domain to initiate differentiation processes beside its ability to activate B cells.

Taking these data into account, we suggest that EBV infected B cells do not have to undergo a classical germinal center reaction to enter the memory B cell pool, but that with the help of EBV proteins they are able to induce processes imitating memory B cell differentiation independent from T cells, thus escaping immune surveillance. These EBV-infected quiescent memory-like B cells eventually enter the germinal center reaction upon antigen-dependent activation. There, re-induction of LMP1 might harbor a substantial risk of malignant transformation.

6 Zusammenfassung

Das Epstein-Barr Virus (EBV) ist ein Gamma-Herpesvirus, welches vor allem humane B-Lymphozyten infiziert. Einige virale Proteine weisen funktionelle Homologien zu zellulären Proteinen auf, die eine wichtige Rolle in der normalen B-Zell-Biologie spielen. So ahmen die latenten Membranproteine (LMP) 1 und 2A den CD40- beziehungsweise B-Zell-Rezeptor nach. Es wurde postuliert, dass EBV-infizierte B-Zellen den normalen Differenzierungsprozess einer T-Zell-abhängigen Immunantwort (der so genannten Keimzentrumsreaktion) durchlaufen, um Persistenz in den langlebigen Gedächtnis-B-Zellen zu erlangen. LMP1 and LMP2A wird dabei eine wichtige Rolle zugeschrieben, da sie B-Zellen mit Wachstums- und Überlebenssignalen versorgen, und somit die negative Selektion EBV-infizierter B-Zellen während der Keimzentrumsreaktion verhindern könnten. LMP1 und LMP2A werden außerdem in von Keimzentrums-B-Zellen abstammenden EBV-assoziierten Lymphomen exprimiert, und es wird vermutet, dass die viralen Proteine zur Tumorentstehung beitragen.

Da es jedoch widersprüchliche Daten über die Rolle der Keimzentrumsreaktion im latenten Infektionszyklus von EBV gibt, bleibt es unbekannt, ob (1) EBV-infizierte B-Zellen die Keimzentrumsreaktion durchlaufen müssen, um Persistenz zu erlangen, und (2) welchen Einfluss die Expression von LMP1 in Keimzentrums-B-Zellen hat.

Im Rahmen dieser Arbeit wurde das Zusammenspiel von EBV und der Keimzentrumsreaktion untersucht, wobei ein besonderer Schwerpunkt auf die Rolle von LMP1 und LMP2A in der normalen B-Zell-Biologie und der Lymphomentstehung gelegt wurde.

In früheren Zellkulturstudien wurde gezeigt, dass die Stimulierung des CD40-Rezeptors und die Expression von LMP1 dieselben Effekte auf B-Zellen ausüben. In transgenen Mäusen jedoch konnte LMP1 den CD40-Rezeptor nur partiell ersetzen, und LMP1 blockierte sogar die Keimzentrumsreaktion. Dies könnte entweder auf unterschiedliche Signaltransduktionsfähigkeiten der beiden Moleküle, oder auf die konstitutive Aktivität von LMP1 zurückgeführt werden. Um die Funktion der LMP1- and CD40-Signaldomänen *in vivo* zu vergleichen, wurde in dieser Arbeit ein trangener Mausstamm generiert, der ein CD40-Ligand abhängiges LMP1-Protein (CD40/LMP1) konditional exprimiert. Wir zeigen, dass die LMP1-Signaldomäne fähig ist, die CD40-Funktion in B-Zellen perfekt nachzuahmen. In CD40-defizienten Mäusen führt die Expression von CD40/LMP1 zu einer normalen B-Zell-Entwicklung, B-Zell-Aktivierung, und T-Zell-abhängigen Immunantwort mit Immunglobulinklassenwechsel und Keimzentrumsreaktion. Daraus schließen wir, dass nicht

die Signaldomäne, sondern die konstitutive Aktivität von LMP1 zur Keimzentrumsblockade führt. Dies wird durch frühere Studien unserer Arbeitsgruppe gestützt, die zeigen, dass ein Liganden-unabhängiges, konstitutiv aktives CD40-Signal (LMP1/CD40) genauso wie LMP1 den Eintritt von B-Zellen ins Keimzentrum verhindern kann. Es blieb jedoch offen, welchen Einfluss ein konstitutives CD40-Signal auf Keimzentrums-B-Zellen ausübt. In der vorliegenden Arbeit wurde die Expression von LMP1/CD40 speziell in Keimzentrums-B-Zellen aktiviert. Wir zeigen, dass ein konstitutives CD40-Signal auch mit bereits etablierten frühen Keimzentren interferiert, und zu einer Lymphoproliferation führt, welche mit hoher Wahrscheinlichkeit eine maligne Transformation darstellt. Die Inzidenz von 100% suggeriert das besonders hohe Risiko von pre-aktivierten B-Zellen durch deregulierte CD40- und LMP1-Signale transformiert zu werden.

Da die konstitutive Aktivität von CD40 and LMP1 mit der Keimzentrumsdifferenzierung von B-Zellen nicht kompatibel sind, LMP1 jedoch in B-Zellen mit Keimzentrumsphenotyp EBV-infizierter Individuen nachgewiesen werden konnte, hinterfragten wir, ob es sich in diesem Fall um echte Keimzentrums-B-Zellen handelte. Wir untersuchten den Einfluss von EBV-Proteinen auf den Phänotyp humaner B-Zellen in Zellkultur und zeigen, dass die Expression von LMP1 und LMP2A zu einem Keimzentrums-Phänotyp der B-Zellen führt. Das Abschalten von EBNA2 und daraus folgend aller EBV-Gene führt nicht zu dem ursprünglichen Phänotyp naiver B-Zellen, sondern zur Expression des Gedächtnis-B-Zell-Markers CD27. Außerdem konnten wir sowohl in den CD40/LMP1-exprimierenden B-Zellen der transgenen Mäuse als auch in den humanen Zelllinien zeigen, dass LMP1 im Gegensatz zu CD40 zu einem Zytokin-unabhängigen Immunglobulinklassenwechsel fähig ist. Dies deutet darauf hin, dass das LMP1-Signal nicht nur B-Zellen aktivieren, sondern auch Differenzierungsprozesse initiieren kann.

Aufgrund dieser Daten postulieren wir, dass EBV-infizierte B-Zellen keine klassische Keimzentrumsreaktion durchlaufen müssen, sondern mit Hilfe von EBV-kodierten Proteinen die Differenzierung zu Gedächtnis-B-Zellen imitieren können. Dieser Prozess verläuft T-Zell-unabhängig, könnte daher EBV-infizierte B-Zellen vor einer immunologischen Abwehrreaktion bewahren, und dem Virus erlauben, in langlebigen Gedächtnis-B-Zell-ähnlichen Zellen zu persistieren. Diese Zellen könnten in seltenen Fällen durch Antigenkontakt in eine Keimzentrumsreaktion eintreten, wo die Expression von LMP1 zu einem besonderen Risiko der malignen Entartung führen könnte.

7 Material

7.1 Plasmids

pTVRosa26 LMP1

Bluescript-vector encoding the *CD40/LMP1* chimeric gene inserted in a targeting cassette, allowing the homologous recombination into the murine rosa26-locus via a short (1kb) and long arm (4kb) of homology (pRosa26-1, Phillipe Soriano, Seattle). Upstream of the transgene a stop-cassette flanked by loxP sites was introduced, containing a transcription and translation termination site (Lasko), the gene encoding the red fluorescent protein (not expressed in this context), and a neomycin-resistance gene flanked by frt sites. Outside the targeting cassette, the vector encodes for diphtheria toxin (DTA) under control of the phospho-glycerate kinase (PGK) promoter for negative selection of non-homologous integrations.

pGK-cre-bpA (Kurt Fellenberg, Institute of Genetics, Köln)

Vector expressing the Cre-recombinase under control of the phospho-glycerate kinase (PGK) promoter.

pRT-1 (Bornkamm *et al.*, 2005)

Tet-On expression plasmid with a tetracycline dependent bidirectional promoter (P_{tet}bi-1) and a reverse tetracycline controlled transcriptional activator (rtTA2^{s}-M2), transcribed from a promoter/enhancer consisting of the mouse heavy chain intron enhancer (Eμ) and the chicken β-actin promoter (CAGp). The plasmid lacks the silencer of the tetracycline dependent promoter, and therefore shows a certain leakiness of the promoter in the absence of tetracycline.

The EBNA1 coding sequence and the origin of replication (oriP) guarantee the episomal maintenance of the plasmid in the cell.

pGEM-T easy vector (Promega)

Vector system with 3'-T overhangs at the insertion site to clone PCR products.

7.2 Bacteria

DH5α

Escherichia coli-Stamm;

Genotyp: F^-, ϕdlacZ Δ M15, endA1, recA1, hsdR17 (r_k^-, m_k^-), supE44, thi-1, gyrA96, relA1, Δ(lacZYA-argF)U169, λ^-

7.3 Cell lines

EREB2-5 (Kempkes *et al.*, 1995b)

EREB2-5 is a conditionally immortalized lymphoblastoid cell line established from primary naïve B cells isolated from human umbilical cord blood. Cells were co-infected with two virions, the so-called P3HR1 virus genome, which has a deletion removing the *ebna2* gene, and a mini-EBV plasmid carrying a chimeric EBNA2 fused to the hormone binding domain of the estrogen receptor gene (ER-EBNA2). Although ER-EBNA2 is constitutively expressed, its function depends on the presence of estrogen. In the absence of estrogen, heat-shock protein 90 binds to the estrogen receptor, leading to incorrect folding of the EBNA2 protein (Briegel *et al.*, 1996) and prevention of nuclear translocation (Francis *et al.*, 1995). In the presence of estrogen, the chimeric ER-EBNA2 protein substitutes for the wild-type EBNA2, leading to the transactivation of the latent EBV genes and to the immortalization of B cells.

Balb/c embryonal stem cells (ES) (B. Ledermann und K. Bürki, Basel)

Used for the generation of the CD40/LMP1^{flSTOP} mouse strain.

7.4 Mouse strains

Balb/c

Used to cross the chimeras generated from injection of ES cells into blastocytes to establish the CD40/LMP1^{flSTOP} mouse strain on a pure Balb/c background.

CD40-/-

Balb/c mice deficient for CD40 by an insertion of a neomycin cassette into the CD40 locus. Used to establish the CD40/LMP1^{flSTOP} mouse strain on a CD40 deficient background.

CD19-Cre (Rickert *et al.*, 1997)

C57BL/6 mouse strain with a homologous recombination of the cre-recombinase gene into the CD19-locus. Crossed to CD40/LMP1^{flSTOP} mice to induce the transgene in B cells.

LMP1/CD40^{flSTOP} (Hömig, 2005)

Balb/c mouse strain with a homologous recombination of the chimeric LMP1/CD40 gene with a loxP-sites flanked STOP-cassette upstream into the rosa26-locus.

Cγ1-Cre (Casola *et al.*, 2006)

C57BL/6 mouse strain with a homologous recombination of the cre-recombinase gene into the Cγ1-locus. Crossed to LMP1/CD40^{flSTOP} mice to induce the transgene in germinal center B cells.

B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (Charles River Laboratories)

C57BL/6 mouse strain expressing the leukocyte marker Ly5.1 instead of Ly5.2.

7.5 Primer

Mouse genotyping

CD40/LMP1

CD40se: 5′-TTGGCTCTTCTGATCTCGC -3′

LMP1: 5′-CATCACTGTGTCGTTGTCCA-3′

LMP1/CD40

Ex1Fw1 LMP1: 5′- AGG AGC CCT CCT TGT CCT CTA -3′

CD40 PCR3: 5′- CTG AGA TGC GAC TCT CTT TGC CAT -3′

CD40-/-

neo rev : 5′-AGG TGA GAT GAC AGG AGA TC-3′

CD40wt rev1: 5′-GAG ATG AGA AGG AAG AAT GGG AAA AC-3′

CD40wt fw1: 5′-GGC AGT AAG ACG ATG TGA CAA CAG AG-3′

CD19-Cre

Cre7: 5′- TCA GCT ACA CCA GAG ACG G -3′

CD19c: 5′- AAC CAG TCA ACA CCC TTC C -3′

CD19d: 5′- CCA GAC TAG ATA CAG ACC AG -3′

Cg1-Cre

IgG1 KpnI fw: 5′-TGT TGG GAC AAA CGA GCA ATC-3′

Cre 13: 5′-GGT GGC TGG ACC AAT GTA AAT A-3′
IgG1 rev3: 5′-GTC ATG GCA ATG CCA AGG TCG CTA G-3′

Somatic hypermutation analysis (mouse)
J558Fr3: 5′-CAG CCT GAC ATC TGA GGA CTC TGC-3′
JHCHint: 5′-CTC CAC CAG ACC TCT CTA GAC AGC-3′

VDJ EREB2-5
VH3se: 5′-GGG GTC CCT GAG ACT CTC CTG TGC AG-3′
JH1,2,4,5as: 5′-ACC TGA GGA GAC GGT GAC CAG GGT-3′
JH3as: 5′-ACC TGA AGA GAC GGT GAC CAT TGT-3′
JH6as: 5′-ACC TGA GGA GAC GGT GAC CGT GGT-3′

Mature Ig transcripts human
V-FR3-se: 5′-GAG GAC ACA GCC GTG TAT TAC TG -3′as
Cμ-as: 5′-CCG AAT TCA GAC GAG GGG GAA AAG GGT T-3′
Cγ1-as: 5′-GTT TTG TCA CAA GAT TTG GGC TC-3′
Cγ2-as: 5′-GTGGGCACTCGACACAACATTTGCG-3′
Cγ3-as: 5′-TTGTGTCACCAAGTGGGGTTTTGAGC-3′
Cα-as: 5′-GGGTGGCGGTTAGCGGGGTCTTGG-3′

Light Cycler
LMP2A
LMP2A-RTse: 5′-ATG ACT CAT CTC AAC ACA TA-3′
LMP2A-RTas: 5′-CAT GTT AGG CAA ATT GCA AA-3′
c-abl
c-abl 1: 5′-GGC CGT GAA GAC CTT GAA GGA G-3′
c-abl 2: 5′-ACC TGC TCA GGC CAA AAT CAG C-3′

7.6 DNA probes

rosa26-probe
A 550 bp fragment was isolated from pRosa26-5-pBS KS (Phillipe Soriano, Seattle) using the enzymes *EcoRI* and *Pac1*.

LMP1-probe

A 495 bp fragment was isolated from pBS-AscI-LMP1 (kindly provided by Cornelia Hömig, GSF) using the enzyme NcoI.

LMP2A-probe

A 372 bp fragment was isolated from pSP64TP1 (kindly provided by Gerhard Laux, GSF) using the enzyme XhoI.

7.7 Antibodies

ELISA

Ig specific rat anti-mouse antibodies (IgM, II/41; IgG1, A85-3; IgG2a, R11-8; IgG2b, R9-91; IgG3, R2-38; IgA, C11-3;) and biotin-conjugated secondary antibodies specific for the different isotypes (IgM, R6-60.2; IgG1, A85-1; IgG2a, R19-15; IgG2b, R12-3; IgG3, R40-82; IgA, C10-1;) were purchased from BD Bioscience.

Flow cytometry

Antibodies against murine B220 (RA3-6B2), CD5 (53-7.3), CD21 (7G6), CD23 (B3B4), CD40 (3/23) , CD43 (S7), CD80 (16-10A1), CD95 (Jo2), CD138 (281-2), Gr1 (RB6-8C5), IgD (11-26c.2a), IgG1 (A85-1) and IgM (R6-60.2) were purchased from BD Biosciences; PNA from Vector Laboratories; anti-CD11c from Miltenyi-Biotech; anti-IgA, anti-IgG2a and anti-IgG2b from Southern Biotech. Biotin conjugates were visualized with PE-streptavidin (BD Biosciences).

Antibodies against human CD10, CD27 (M-T271), CD77 (5B5), CD38 (HIT2) and IgM (G20-127) were purchased from BD Biosciences, anti-human CD21 (IOb1a) from Immunotech.

Anti-BrdU antibody (3D4) was purchased from BD Biosciences.

Immunohistochemistry

Cryosections

peroxidase conjugated anti-mouse IgM (Sigma)

rat anti-mouse CD3 (kindly provided by E. Kremmer, GSF Munich)

biotin conjugated PNA (Vector Laboratories)

biotin conjugated mouse anti-rat IgG1 (Jackson Laboratories)

Paraffinsections

anti-mouse B220 (BD Pharmingen)

anti-mouse CD3 (DakoCytomation)

Immunofluorescence

mouse anti-LMP1 CS1-4 (Dako)

Cy3 conjugated anti-mouse IgG

Western blotting

Primary antibodies

α-CD40 human	rabbit	1:500	Santa Cruz Biotechnologies
α–LMP1 CS1-4	mouse	1:400	Dako
α–AID 5G9	rat	1:5	Elisabeth Kremmer, GSF
α–actin	mouse	1:1000	Santa Cruz Biotechnologies
α–tubulin	mouse	1:20000	Promega

Secondary antibodies

α–rabbit-IgG-HRP	goat	1:5000	Santa Cruz Biotechnologies
α–mouse-IgG-HRP	goat	1:5000	Promega
α–rat-IgG-HRP	goat	1:10000	Jackson Immuno Research

7.8 Software

Adobe Photoshop

Adobe Illustrator

CELLQuest Becton Dickinson

Clone Manager 6

DNAPLOT

Mac Vector

Microsoft Internet Explorer

Microsoft Excel 2000

Microsoft Powerpoint

Microsoft Word XP

OpenLab Improvision

SeqMan

TINA Bas

8 Methods

All standard methods not described in detail were performed according to (Sambrook and Russel, 2001) or the manufacture's protocols.

8.1 Mice

8.1.1 Generation of the transgenic mouse line CD40/LMP1

The *CD40/LMP1* chimeric gene was generated by fusing the murine CD40 cDNA encoding for the first 215 aminoacids (aa) (amplified by RT-PCR from murine splenic cell RNA) to the cDNA of LMP1 encoding for the terminal 200 aa. To insert the CD*40/LMP1* fusion gene into the *rosa26*-locus the vector pRosa26-1 was used (Soriano, 1999). Before introducing *CD40/LMP1*, pRosa26-1 was modified by introducing a loxP-flanked region, consisting of a stop cassette containing a transcription and translation termination site (Lakso *et al.*, 1992), the gene encoding the red fluorescent protein (not expressed in this context), and a neomycin-resistance gene flanked by frt sites. The *CD40/LMP1* fusion gene was cloned downstream of the stop cassette. The final targeting vector was sequenced, linearized and electroporated into BALB/c-derived embryonic stem (ES) cells. The targeted ES cells were screened for homologous recombination by Southern-blot analysis and a subset was transfected with a Cre-expression vector (pGK-cre-bpA, kindly provided by Kurt Fellenberg) to test Cre-loxP mediated deletion of the stop cassette. The DNA was digested with EcoRI and hybridized with a specific radioactive labeled *rosa26*-probe (Soriano, 1999). Recombinant ES cells containing the loxP flanked region were injected into C57BL/6 blastocysts, which were then transferred into foster mothers to obtain chimeric mice.

8.1.2 Mice crossings

Mice carrying the *CD40/LMP1*^{flSTOP} allele were crossed to the CD19-Cre mouse strain (C57BL/6 background) to generate mice expressing the transgene in early B cell stages in the bone marrow. Offspring of this crossing were bred to CD40-/- mice (Balb/c background) to generate CD40/LMP1 expressing mice on a CD40-deficient background (CD40/LMP1+//CD40-/-).

Mice carrying the *LMP1/CD40*^{flSTOP} allele (Balb/c background) were crossed to the Cγ1-Cre mouse strain (C57BL/6 background) to generate mice expressing the transgene in early germinal center B cells.

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.
Mice were analyzed at 8-16 weeks of age unless stated otherwise.

8.1.3 Mouse immunizations

NP-CGG

8-12 week old mice were immunized intraperitoneally with 100 µg alum-precipitated nitrophenylacetyl chicken gamma globulin (NP-CGG) (Biosearch Technologies). For precipitation, one volume of antigen was mixed with one volume of 10% (w/v) $KAl(SO_4)_2$ and pH was adjusted to pH 6.5 with 1 N NaOH. After 30 min. incubation on ice, the antigen was centrifuged for 10 min. at 4.200 rpm (Minifuge Sigma) and washed three times in sterile PBS. Finally, antigen was resuspended in 200 µl sterile PBS and injected intraperitoneally (i.p.).

Sheep red blood cells (SRBC)

Defibrinated sheep blood (Oxoid) was washed three times in sterile PBS and diluted in PBS to a final concentration of 10^9 cells/ml. $2x10^8$ red blood cells in 200 µl PBS were injected i.p. To purchase around 10^{10} cells, 3 ml blood was washed in 50ml PBS.

8.1.4 Preparation of primary lymphocytes from mice

Mice were euthanized by CO_2 gassing for 2 min. and subsequently dissected. Spleen and lymph nodes were taken out as entire organs and maintained in medium (1x RPMI 1640 (Gibco), containing 5% (v/v) FCS, 1% (v/v) Penicillin-Streptomycin, 1% (v/v) sodium pyruvate, 1% (v/v) L-Glutamin (all purchased from Gibco)). Tissues were passed through a capillary cell strainer (Becton Dickinson) to receive single cell suspensions. To isolate cells from the bone marrow, leg bones were dissected, cut, and rinsed with medium. Spleen and bone marrow cells were depleted of erythrocytes by lysis (3 min. incubation with a fresh 1:9 mixture of 170 mM Tris/HCl, pH 7.65, and 155 mM NH_4Cl).
Isolation of B cells was performed by Magnetic Cell Separation (MACS) (Miltenyi Biotec) according to the manufacture's protocol, using either anti-CD43-Beads and LD Columns or anti-CD19-Beads and LS columns (all Miltenyi Biotec).

8.1.5 In vivo BrdU assay

14 days post-immunization with 100 µg NP-CGG mice were injected intraperitoneally with 150 µl BrdU solution (10mg/ml, BD Biosciences) and sacrificed 2 and 6 hours later. Splenocytes were purified and stained using the APC BrdU Flow kit (BD Biosciences).

8.1.6 Isolation of germinal center B cells and analysis of somatic hypermutation

14 days post-immunization with 100 µg NP-CGG, GC B cells from spleen were purified directly following incubation with PNA-FITC, anti-CD95-PE and anti-B220-APC on a FACSAria cell sorter (BD Biosciences) and sorted into a naïve fraction ($B220^+CD95^-PNA^-PI^-$) and a GC fraction ($B220^+CD95^+PNA^+PI^-$). PCR was performed of DNA of 40000 cell equivalents using the Expand High fidelity PCR system (Roche) and primer J558Fr3, which anneals in the framework 3 region of most V_HJ558 genes, and primer JHCHint, which hybridizes in the intron 3' of exon J_H4 (Jolly *et al.*, 1997). The 600 bp PCR product bearing the JH4 segment of the IgH was cloned into pGEM-T Easy vector (Promega) and sequenced. A stretch of 500 bp intron sequence immediately downstream of the J_H4 element was analyzed for somatic mutations using the SeqMan and MacVector software.

8.2 Cell culture

8.2.1 Primary murine B cells

CD43- and IgG1-depleted splenic B cells (Miltenyi-Biotech) were cultured in B cell medium (1x RPMI (Gibco), 10% (v/v) FCS (Biochrom KG), 1% (v/v) L-Glutamine, 1% (v/v) sodium pyruvate, 1% (v/v) Pen/Strep, 1% (v/v) non-essential amino acids (all purchased from Gibco), 50mM 2 mercaptoethanol (Sigma)) for 5 days in 96-well plates ($5x10^5$/200 µl/well). Stimuli added to the cultures included anti-CD40 antibody (2,5 µg/ml, clone 1C10, Biolegends) and IL-4 (60ng/ml; R&D Systems). For CFSE labeling, B cells were incubated in serum-free RPMI media containing 5-(and 6)-carboxyfluorescein diacetate N-succinimidyl ester (CFDA SE = CFSE; final concentration 5 µM; Molecular Probes) for 10 min. at 37°C.
For mixed B cell cultures, Ly5.1 expressing B cells were cultured 1:1 together with B cells of CD40/LMP1+//CD40-/- or CD40+/+ mice expressing Ly5.2.

8.2.2 Bone marrow derived dendritic cells from the mouse

BM-derived cells were depleted of erythrocytes by lysis, and cultured in DC medium (1x RPMI (Gibco), 20% (v/v) FCS (Biochrom KG), 1% (v/v) L-Glutamine, 1% (v/v) sodium pyruvate, 1% (v/v) Pen/Strep, 1% (v/v) non-essential amino acids (all purchased from Gibco), 50 mM 2 mercaptoethanol (Sigma), Granulocyte/Macrophage colony stimulating factor (GM-CSF) (kindly provided by Ralf Mocikat, GSF) for seven days in 6-well plates ($1{,}5x10^6$/3 ml/well). Every two days, half of the medium was replaced by fresh warm medium (days 2 and 4) or cells were splitted 1:2 (day 6), respectively. At day 7, half of the medium was replaced, and LPS added (1 µg/ml) to induce maturation of DCs. After 12 hours, un-stimulated and LPS-stimulated cells were stained for CD11c, CD40 and CD80 to perform FACS analysis.

8.2.3 EREB2-5 cell line

EREB2-5 cells were cultured in 1x RPMI (Gibco), supplemented with 10% (v/v) FCS (Biochrom KG), 1% (v/v) L-Glutamine, 1% (v/v) sodium pyruvate, 1% (v/v) Pen/Strep (all purchased from Gibco) and 1µM 1.2-estrogen. In order to remove estrogen, cells were washed three times in 1x RPMI, 10% (v/v) FCS. Between the second and third washing step, cells were incubated in 1x RPMI, 10% (v/v) FCS for 20 min. at RT, to increase the efficiency of the estrogen removal.

Generation of stable transfected EREB2-5 cells

10^7 cells, supplied with fresh medium 24 hours prior to transfection, were washed once in cold serum-free 1x RPMI, resuspended in 250 µl serum-free 1x RPMI and transferred to a 4mm electroporation cuvette (Biorad, USA). 20 µg of plasmid DNA was added and introduced into the cells by electroporation (1mF, 230V; Gene Pulser, Biorad, USA). After electroporation, cells were subsequently supplied with 500 µl ice-cold FCS and 10 min. later with 10ml 37°C warm medium to a final concentration of 20% FCS, and transferred to the incubator. Selection of cells carrying plasmids was started at day 2 after transfection by adding hygromycin B to the culture medium (75 µg/ml; Invitrogen, Germany). Cells were cultured for 20 days under hygromycin B selection before they were used for experiments.
To induce the promoter of the pRT-1 vector, doxycycline was added to the culture medium to a concentration of 100 ng/ml.

In vitro BrdU assay

$2x10^6$ cells were cultured for 4 hours in 5 ml B cell medium with 5 µl BrdU (10mM). Subsequently, cells were washed twice in PBS, resuspended in 300 µl PBS, and 700 µl Ethanol (100%) was added drop by drop while gently vortexing to fix and permeabilize the cells. Cells were incubated 30 min. on ice or stored at -20°C. Cells were washed twice with PBS, and then resuspended in 2M HCl and incubated for 30 min. at 37°C to make the incorporated BrdU in the DNA accessible. After three washing steps with PBS, cells were blocked with 10% FCS in PBS for 45 min. at RT, and then incubated with anti-BrdU antibody for 15 min. at RT. After a final washing step with PBS cells were resuspended in PBS containing 7-AAD to stain for DNA content, and FACS analysis was performed.

8.2.4 Primary human B cells

Primary human B cells were isolated from fresh adenoids. Adenoids were passed through a strainer in 1x RPMI (Gibco), supplemented with 10% (v/v) FCS (Biochrom KG), 1% (v/v) L-Glutamine, 1% (v/v) sodium pyruvate, 1% (v/v) Pen/Strep (all purchased from Gibco). Lymphocytes were isolated by Ficoll gradient, transferred to PBS/Versen (1:5000) and washed three times in PBS/Versen.

In parallel, 10 ml sheep blood was diluted in 40ml PBS/Versen and washed twice by centrifugation without brake. Sheep red blood cells (SRBC) in the pellet were used to deplete T cells. Lymphocytes were mixed with SRBC, and T cells bound to SRBC via the adhesion molecule CD2 could be separated from B cells by Ficoll gradient.

8.3 Fluorescence-activated cell sorting (FACS)

Single-cell suspension from various lymphoid organs or cultured cells were washed in FACS buffer (PBS, 0,5% (w/v) BSA). Subsequently, $1x10^6$ primary cells or $1x10^5$ cell culture cells per sample were labeled with a combination of FITC-, PE-, and APC-conjugated monoclonal antibodies, diluted in FACS buffer, for 20 min. on ice in the dark. Labeled cells were washed once more in FACS buffer and resuspended in 100 µl FACS buffer containing propidium iodide (PI) for analysis. All analyses were performed on a FACSCalibur™ (BD Biosciences) and results were analyzed using CELLQuest™ software.

8.4 Immunohistochemistry

Cryosections

Spleens were embedded in OCT Tissue-Tek (Sakura), frozen on dry ice and cut with 8µm thickness. The sections were thawed, air dried, fixed in acetone, incubated with Avidin/Biotin blocking kit (Vector), and stained with peroxidase conjugated anti-mouse IgM (Sigma), rat anti-mouse CD3 (kindly provided by E. Kremmer, GSF Munich), or biotin conjugated PNA (Vector Laboratories). Anti-CD3 antibody was detected using biotin conjugated mouse anti-rat IgG1 (Jackson Laboratories). Biotinylated reagents were detected using streptavidin coupled alkaline phosphatase (Sigma). Enzyme reactions were developed with alkaline phosphate or peroxidase substrate kit (Vector). All incubation steps were performed at 22°C in humidified chamber, followed by three washing steps with PBS. Slides were analyzed with a Zeiss microscope; pictures were obtained with a RS Photometrics digital camera and processed with Openlab from Improvision and Adobe Photoshop software.

Paraffinsections

Immunohistochemical staining of paraffin sections was performed on an automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the company's protocols. Antigen retrieval was performed with a microwave pressure cooker in 0.01M citrate buffer (pH 6.0). Incubation with the primary antibodies was performed overnight at RT. The rest of the procedure was completed on the Ventana immunostainer.

8.5 Immunofluorescence

Cells were fixated on microscopy slides using a 1:1 solution of methanol and acetone. Slides were incubated in a blocking solution (PBS/10% FCS) for 1 hour at RT, and then incubated with the LMP1-specific antibody CS1-4 (Dako, 1:1000 in PBS/10% FCS) overnight at 4°C. To detect anti-LMP1, slides were incubated with anti-mouse Cy3-antibody (1:100) for 1 hour at RT. All incubation steps were performed in a humidified chamber, followed by three washing steps with PBS/4% FCS.

8.6 Enzyme-linked immunosorbent assay (ELISA)

To determine Ig isotype concentrations and NP-specific antibodies, microtiter plates (Nunc) were coated with Ig specific rat anti-mouse antibodies or with NP3-BSA or NP17-BSA (Biosearch Technologies), respectively, in 0.1 M NaHCO3 buffer (pH 9,2) at 4°C overnight.

Subsequently, wells were blocked with PBS, 1% (w/v) milk powder solution at RT for 30 min. Serially diluted serum samples were applied to the wells and incubated for 1 hour at RT, then incubated 30 minutes at RT with biotin-conjugated secondary antibodies specific for the different isotypes, followed by the incubation with streptavidin coupled alkaline phosphatase (SA-AP) at RT for 30 minutes. The amount of bound SA-AP was detected by incubation with O- phenyldimine (Sigma) in 0,1 M citric acid buffer containing 0,015% H_2O_2. Following each incubation step, plates were washed three times with PBS. The OD at 405 nm was measured with a microplate reader (Photometer Sunrise RC; Tecan), and antibody concentrations were determined by comparison with isotype specific standards (IgM, G155-228; IgG1, MOPC-31C; IgG2a, G155-178; IgG2b, MPC-11; IgG3, A112-3; M18-254; BD Bioscience).

Anti-Cardiolipin-ELISA

To detect anti-cardiolipin antibodies in serum from un-immunized mice, an ELISA kit from Alpha Diagnostics Int., USA, was used according to the manufacture's protocol. Serum was diluted 1:100 and duplicates of each sample were tested in one experiment. The OD of the negative control serum was set two times to define positive and negative tests.

8.7 RNA isolation and analysis

Total RNA isolation was performed using TRIzol reagent (Invitrogen), which is a solution of phenol and guanidine thiocyanate. Each 1 x 10^7 cells were resuspended in 1ml TRIzol for lysis and incubated for 10 min. at RT. After this step, samples could be shock frozen on dry ice and stored at -80 °C for at least one month. For phase separation, the homogenates were supplemented with 0.2ml chloroform per 1ml TRIzol, subsequently vortexed for 15 sec., incubated at RT for 2 to 15 min. and centrifuged at 12.000 x g for 15 min. at 4 °C. Following centrifugation, the mixture separated into a red phenol-chloroform phase at the bottom of the tube, a whitish interphase and a colorless aqueous phase in the upper part, which contained the RNA. The aqueous phase was carefully transferred to a new tube and RNA was precipitated by adding 0.5 ml 100% (v/v) isopropanol per 1 ml used TRIzol. Samples were vortexed, incubated for 5 min. at RT, and then centrifuged at 12.000 x g for 8 min. at 4 °C to pelletize precipitated RNA. After one washing step of the RNA pellet with 75% (v/v) ethanol, it was briefly air-dried and subsequently dissolved in DEPC-H_2O by pipetting several times and incubating for 15 min. at 55-60 °C. The RNA concentration was determined by measuring the absorbance at 260 nm using a Bio-Photometer (Eppendorf, Hamburg). The ratio of the readings at 260 nm and 280 nm (OD_{260}/ OD_{280}) provided an estimation of the

purity of the RNA preparation, with respect to contaminants that absorb UV, such as proteins. Pure RNA has an OD_{260}/OD_{280} ratio of > 1.95.

Agarose gel electrophoresis of RNA

RNA electrophoresis is performed under denaturing conditions in 2.2 M formaldehyde according to Maniatis (Maniatis *et al.*, 1982), using the MOPS (4-morpholinopropanesulphonic acid) buffer system. RNA samples were heated for 10 min. at 60 °C to denature secondary structures of the RNA, subsequently cooled on ice for 1 min., and loaded on a RNA agarose gel (5% (v/v) formaldehyde, 1% (w/v) agarose, 1x MOPS) to electrophoretically separate it. The 18S (~1.9 kb) and the 28S (~4.8 kb) rRNA bands visualized by UV irradiation served as a loading and quality control of the RNA.

Northern Blot analysis

After separation on a RNA agarose gel as described, the gel was incubated for 12 min. in 1xSSC, 0,05 M NaOH, and subsequently in 10xSSC two times for 20 min. to get rid of the formaldehyde. RNA was transferred to a nitrocellulose membrane (Hybond-N, Amersham) in 10xSSC. After the transfer, the membrane was washed for 5 min. in water and then in 10xSSC, before it was baked for two hours at 80°C to fix the RNA. Pre-hybridization was performed in Churchpuffer (7% (w/v) SDS, 10 mM EDTA pH8.0, 250 mM Na_2HPO_4/NaH_2PO_4) at 65°C for at least 3 hours. Probes were labeled with 50 µCi α32-dCTP applying ´Random Prime Labeling Kit´ (Amersham Bioscience) and the membrane was incubated with the radioactive labeled probe for another 16 hours at 65 °C. Afterwards the membrane was rinsed in pre-heated 1x SSC, 1% (w/v) SDS at 65 °C. Bands on the membrane were visualized by autoradiography, using radiosensitive films (Biomax MS PE Applied Biosystems, KODAK).

cDNA synthesis

1 µg total RNA was reverse transcribed with > 10U of viral AMV Reverse Transkriptase, using 1st Strand cDNA Synthesis Kit for RT-PCR [AMV] (Version 3, August 2004, Roche Diagnostics) with 0.02 A_{260} units (0.8 µg) Oligo-$p(dT)_{15}$ primers according to manufacturer's instructions, whereas each reaction was performed in a final volume of 10 µl. The resulting first strand cDNA was stored at –20 °C and was used as a template for RT-PCR.

RT-PCR

RT-PCR of mature Ig transcripts was performed on a T3 Thermocycler (Biometra) using 1 ng cDNA per reaction. The amplification cycle number ranged between 30 and 40. Bands were visualized on a 1-2% agarose gel.

Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler FastStart DNA Master SYBER Green (Roche Diagnostics). cDNA was diluted in a ratio of 1:10 before use and 1 µl was added to 9 µl of LightCycler mastermix according to the manufacturer's protocol. Analyses were performed on a LightCycler instrument (Roche Diagnostics). Dilution series (10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}) of former LightCycler amplification products were prepared to generate calibration curves, needed for the determination of PCR efficiencies later on. As negative control, template DNA was replaced by PCR-grade water. Dilution series and negative control were carried out for each run. In order to document the specificity of the desired PCR products, samples were electrophoretically separated on a 2% (w/v) agarose gel and analyzed on a UV luminescent screen. Additionally, LightCycler melting curve analysis was performed. The copy number of analyzed gene products was normalized to c-abl, a gene known to be equally expressed in EREB2-5 cells grown under different conditions (personal communication Martin Schlee).

LightCycler run protocol:

Starting temperature:	95 °C	10 min.
Cyclic denaturation:	95 °C	1 sec.
Cyclic annealing:	54 to 65 °C	10 sec.
Cyclic elongation:	72 °C	1 sec. per 25 bps
Melting	70 to 97 °C	10 sec
Transitionrate	0.1 °C/sec.	
Cooling	40 °C	15 sec.
Cycle number:	55	

8.8 Western blot analysis

For protein extraction, cells were lysed in hot 2x Laemmli sample buffer (2.5% (w/v) SDS, 20% (v/v) glycerin, 0.12 M Tris pH6.8) containing protease inhibitors (Protease Inhibitor Complete Mini Tablets, Roche). Samples were boiled at 100°C for 5 min. and mixed well by pipetting. Lysates were subsequently used or frozen at –80°C. Protein concentration was quantitated using a Bradford reagent (DC protein assay; Bio-Rad) and a bovine serum albumin standard curve.

Protein mixtures were separated by discontinuous SDS-PAGE (Laemmli, 1970). 2/10 volume of sample buffer (Bromphenol blue solution, 0.5 M DTT) was added to cell lysates and boiled for 5 min. at 100°C to denature proteins. Samples were subsequently loaded an a SDS polyacrylamide gel, composed of 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), in which charged proteins are focused, and a resolving gel (10% (v/v) acrylamide, 3.75mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.004% (w/v) TEMED), in which proteins are separated according to molecular weight. For size determination of separated proteins, a protein ladder (Pre-Stained Protein Ladder; Invitrogen) was used. Electrophoresis was accomplished in Laemmli running buffer (25 mM Tris base, 0.2 M glycine, 0.1% SDS) at 100 V, using a Bio-Rad electrophoresis chamber.

After separation, proteins were transferred onto a polyvinylidenfluoride (PVDF) membrane (Hybond-P, Amersham) by "wet transfer" in transfer buffer (25 mM Tris base, 0.2 M Glycine, 20% (v/v) methanol, in H_2O), utilizing a Bio-Rad-Tank. Proteins were transferred for 1.5 h at 100V on a magnetic stirrer. To verify protein transfer, the membrane was stained with Ponceau S solution (2% PonceauS, 30% Trichloroacetic acid, 30% Sulfosalicyl acid).

Membranes were incubated for 1 h in PBS, 5% (w/v) milk powder solution with slight shaking to block unspecific reactions. For primary antibody incubation, membranes were rolled over night at 4°C with the primary antibody dilution in PBS, 1% (w/v) milk powder solution. The membrane was washed three times for 5 min. in PBS, 1% (w/v) milk powder solution and subsequently incubated with secondary antibody conjugated to HRP, diluted in PBS, 1% (w/v) milk powder solution for 1.5 h at RT. After three washing steps as before, the membrane was shortly rinsed with water and proteins were visualized using Enhanced Chemiluminescence (ECL)-System (Amersham). Signals were detected by the exposition of photosensitive films (Amersham) using a M35 X-OMAT processor (Kodak).

8.9 Southern Blot analysis

ES cell DNA was digested with EcoRI and separated on a 0.8% agarose gel overnight. Then the gel was incubated in 0.25 N HCl for 20 min., shortly rinsed with water and incubated for 40 min. in alkaline transfer buffer (0.4 M NaOH, 0.6 M NaCl), incubation steps performed on a shaker. Subsequently, the DNA was blotted from the gel to a nitrocellulose membrane (Hybond-N, Amersham) in transfer buffer overnight. After blotting, the membrane was rinsed

in 2xSSC (0.3 M NaCl, 0.03 M NaCitrate; pH6,5) for neutralization. DNA fragments were fixed on the membrane by heat (2 h, 80°C).

To block non-specific binding, the membrane was pre-hybridized in pre-heated hybridization solution (1 M NaCl, 50 mM Tris, pH7.5, 10% (w/v) dextran sulfate, 1% (w/v) SDS, 250 µg salmon sperm DNA/ml) for at least 3 hours at 64°C. 50-100 ng DNA probe (*rosa26*-probe (Soriano, 1999)) were labeled with 50 µCi α32-dCTP applying ´Random Prime Labeling Kit´ (Amersham Bioscience) and the membrane was incubated with the radioactive labeled probe for another 16 hours at 65°C. Afterwards the membrane was rinsed in pre-heated 2xSSC, 0.1% (w/v) SDS at 65°C. Bands on the membrane were visualized by autoradiography, using radiosensitive films (Biomax MS PE Applied Biosystems, KODAK).

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Education and Career

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PhD thesis at the GSF - National research center for environment and health, Institute of Clinical Molecular Biology and Tumor Genetics in Munich, Germany.
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Studies of biology at the University of Vienna, Austria.
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Research work at the Institute of Cancer Research, University of Vienna.
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"B cell specific LMP1 signaling perfectly mimics CD40 in vivo and harbors unique features to induce class switch recombination"

Cornelia Hömig, **Julia Rastelli**, Stefano Casola, Caroline Hojer, Werner Müller, Leticia Quintanilla-Martinez, Andreas Gewies, Jürgen Ruland, Klaus Rajewsky and Ursula Zimber-Strobl

"Constitutive CD40 signaling in B cells leads to the activation of the non-canonical NFκB-pathway and promotes lymphomagenesis"