Technische Universität München
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Functional role of Bcl10 and Malt1 in signal transduction from the FcεRI in mast cells and the LPA receptor in murine embryonic fibroblasts

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Für meine Familie
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List of abbreviations

%	percent
(w/v)	weight in volume
(v/v)	volume in volume
α	anti
Δ	delta (difference)
λ	wavelenght
µg	10^{-6} gram (microgram)
µl	10^{-6} liter (microliter)
µmol	10^{-6} mol (micromol)
µM (µmol/l)	10^{-6} mol/liter
A	alanine/Ampère
aa	amino acid
ab	antibody
a.d.	aqua destillata
ADP	adenosine diphosphate
Akt/PKB	V-Akt murine thymoma viral oncogene homologue 1/protein kinase B
APC	antigen-presenting cell
appr.	approximately
APS	ammoniumperoxodisulfate, ammoniumpersulfate
ATP	adenosine triphosphate
bp	basepairs
Bcl10	B cell lymphoma 10
Bcl-X_L	B cell lymphoma-extra long
BM	bone marrow
BMMC	bone marrow-derived mast cell
BSA	bovine serum albumin
C	Celsius
caspase	cysteinyl-aspartate-specific protease
CD	cluster of differentiation
cDNA	complementary DNA
C-terminal	carboxy-terminal
d	day
D	aspartate/Dalton

DC	dendritic cell
ddH_2O	bidistilled water
del	deletion
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynuleoside-5'-triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
E	extinction
E. coli	*Escherichia coli*
e.g.	for example (from latin "*exempli gratia*")
ECL	enhanced chemiluminescence
EDTA	ehtylenediamine-N,N,N',N'-tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIA	enzyme-linked immunoassay
ELISA	enzyme-linked immunosorbent assay
EMSA	electromobility shift assay
Erk	extracellular signal-regulated kinase
et al.	*et alteri*
EtBr	ethidium bromide
FACS	fluorescence activated cell sorting
FCS	fetale calf serum
FSC	forward scatter
g	gravitation (9.81 m/s^2)/gram
GAPDH	glycerolaldehyde dehydrogenase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	human/hour
H. pylori	*Helicobacter pylori*
HEPES	N-(2-hydroxyethyl)-piperacine-N'-2-ethanesulforic acid
HCl	hydrochloric acid
HRP	horseradish peroxidase
IκB	inhibitor of κB

IB	immunoblot
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IKK	inhibitor of κB kinase
IL	interleukin
Iono	ionomycin
IP	immunoprecipitation
IRES	internal ribosomal entry site
i.p.	intraperitoneally
i.v.	intravenously
kb	kilo bases, 1000 basepairs
kD, kDa	kilodalton
kg	kilogram
KOH	potassium hydroxide
l	liter
LN	lymph node
log	logarithm
LPA	lysophosphatidic acid
$LTC_4/D_4/E_4$	leukotriene $C_4/D_4/E_4$
LTR	long terminal repeat
m	murine/monoclonal
M	molarity/methionine/marker
mA	10^{-3} Ampere/milliampere
MALT	mucosa-associated lymphoid tissue
Malt1	mucosa-associated lymphoid tissue protein 1
MAPK	mitogen-activated protein kinase
mg	10^{-3} g, milligram
MHC	major histocompatibility complex
min	minute
MIP-2	macrophage inflammatory protein 2
ml	10^{-3} liter, milliliter
mM (mmol/l)	10^{-3} mol/l
mRNA	messenger RNA
n	number
NaCl	natrium chloride

NF-κB	nuclear factor κB
NF-Y	nuclear factor Y
ng	10^{-9} gram, nanogram
nm	10^{-9} meter, nanometer
nM (nmol/l)	10^{-9} mol/liter
NP-40	nonylphenoxypolyethoxyethanol, Nonidet P-40
N-terminal	amino-terminal
OD	optical density
p	phosphorylated
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pg	10^{-12} gram, pikogram
PE	phycoerythrine
pH	pondus hydrogenii
phospho	phosphorylated
PI	propidium iodide
PI3K	phosphoinositol-3-kinase
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
pmol	10^{-12} mol, picomol
PRR	pattern recognition receptor
PVDF	polyvinyl difluoride
R	arginine
rm	recombinant murine
rmIL-3	recombinant murine interleukin 3
rmSCF	recombinant murine stem cell factor
RNA	ribonucleic acid
rpm	rounds per minute
RT	reverse transcription/room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SCF	stem cell factor

SD	standard deviation
SDS	sodiumdodecylsulfate
SDS-PAGE	sodiumdodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of means
SH	*Src* homology
SSC	sideward scatter
ssRNA	singlestranded RNA
T	temperature
TAE	Tris-acetate EDTA
Taq	*Thermophilus aquaticus*
TBE	Tris-borate EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
TLR	Toll-like receptor
T_m	melting temperature
TNF-α	tumor necrosis factor-α
Tris	Trishydroxymethylaminomethane
Triton® X-100	t-octylphenoxypolyethoxyethanol
U	unit
UV	ultraviolet
V	volume/volt
wt	wildtype
Y	tyrosine

1 Introduction

1.1 The immune system

The immune system (referring to the Latin word *immunis* = “free”, “untouched”, “intact”) is the system of defence within an organism, which it protects against infection. It identifies and kills invading pathogens ranging from viruses to parasitic worms and distinguishes them from the organism’s normal cells and tissues. The immune system of vertebrates is made up of a complex and dynamic interaction of several organs, cell types and proteins (Janeway, Travers *et al.* 2001).

1.1.1 Innate immunity

Early in phylogeny, the relatively simple and non-specific *innate immunity* evolved, which is represented by anatomical and physiological barriers, cell-mediated defence via phagocytosis, inflammatory responses or the biochemical cascade of the complement system (Janeway, Travers *et al.* 2001; Janeway and Medzhitov 2002). If a microorganism succeeds in overcoming the epithelial barrier of the body, it will immediately be identified by innate immune cells, which respond to it in a vindicative way. But, in contrast to the *adaptive immunity* (see chapter 1.1.2), these responses do not confer long-lasting or protective immunity to the host.

1.1.1.1 Cells of the innate immune system

The cell types of innate immunity mostly belong to the group of white blood cells known as *leukocytes* and can be roughly classified - according to the content of intracellular granules - into ***granulocytes*** and ***agranulocytes*** (Janeway, Travers *et al.* 2001).
Granulocytes comprise the innate immune cells neutrophils, eosinophils and basophils. They are named after their numerous intracellular vesicles with bactericidal substances, inflammatory mediators (e.g. histamine) or basic proteins and can attack and destroy bacteria through chemotaxis and phagocytosis (Alberts, Johnson *et al.* 2002; Janeway and Medzhitov 2002).
In contrast, lymphocytes, monocytes and macrophages belong to the group of agranulocytes and are characterized by the absence of intracellular granules.
The most important innate immune cells and their function shall be described in the following paragraphs.

Phagocytes are cells that engulf pathogens or foreign particles and subsequently digest them through a process called phagocytosis. This group comprises the *macrophages* (from the Greek expression for “large eating cell”), which mature from monocytes and can move across the cell membrane outside the vascular system. They can present ingested parts of the microorganism, which have been degraded to single peptides or epitopes on the cell surface. This renders the macrophage an antigen-presenting cell (APC) that is able to activate cells of the adaptive immune system (see chapter 1.1.2). *Neutrophils* constitute 50-60% of total circulating leukocytes and contain a variety of toxic substances and strong oxidizing agents to inhibit growth of or kill pathogens. They are particularly involved in inflammatory processes. The third phagocytic cell group are the *dendritic cells (DCs)*, which mainly reside in tissues that are in contact with the external environment (Janeway and Medzhitov 2002). They are involved in innate immunity in particular through the process of antigen presentation, and therefore serve as an important link between innate and adaptive immunity.

Mast cells (MCs) are a type of innate immune cells found in mucous membranes and connective tissues and are not only associated with the defence against pathogens and wound healing, but also with allergic and anaphylactic reactions. The latter are induced through the release of proinflammatory mediators like histamine, serotonin or heparin and lipid mediators from their intracellular granules. A more detailed description of mast cells and their role in the immune system will be given in section 1.4.

The family of *basophils* and *eosinophils* is directly related to the neutrophils as another main group of granulocytes. They are responsible for the defence against parasites and important in allergic reactions and asthma. As such, basophils - like MCs described above - release histamine, proteolytic enzymes and lipid mediators. Eosinophils also contain histamine and, furthermore, proteins like peroxidases, RNases and DNases and reactive oxygen species.

The group of innate immune cells named *natural killer (NK) cells* is able to distinguish foreign pathogens from healthy body cells, because the latter all possess the major histo-compatibility complex (MHC) on their surface. Thus, foreign cells that do not contain the MHC will be recognized and destroyed. Upon contact with a cell lacking the MHC class I complex - a so-called “missing self” - NK cells can kill invading bacteria without having been in contact with the pathogen before. Moreover, virus-infected somatic cells or tumour cells, which possibly have lost their MHC class I complex, are destroyed by NK cells.

1.1.1.2 Innate immunoreceptors

Innate immune cells are able to recognize pathogen-associated molecular patterns (PAMPs) through a variety of germline-encoded receptors. The latter are referred to as *pattern recognition receptors* (PRRs) and can be membrane-bound, cytoplasmatic or secreted.

Membrane-bound PRRs, amongst others, contain the major group of *Toll-like receptors* (TLRs), which recognize a wide range of PAMPs, e.g. lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4) and peptidoglycan from Gram-positive bacteria (TLR2), bacterial flagellins (TLR5) and bacterial DNA (TLR9) or viral dsRNA (TLR3) (Medzhitov 2001; Barton and Medzhitov 2003). Other membrane-bound PRRs, the *lectins*, are glycoproteins or carbohydrate-binding proteins that are able to bind carbohydrate moieties free in solution or bound to a pathogen and thereby can agglutinate cells or precipitate glycoconjugates.

Cytoplasmatic PRRs are e.g. *NOD-like receptors* (NLRs), in which NOD stands for nucleotide-binding oligomerisation domain, and they include the two major subfamilies called NODs and NALPs (NACHT, leucine rich repeat and pyrin domain containing). *RNA helicases* are responsible for the recognition of viral double-stranded RNA and other single-stranded RNA molecules. Both groups contain N-terminal caspase recruitment domains (CARD) for intracellular signal transduction.

Secreted receptors like the mannan-binding lection (MBL) do not remain associated with the producing cell and recognize phospholipids or sugar groups on the surface of microorganisms, but also bind to nucleic acids or non-glycosylated proteins.

Each receptor binds to a small range of conserved molecules from a group of pathogens and upon ligand = PAMP binding, the membrane-bound pattern-recognition receptors are triggered to activate phagocytosis and the cytoplasmatic PRRs often form oligomers, which activate inflammatory caspases. This leads to the activation of specific signalling pathways like intracellular bactericidal mechanisms or the production of inflammatory cytokines (e.g. TNF-α, IL-1, IL-12) and the enhancement of the cells' antimicrobial killing mechanisms and antigen-presenting capacity (Janeway and Medzhitov 2002).

1.1.2 Adaptive immunity

The complex *adaptive* or acquired *immune system* evolved from innate immunity and is characterized by the ability to adapt to novel or modified pathogens. Cells of the adaptive immune system are able to identify specific antigens of the invading microorganisms by specific *antigen-receptors* (see section 1.1.2.2).

1.1.2.1 Cells of the adaptive immune system

Besides APCs like dendritic cells, which are also involved in innate immune responses (see section 1.1.1.1), two other main groups of cells represent the essential elements of the adaptive immune system.

The different types of ***T lymphocytes*** originate in the bone marrow and mature in the **t**hymus ("T" lymphocytes) and provide for cell-mediated immune responses. They are grouped according to specific CD proteins (**c**luster of **d**ifferentiation) on their cell surface and their resulting function (Janeway, Travers *et al.* 2001; Alberts, Johnson *et al.* 2002).

T helper cells (T_h cells) carry the CD4 protein and recognize antigens presented by APCs on MHC class II complexes via their specific T cell receptor (TCR). This activates the T helper cells to secrete cytokines enhancing the immune response and activating B lymphocytes (see below).

Regulatory T cells (T_{reg} cells), also known as T suppressor cells, additionally bear CD25 on their surface and suppress immune responses of other cells by inducing tolerance to self-antigens and thereby, maintain the homeostasis of the immune system.

Cytotoxic T cells recognize antigens presented on MHC class I molecules, e.g. from infected somatic cells, and adhere to these cells via their TCR and the CD8 receptor. Fast proliferation and the secretion of cytotoxic substances like cell death-inducing enzymes (granzymes) and pore-forming proteins (e.g. perforin) lead to the death of infected or mutated cells and make them the most potent killer cells of the immune system.

An additional central function of T cells is the support and activation of ***B lymphocytes***. The latter belong to the family of leukocytes, originate in the **b**one marrow ("B" lymphocytes) and are responsible for the humoral immunity (from the Latin word *humour* = "fluid"). If a B cell binds to an antigen matching its unique B cell receptor (BCR) and

receives an additional signal from a T_h cell, it gets activated via T_h cytokines to proliferate and further differentiate either into a *plasma B cell* or a *memory B cell*.

Plasma B cells produce and secrete large amounts of specific antibodies directed against pathogens, which on the one hand immobilise the pathogens and on the other hand mark them for the destruction through other immune cells.
Memory B cells are long-living cells and can quickly respond to the same antigen upon a second exposure with the pathogen as an adaptation to the infection, which, together with the preservation of specific antibodies, is a main characteristic of adaptive immunity.

Despite its great variability and this denotative adaptability, the adaptive immune system cannot replace the innate immune system, but instead they collaborate. Only this coordinated interaction of adaptive and innate immune responses enables the complex immune reaction of an organism.

1.1.2.2 ITAM-containing multi-subunit immunoreceptors

A major group of immunoreceptors of the adaptive immune systems is composed of several subunits, which contain one or more immunotyrosine-based activation motifs (ITAMs). The ITAM is a specific consensus sequence of amino acids (Tyr-X-X-Leu/lle) occurring twice in close succession spaced by six to eight amino acid residues. When the tyrosine (Y) residue is phosphorylated by receptor-proximal tyrosine kinases of the Src (sarcoma) family, the ITAMs function as “on and off” switches linking the immunoreceptor to an intracellular signalling cascade proceeding via multiple adaptor molecules (Isakov 1997).
This immunoreceptor family contains important antigen receptors like the TCR and BCR, whose great variety is generated by somatical rearrangement of *TCR* and *BCR* genes during early stages of T cell and B cell development, respectively, in contrast to the germline-encoded and therefore relatively invariant innate immunoreceptors. Though the multi-subunit Fc receptors are mainly found on innate immune cells, they shall nevertheless be covered in this section, for they also are able to confer antigen-specificity.

1.1.2.2.1 T cell receptor

The T cell receptor (TCR) on the surface of T lymphocytes (figure 1a) is responsible for the recognition of MHC-bound antigens. It is composed of a heterodimer of a clonally

variable α and β chain for antigen binding, each chain belonging to the immunoglobulin superfamily. This αβ complex interacts with additional invariant accessory molecules, in particular the CD3 complex consisting of two ε, one γ and one δ chain or a homodimer of ζ chains (see figure 1a), which transduce signals via their cytoplasmatic ITAMs to the cell interior (van Oers, Love *et al.* 1998).

1.1.2.2.2 B cell receptor

The B cell receptor (BCR) complex on the surface of B lymphocytes is composed of a Y-shaped cell surface immunoglobulin consisting of the variable antigen-binding part of the heavy and light immunoglobulin chains and the constant region (see figure 1b). Since this antibody cannot itself generate a signal, it is attached to antigen-non-specific accessory molecules (disulfide-linked Igα and Igβ chains) carrying out the signalling function through a single ITAM in each case (Takata and Kurosaki 1996; Jiang, Craxton *et al.* 1998; Craxton, Jiang *et al.* 1999).

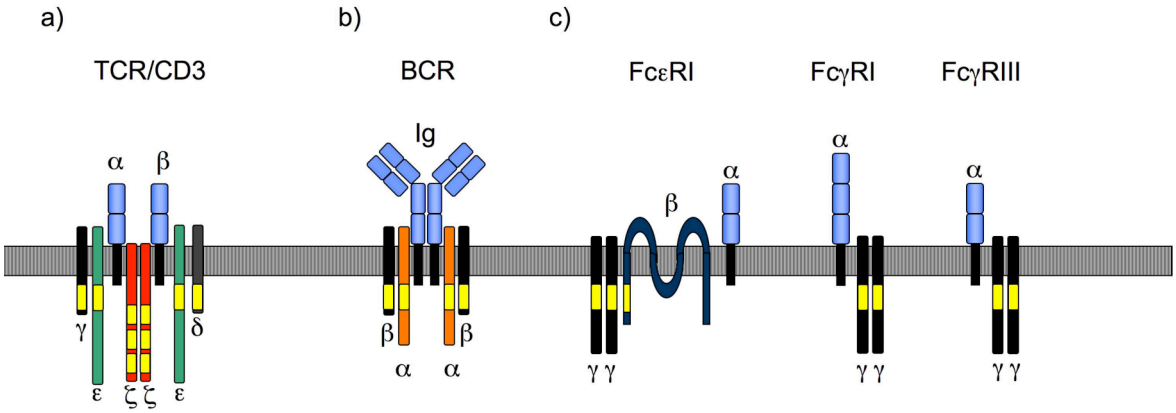


Fig. 1: Domain structure of antigen-binding immunoreceptors with ITAMs

The figure displays the various domains of immunoreceptors in the cell membrane (black&white-striped). Yellow rectangles represent signal-transducing ITAMs. (a) The TCR consists of the antigen-binding α and β chain and the signal-transducing subunits with ITAMs in the cytoplasmatic portion: a homodimer of ζ chains and the CD3 complex composed of two ε chains, one γ and one δ chain. (b) The BCR is composed of the invariant heavy and light immunoglobulin chains (Ig) and in each case two α and β chains. (c) Fc receptors, such as the IgE receptor FcεRI or the IgG receptors FcγRI or FcγRIII, consist of an Ig-binding α chain, a homodimer of ITAM-containing γ chains and in case of the FcεRI a membrane-spanning β chain.

1.1.2.2.3 Fc receptors

Fc receptors constitute the third big class of antigen-binding immunoreceptors and are mainly found on the surface of innate immune cells, such as NK cells, macrophages, neutrophils, dendritic cells and mast cells. The name is derived from their binding

specificity for the Fc part (Fragment, crystallisable) of an antibody (figure 2), and they bind to antibodies attached to infected cells or invading pathogens. This activity stimulates phagocytic or cytotoxic cells to destroy these microorganisms by phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC).

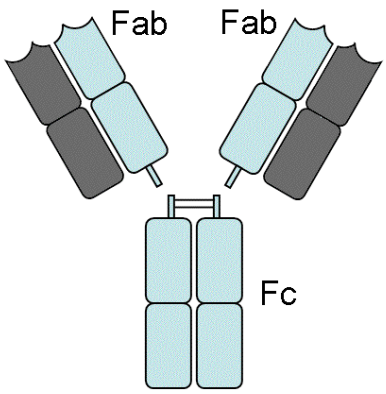


Fig. 2: Portions of an antibody (from Janeway 2001)

An antibody is made up of four protein chains, two heavy chains (blue) and two light chains (grey). They are linked by disulfide bonds and form two **Fab** (fragment, antigen-binding) regions and one **Fc** (fragment, crystallisable) region. Fc receptors bind to the Fc fragment.

All different types of Fc receptors are multichain immunoreceptors composed of an immunoglobulin-binding α-chain and signal-transducing β- and γ-chains (figure 1c). Their classification is based on the type of antibody they recognize: Fc gamma receptors (e.g. FcγRI, FcγRIII) bind to the most common class of immunoglobulin G (IgG) antibodies, Fc alpha receptors (FcαR) bind to IgA and those that bind to IgE are called Fc epsilon receptors (e.g. FcεRI, FcεRIII). An important receptor on granulocytes involved in allergic reaction is the high affinity IgE receptor FcεRI, which will be addressed in chapter 1.4 (Fridman 1991).

After antibody binding to the ligand-binding motif and antigen-mediated crosslinking of Fc receptors, they generate intracellular signals through the γ-chain ITAMs. If only one YXXL/I motif is present in an Fc receptor, this is not sufficient to activate cells, but instead represents an immunotyrosine-based inhibitory motif (ITIM), which controls the activities of numerous phosphatases to inhibit Fc receptor signalling.
Once the Fc receptor molecules are crosslinked, their phosphorylated ITAMs subsequently recruit and activate the receptor-proximal tyrosine kinases ZAP70 (ζ chain-associated protein of 70 kD) or Syk (spleen tyrosine kinase) to induce activation of multiple downstream signalling pathways (Turner and Kinet 1999). Important target proteins of ZAP70 and Syk in FcεRI signal transduction are phosphatidyl-inositol-3-kinase (PI3K) to induce Akt or Rac activation, the molecular adaptor Grb2 (growth factor receptor-bound protein 2), which triggers Ras and Erk (extracellular signal-regulated

protein kinase) activation, and phospholipase γ (PLCγ), generating second messengers to activate protein kinase C (PKC) isoforms (Costello, Turner *et al*. 1996).

1.1.3 Multiple cell activation pathways

Activation of the previously described multichain immunoreceptors is crucial for the formation, differentiation and activation of immune cells and their responses to infectious agents. Due to this great variety of required effector functions, immunoreceptor activation induces a multitude of intracellular signalling cascades, primarily induced by the receptor-proximal signalling molecules such as transmembrane adaptors, finally leading to the induction of the appropriate cellular responses. These can on the one hand be represented by non-transcriptionally regulated cell activation mechanisms, but on the other hand by the transcriptional induction of proteins involved in immunoregulatory pathways.

Cell activation mechanisms, which are not transcriptionally regulated, include for example the release of preformed mediators from intracellular vesicles, the induction of chemotaxis through integrin activation and integrin-mediated adhesion or cytoskeletal rearrangements.

In contrast, there is a multitude of signalling pathways leading to the activation of central immunoregulatory transcription factors. A major group are the mitogen-activated protein kinase (MAPK) signalling cascades via multiple protein phosphorylation steps, which are induced amongst others by growth factors, mitogens, stress, and inflammatory cytokines. At the end, they induce various transcription factors, such as activating protein 1 (AP-1), signal transducers and activators of transcription (STATs), nuclear factor of activated T cells (NFAT), Myc/Max or nuclear factor κB (NF-κB), all of them leading to the expression of cytokines, chemokines or enzymes controlling immune regulation, inflammation, proliferation and many more.

1.2 The transcription factor NF-κB

1.2.1 Function in the immune system

The mammalian family of transcription factors NF-κB plays a central role in the regulation of innate and adaptive immune responses and its members control the expression of numerous cytokines and antimicrobial effectors as well as of genes that regulate differentiation, survival and proliferation of various immune cells (Ghosh, May *et al.* 1998;

Ghosh and Karin 2002; Karin and Lin 2002; Karin and Greten 2005). NF-κB proteins can be activated by a multitude of immunoreceptors, such as TLRs (see chapter 1.1.1.2), antigen-specific receptors like the TCR, BCR or Fc receptors (see chapter 1.1.2.2), but also stimulation of G protein-coupled receptors (GPCRs, see chapter 1.5) can lead to prominent NF-κB induction (Ye 2001; Weil and Israel 2004). Thus, NF-κB is a key regulator in the differentiation and proliferation of immune cells like T and B cells, but also the activation of dendritic cells or macrophages and the homeostasis of non-immune cells depends on NF-κB-derived signals (Li and Verma 2002).

In addition, NF-κB is a master regulator in inflammation and cancer (Karin and Greten 2005), and it also provides a mechanistic link between both, because NF-κB activation in response to infectious agents leads to the production of proinflammatory cytokines (e.g. TNF-α, IL-6, IL-8) from inflammatory cells. Taking these functions together, NF-κB can be designated a central regulator of the immune system.

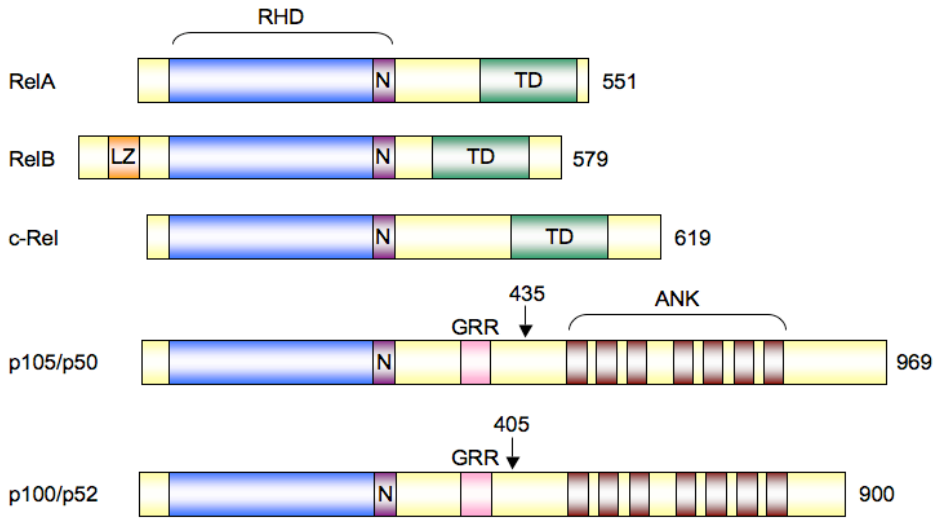


Fig. 3: Mammalian NF-κB family members (after Li and Verma 2002)

The nuclear factor-κB (NF-κB) family comprises five members: RelA (p65), c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2). They contain a structurally conserved amino-terminal Rel-homology domain (RHD), which contains the dimerisation (blue), nuclear-localization (N, purple) and DNA-binding domains. c-Rel, RelB and RelA proteins additionally have a carboxy-terminal non-homologous transactivation domain (TD, green). RelB furthermore has a leucine-zipper motif (LZ, orange) at its N-terminus. p105 and p100 contain RHDs at the amino terminus and ankyrin (ANK) repeats (brown) at the C-terminus. Proteolytic processing of p105 and p100 at residues 435 and 405 (as indicated by arrows), respectively, generates the active p50 and p52 NF-κB proteins. The glycine-rich region (GRR, pink) and C-terminal sites of inducible phosphorylation are required for processing. The size of each human protein is shown on the right (number of amino acids).

1.2.2 Structure and activation

The family of NF-κB transcription factors consists of five subunits (figure 3): RelA (p65), RelB, c-Rel, p50 (p102/NF-κB1) and p52 (p100/NF-κB2), which can be assembled into homo- or heterodimers via their Rel homology domain (RHD) to form active NF-κB complexes, but the predominant NF-κB heterodimer in most of the immune cells is p50/RelA (p65) (Ghosh, May *et al.* 1998; Li and Verma 2002; Hayden and Ghosh 2004). In resting cells, the NF-κB complex is retained in an inactive state in the cytoplasm through the binding to inhibitory proteins, the IκB proteins (inhibitors of κB).

There is a ***classical*** (or ***canonical***) NF-κB activation pathway (figure 4a) occurring through the IκB kinase (IKK) complex, which is composed of the two catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ/NEMO. This complex can be activated by various stimuli and phosphorylates IκB on N-terminal serine (Ser)- and threonine (Thr)-residues. This targets the inhibitor to polyubiquitination and fast degradation by the proteasome. Subsequently, active NF-κB dimers, in particular p50/RelA, are free to translocate to the nucleus to induce gene transcription of inflammatory mediators, cytokines and chemokines for innate and adaptive immune responses and the expression of pro-survival proteins.

Additionally, an ***alternative*** or ***non-canonical*** pathway for NF-κB activation exists, which is induced through selected TNF-receptor superfamily members. It is under the control of the NF-κB-inducing kinase (NIK) and specifically activates IKKα homodimers to phosphorylate the p52 precursor p100, resulting in proteolytic degradation of its IκB-like domain and subsequent release of active p52/RelB dimers to the nucleus (figure 4b) (Ruland and Mak 2003). This non-canonical pathway is important for the generation of secondary lymphoid organs, B cell maturation and survival and adaptive humoral immunity, but is not directly involved in innate immunity and inflammation (Dixit and Mak 2002; Luo, Kamata *et al.* 2005; Karin 2006).

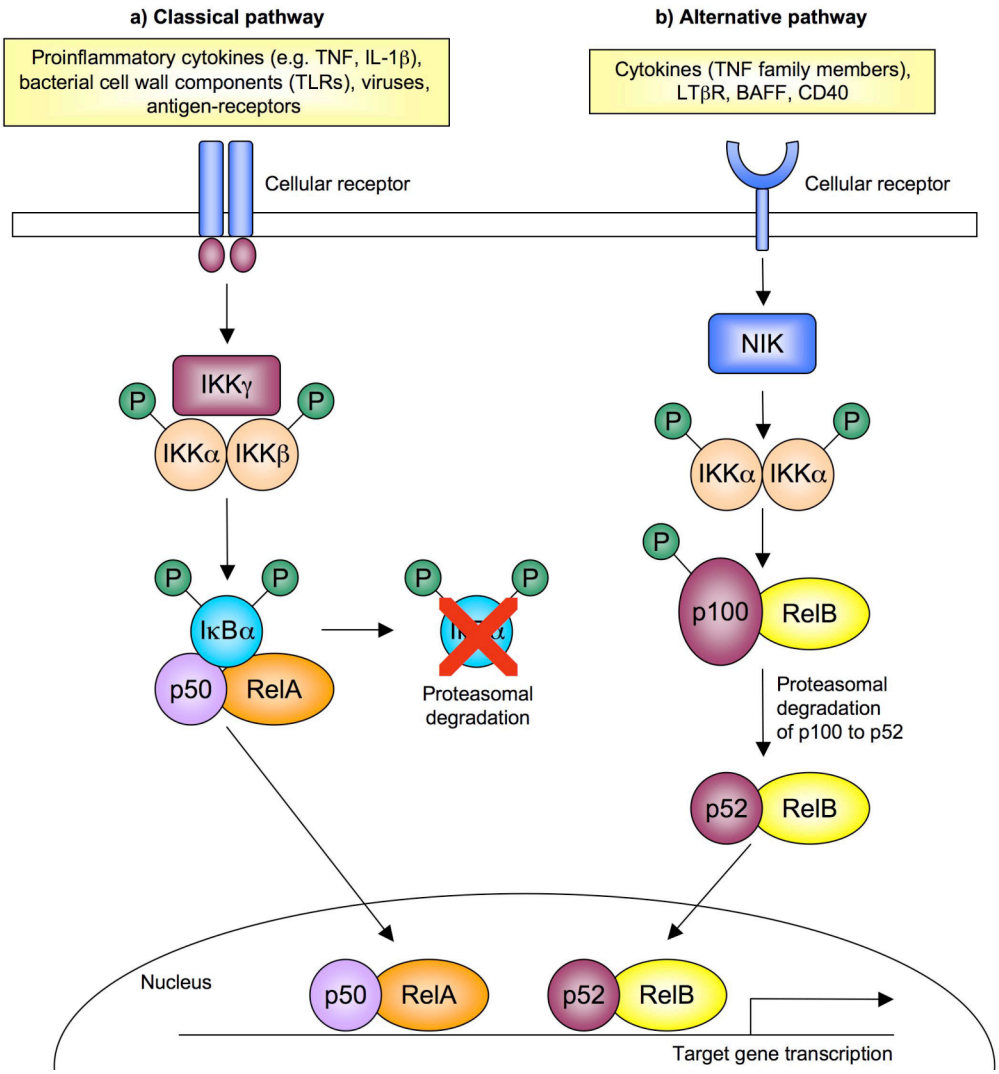


Fig. 4: Classical and alternative NF-κB signalling pathway (after Karin and Greten 2005)

a) The classical, canonical NF-κB pathway is activated by a variety of inflammatory signals and leads to the IKKα/β-mediated phosphorylation and degradation of IκB-α, finally resulting in coordinate expression of multiple inflammatory and innate immune genes by p50/RelA dimers.
b) The alternative, non-canonical NF-κB pathway is activated by lymphotoxin β receptor (LTβR), B cell-activating factor belonging to the TNF family (BAFF) and CD40. It is strictly dependent on IKKα homodimers phosphorylating p100, which induces its proteasomal degradation and the translocation of active p52/RelB dimers. The alternative pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs and adaptive humoral immunity.

1.2.3 Specific adaptors in NF-κB activation

The molecular factors controlling NF-κB activation downstream of the various immunoreceptors and upstream of the IκB/IKK complex are cell type- and stimulus-specific, because different receptors assemble a unique set of adaptor molecules for specific NF-κB activation pathways.

1.2.3.1 Adaptors in TCR-mediated NF-κB activation

One of the key signalling proteins in TCR-mediated NF-κB activation (figure 5, left pathway) is the ζ-chain-associated protein of 70 kD (ZAP70), a kinase of the spleen tyrosine kinase (Syk) family (Salojin, Zhang *et al.* 1999; Isakov, Wange *et al.* 1995). It couples to TCR ITAMs and influences downstream events by phosphorylating the adaptor protein linker of activated T cells (LAT) and Src homology (SH) 2 domain-containing leukocyte protein of 76 kD (SLP-76) (Kuhne, Lin *et al.* 2003). These play an essential role in organising multimolecular signalling complexes in the lipid raft components of the cell membrane (Zhang, Sloan-Lancaster *et al.* 1998; Herndon, Shan *et al.* 2001).

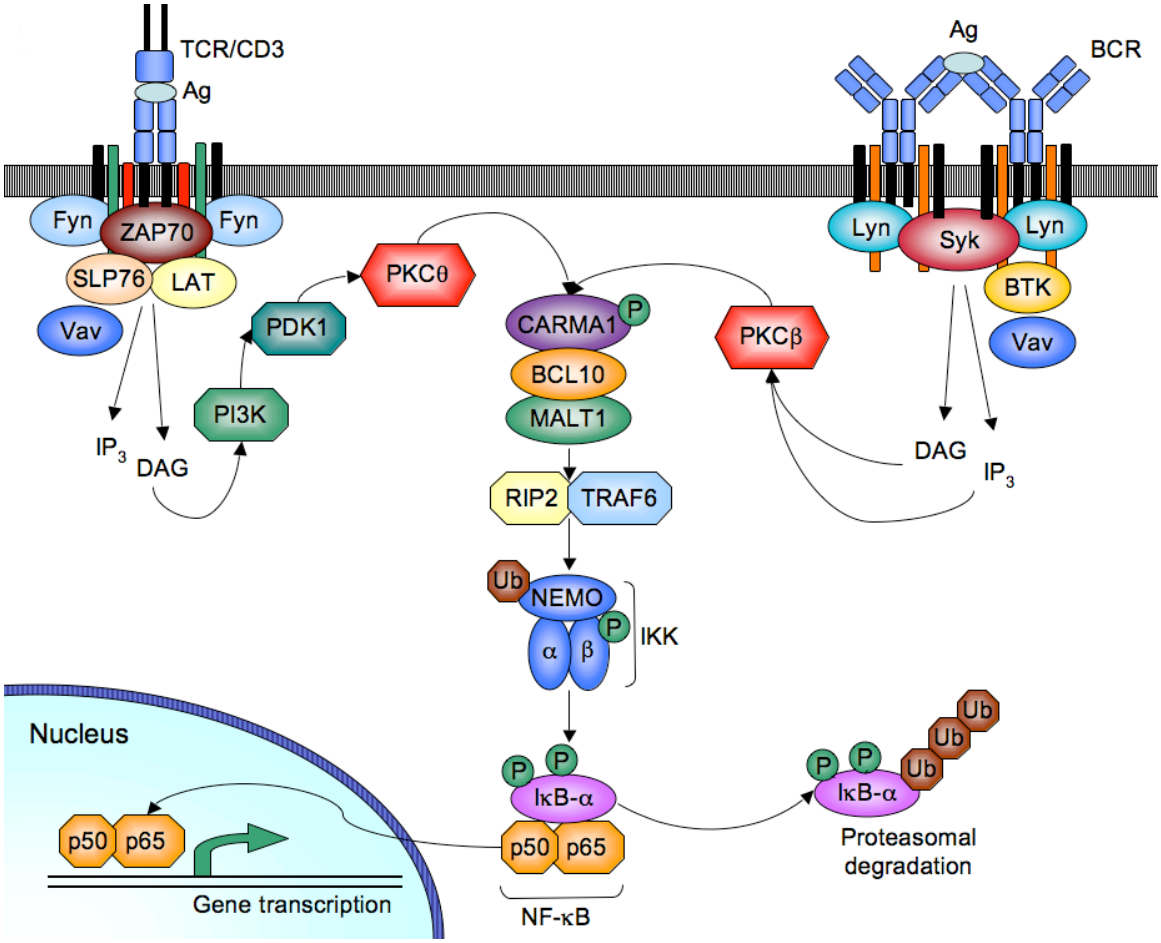


Fig. 5: TCR- and BCR-receptor-specific adaptors for NF-κB activation

TCR-mediated NF-κB activation (left side) essentially involves the ζ chain-associated protein of 70 kD (ZAP-70) and the adaptor Vav to generate second messengers (IP_3 and DAG) and to induce PI3K (phosphatidyl-inositol-3 kinase) to activate phosphoinositide-dependent kinase 1 (PDK1). This kinase phosphorylates PKCθ, which subsequently recruits the essential adaptor complex CARMA1/BCL10/MALT1 to activate IKKγ/NEMO via RIP2/TRAF6. BCR-mediated NF-κB activation (right side) occurs through receptor-associated spleen tyrosine kinases (Syk), Bruton's tyrosine kinase (BTK) and the adaptor Lyn to generate second messengers and finally induce PKCβ activation and the recruitment of CARMA1/BCL10/MALT1 for IKK-mediated NF-κB induction.

Importantly, TCR signalling is enhanced by costimulation of the transmembrane CD28 complex (not shown in figure 5), which also induces activation of effectors like the tyrosine kinase ZAP-70 and then activates downstream adaptors.

Another protein in the TCR-induced signalling complex is the guanine nucleotide exchange factor (GEF) Vav1 (Costello, Walters *et al.* 1999), which undergoes rapid tyrosine-phosphorylation after TCR ligation and leads to the generation of second messengers and the activation of phosphatidyl-inositol-3-kinase (PI3K) for induction of 3-phoshoinositide-dependent kinase (PDK1), which recruits and phosphorylates PKCθ (Bi, Tanaka *et al.* 2001; Isakov and Altman 2002; Lee, D'Acquisto *et al.* 2005; Hayden, West *et al.* 2006). Further downstream, PKCθ subsequently activates the essential adaptor complex composed of CARMA1 (caspase-recruitment domain (CARD)-containing membrane-associated guanylate kinase (MAGUK) region), B cell lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue 1 (MALT1) (Gaide, Martinon *et al.* 2001; Ruland, Duncan *et al.* 2001; Ruland, Duncan *et al.* 2003; Lin and Wang 2004; Thome 2004). The CARMA1/BCL10/MALT1 (CBM) complex recruits the ubiquitin ligase TRAF6 (TNF receptor-associated factor 6), which subsequently ubiquitinates and activates the IKK complex to induce IκB-α degradation and NF-κB translocation (Sun, Deng *et al.* 2004). An important T cell-derived NF-κB-dependent gene is interleukin 2 (IL-2), which is the major growth-promoting factor for lymphocytes. Recently, the CARD-containing Ser/Thr kinase RIP2 (receptor-interacting protein 2) has also been found to associate with BCL10 and induce its phosphorylation (Ruefli-Brasse, Lee *et al.* 2004).

1.2.3.2 Adaptors in BCR-mediated NF-κB activation

Receptor-proximal events of BCR-mediated NF-κB induction (figure 5, right pathway) are highly analogous to those of the TCR. The ITAM-containing BCR Igα and Igβ chains are coupled to membrane-bound receptor-proximal kinases Lyn and Fyn, which amongst others activate Syk and Btk (Bruton's tyrosine kinase) (Khan, Alt *et al.* 1995; Bajpai, Zhang *et al.* 2000). Second messengers and proteins of the Vav family finally activate PKCβ (Kawakami, Kitaura *et al.* 2000; Saijo, Mecklenbrauker *et al.* 2002), which subsequently recruits the CARMA1/BCL10/MALT1 complex to induce IKK activation as in the TCR pathway described above (Weil and Israel 2004; Hayden, West *et al.* 2006).

Genetic knockout studies have shown that in the absence of either BCL10 or MALT1 antigen-receptor induced activation of NF-κB in T cells or B cells is not possible, although

MALT1 has a differential role in B cells (Ruland, Duncan *et al.* 2001; Gaide, Favier *et al.* 2002; Ruland, Duncan *et al.* 2003). Individual structure and function of BCL10 and MALT1 known from lymphocyte signalling will be covered in the next section.

1.3 BCL10 and MALT1

1.3.1 Identification from MALT lymphoma

The human proteins BCL10 and MALT1 were originally identified from chromosomal translocation breakpoints in patients with MALT (mucosa-associated lymphoid tissue) lymphoma (Isaacson and Du 2004). In the t(1;14)(p22;q32) breakpoint, the *BCL10* gene is translocated to the immunoglobulin (Ig) heavy chain locus, leading to protein truncations caused by frameshift mutations. The overexpression of truncated BCL10 results in anti-apoptotic activity and constitutive NF-κB activation, thus having a twofold lymphomagenic effect (Willis, Jadayel *et al.* 1999; Zhang, Siebert *et al.* 1999).

The translocation t(11;18)(q21;q21) is the most common chromosomal abnormality in low-grade MALT lymphoma and leads to the fusion of the *cIAP2* (cellular inhibitor of apoptosis protein 2, also known as *apoptosis inhibitor 2* (*API2*)) gene to the *MALT1* gene (Akagi, Motegi *et al.* 1999; Dierlamm, Baens *et al.* 1999). The overexpression of the cIAP-MALT1 fusion protein strongly activates NF-κB (Uren, O'Rourke *et al.* 2000; Lucas, Yonezumi *et al.* 2001; Zhou, Du *et al.* 2005), thereby increasing anti-apoptotic effects and conferring a survival advantage and antigen-independent proliferation of lymphocytes, which are critical factors in lymphoma progression (Dierlamm, Baens *et al.* 1999; Stoffel, Chaurushiya *et al.* 2004).

1.3.2 Structure and function

1.3.2.1 Bcl10

Mammalian Bcl10 is the cellular homologue of the *equine herpesvirus-2 E10* gene and displays ubiquitous expression throughout the entire development with particularly high expression levels in lymphoid tissues and the embryonic central nervous system (Costanzo, Guiet *et al.* 1999; Koseki, Inohara *et al.* 1999; Srinivasula, Ahmad *et al.* 1999; Ye, Dogan *et al.* 2000).

Human and murine Bcl10 show 91% sequence homology and murine Bcl10 is a 26 kDa protein composed of 233 amino acids. It consists of an N-terminal caspase recruitment domain (CARD), mediating self-oligomerisation and interaction with other CARD-containing proteins, and a C-terminal serine- and threonine-rich region, which can be phosphorylated upon cellular activation (Thome 2004; Gewies and Ruland 2005). The domain structure of Bcl10 is shown in figure 6b.

1.3.2.2 Malt1

Mammalian Malt1 is a 92 kDa protein composed of 821 amino acids and contains an N-terminal death domain (DD), two immunoglobulin (Ig)-like domains mediating the interaction with a short amino acid motif following the Bcl10-CARD, a C-terminal caspase-like domain and a third, recently identified Ig-like domain (Uren, O'Rourke *et al.* 2000; Zhou, Du *et al.* 2005). The direct interaction partners of the Malt1-DD and the caspase-like domain are not yet identified, but these domains are supposed to mediate downstream activation of the IKK complex and probably also p38 and Jnk activation (Thome 2004). The domain structure of Malt1 is depicted in figure 6c.

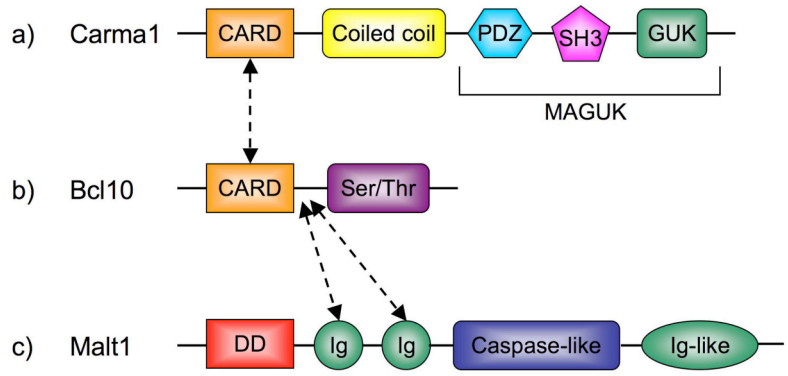


Fig. 6: Domain structure of Carma1, Bcl10 and Malt1 (after Thome 2004)

a) Carma1 contains an N-terminal caspase recruitment domain (CARD; orange) mediating direct binding to Bcl10 via CARD-CARD interaction. The molecular function of the following coiled-coil motif (yellow) is not completely understood. The C-terminal PDZ–SH3–GUK array characterizes Carma1 as a member of the membrane-associated guanylate kinase (MAGUK) family, and might allow binding to the C-terminus of a transmembrane protein (through the PDZ domain; turquoise) and oligomerisation through intermolecular SH3 (pink)–GUK (green) interactions. b) Bcl10 is composed of an N-terminal caspase recruitment domain (CARD; orange) and a C-terminal serine/threonine (Ser/Thr)-rich region (magenta). c) Malt1 consists of an N-terminal death domain (DD; red), three immunoglobulin (Ig)-like domains (green) and a C-terminal caspase-like domain (blue). The two middle Ig domains mediate binding to Bcl10 via a short motif of amino acids following the Bcl10-CARD. The Malt1-DD and the caspase-like domain probably induce downstream cell activation. For details see text.

1.3.3 The Bcl10/Malt1 complex in lymphoid cells

Bcl10 and Malt1 can directly bind to each other via the Bcl10-CARD and the Ig-domains of Malt1 (Uren, O'Rourke *et al.* 2000; Lucas, Yonezumi *et al.* 2001), and this Bcl10/Malt1 complex has recently been shown to be an essential regulator of T cell and B cell receptor-mediated NF-κB activation, although Malt1 is dispensable for NF-κB activation in B cells (Ruland, Duncan *et al.* 2003). Bcl10 and Malt1 fulfil their function in complex with a protein of the Carma family (CARD and MAGUK-like region) (Ruland, Duncan *et al.* 2001). In lymphocytes and probably in other immune cells, this function is accomplished by Carma1 (CARD11/Bimp3) (figure 6a), whereas Carma3 (CARD10/Bimp1) seems to function in non-immune cells like fibroblasts and Carma2 (CARD14/Bimp2) expression is restricted to the placenta (Bertin, Wang *et al.* 2001; Gaide, Martinon *et al.* 2001; McAllister-Lucas, Inohara *et al.* 2001; Gaide, Favier *et al.* 2002; Wang, You *et al.* 2002; Thome 2004).

It is known that Bcl10 and Malt1 are ubiquitously expressed in most tissues and their role in lymphoid cells has been clearly described during the last years. However, a functional role for Bcl10 and Malt1 in non-lymphoid cells (myeloid and non-immune cells) and signalling pathways from other immunoreceptors beyond the TCR or BCR has not been reported up to now. Consequently, the central aim of this study was to investigate the possible involvement of the Bcl10/Malt1 complex in signal transduction from two other receptor families, namely the FcεRI as a representative for Fc receptors and the LPA receptor as a representative for G protein-coupled receptors (GPCRs).

1.4 The FcεRI and the role of mast cells in immunity

Fc receptors for the different Ig isotypes are mainly expressed on immune cells of the myeloid lineage, such as mast cells, dendritic cells or macrophages (see sections 1.1.1 and 1.1.2.2.3). One of the best characterized Fc receptors is the high affinity IgE receptor FcεRI expressed on basophils and particularly on mast cells (see figure 1c), for which it is the most important receptor for immunological cell activation (Galli, Kalesnikoff *et al.* 2005).

Mast cells have been discovered by Paul Ehrlich in 1876 through the staining of connective tissue with anilin dyes and since then have been studied extensively. Mast cells derive from $CD13^+$ $CD34^+$ $CD177^+$ hematopoietic progenitors in the bone marrow

and upon maturation, they migrate to vascularized connective tissues including the skin, mucosa and airways, where they build a first line of defence against invading pathogens (Metcalfe, Baram *et al.* 1997). They contain intracellular granules in which preformed and newly synthesized mediators are stored. Extracellular crosslinking of Fcε receptor-bound IgE molecules with a specific antigen leads to intracellular mast cell activation, thereby inducing a variety of physiological effector functions:

(1) degranulation with the immediate release of preformed inflammatory mediators, such as mast cell-specific proteases stored in intracellular granules (e.g. chymase, tryptase),
(2) *de novo* synthesis and release of arachidonic acid metabolites (e.g. leukotrienes LTC_4, LTD_4, LTE_4 or prostaglandins) and
(3) the expression and secretion of proinflammatory chemokines, cytokines and growth factors occurring hours after activation (Galli, Nakae *et al.* 2005). Those include interleukin-1 (IL-1), IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL- 11, IL-13, IL-16, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) (Metcalfe, Baram *et al.* 1997).

Physiologically, mast cells are involved in innate and adaptive immunity, host defense against parasites, immunomodulation, tissue repair and angiogenesis (Metcalfe, Baram *et al.* 1997; Mekori and Metcalfe 2000; Wedemeyer, Tsai *et al.* 2000). Furthermore, the immediate and late phase outcomes of mast cell activation are the main causes of IgE-dependent acute and chronic allergic reactions (e.g. hay fever), and particularly IgE-induced chemokine and cytokine secretion (TNF-α, IL-6, MIP-2) from mast cells plays a central role in chronic inflammatory diseases like asthma or artherosclerosis. In the development of asthma, mast cells infiltrate airway smooth muscles and the release of specific proteases and T-helper 2 (T_h2) cytokines (e.g. IL-4, IL-13) is critical for the development of airway inflammation, mucus production and bronchial hyperresponsiveness (Bradding 2003; Fireman 2003).
Moreover, the IgE-induced secretion of T_h2-type cytokines significantly contributes to acquired immunity against certain pathogens, for it induces a positive feedback loop and activates plasma B cells to produce more antigen-specific IgE antibodies. However, mast cells also are involved in innate immune responses and can be directly or indirectly activated by bacterial products to release proinflammatory mediators that induce structural and functional changes in tissues and inflammation (Galli, Maurer *et al.* 1999; Wedemeyer, Tsai *et al.* 2000; Maurer, Theoharides *et al.* 2003; Galli, Nakae *et al.* 2005).

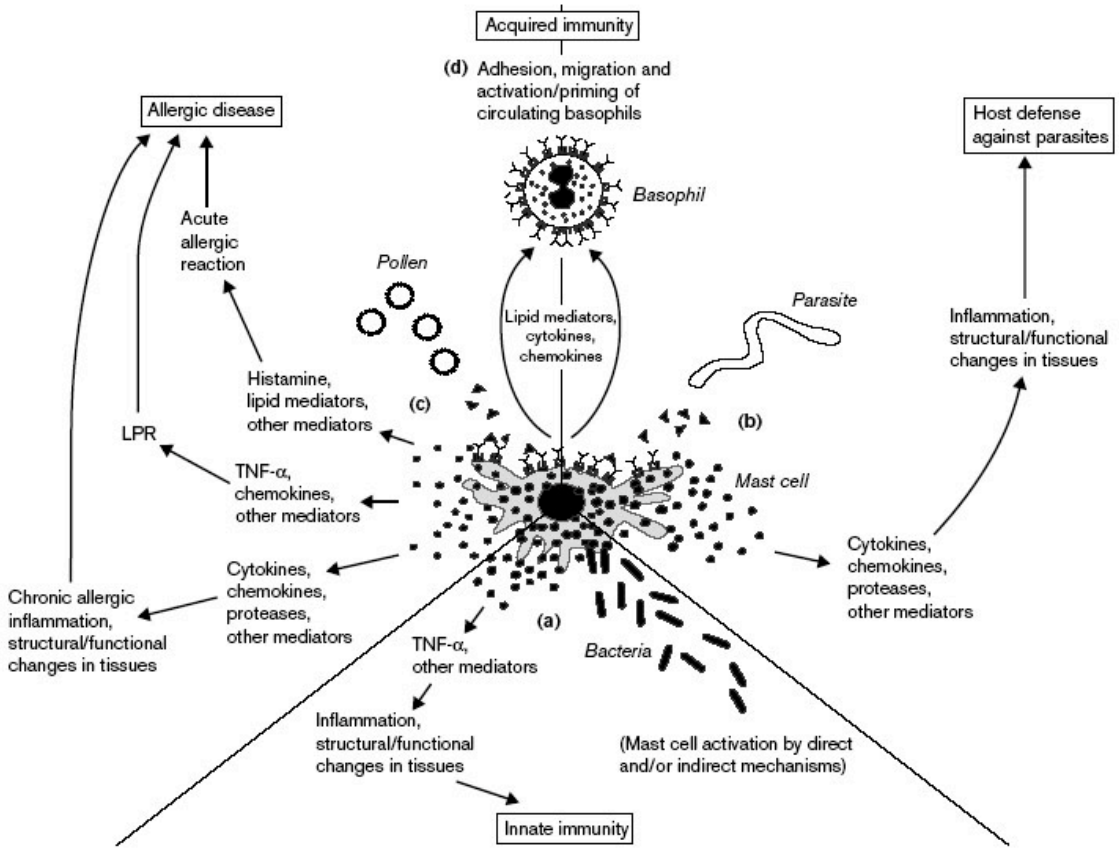


Fig. 7: Central roles of mast cells in allergic reactions and innate and acquired immune responses (from Wedemeyer *et al.* 2000)

Mast cells can exhibit protective functions (a) in the context of innate immunity (e.g. against bacteria) as well as (b) in acquired, IgE-associated responses against parasites by releasing TNF-α and other mediators that orchestrate local inflammatory responses with beneficial effects for the host. (c) However, when IgE-dependent mast cell activation is induced in response to otherwise innocuous environmental antigens (e.g. allergens derived from pollen), this can contribute to the expression of allergic diseases. These are on the one hand immediately induced by rapidly released mediators like histamine (e.g. hay fever), but on the other hand are induced by late phase reaction and release of cytokines and chekomines, which leads to chronic allergic inflammation (e.g. in asthma). (d) In IgE-associated responses, mast cell activation can induce the recruitment of leukocytes, including basophils, to the site of inflammation.

Recent studies have shown an active role in antigen presentation to T cells and direct interaction between mast cells and B cells providing signals for specific IgE production has also been demonstrated (Wedemeyer, Tsai *et al.* 2000; Gauchat, Henchoz *et al.* 1993). The central role of mast cells in IgE-mediated allergic reaction as well as innate and acquired immunity is summarized in figure 7.

IgE/FcεRI-dependent activation of mast cells induces a variety of different transcription factors and thereby also a multitude of cytokines and chemokines. Many of the proinflammatory mast cell cytokines depend on the transcriptional activity of NF-κB, e.g. IL-4, IL-6 or TNF-α, which are in particular involved in chronic allergic diseases. However, specific regulatory mechanisms of unique FcεRI-mediated signalling pathways, including the adaptors for IgE-induced NF-κB activation, were unknown up to now.

1.5 G protein-coupled receptors

1.5.1 Structure and signalling proporties

The G protein-coupled receptors (GPCRs), also known as *seven transmembrane receptors*, represent the largest family of cell surface receptors and are involved in the majority of stimulus-response pathways, ranging from intercellular communication to physiological senses, but also in immune regulation. This diversity of functions is matched by the wide range of ligands, which bind to receptors of the GPCR family, such as hormones, neurotransmitters, peptides or cytokines, but also photons and Ca^{2+} ions activate GPCRs. A classification of GPCRs can be conducted either according to their function or to their phylogenic relation. However, in contrast to the TCR, BCR and Fc receptors described in section 1.1.3, they do not confer antigen specificity (Milligan and Kostenis 2006).

GPCRs consist of mostly seven transmembrane α-helices connected by extra- and intracellular loops. Characteristically, the receptor is coupled to a heterotrimeric G protein consisting of an α, β and γ subunit on the cytoplasmatic side, which is responsible for the activation of intracellular signalling cascades (Kristiansen 2004).

Upon ligand binding, a conformational change in the extracellular domain of the receptor is submitted to the cytoplasmatic G protein and mechanically activates guanine nucleotide exchange factors (GEFs). These mediate the exchange of the Gα-bound GDP to an energy-rich GTP, thereby triggering the activation of the heterotrimeric G protein and its dissociation into the α and $\beta\gamma$ subunit, both parts subsequently inducing different downstream effectors like adenylate cyclases or phospholipases for the generation of second messengers (Kristiansen 2004). Inactivation of the receptor is accomplished through GTP → GDP hydrolysis by the Gα-intrinsic GTPase activity and dissociation of the ligand. Through subsequent reassociation of the inactive Gα - GDP subunit with the $\beta\gamma$ subunit, the GPCR is prepared to start a new activation cycle (see figure 8).

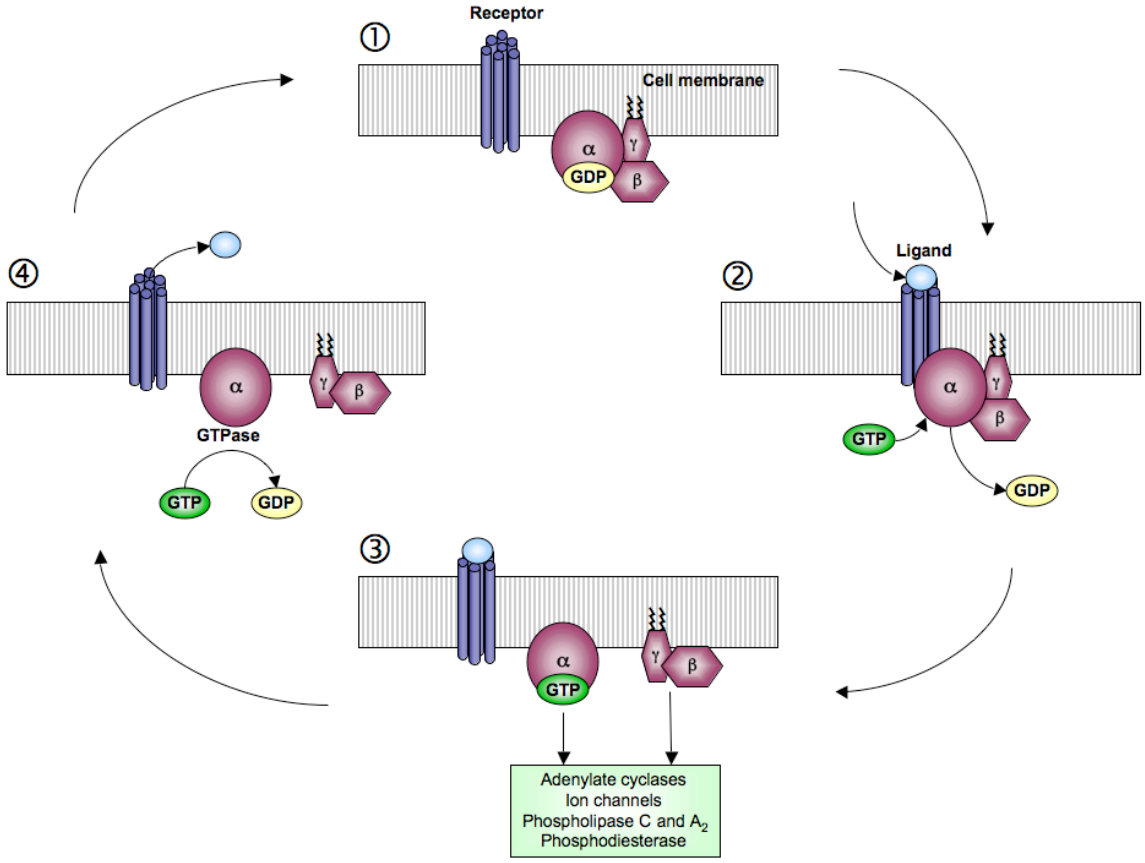


Fig. 8: Schematic illustration of GPCR signalling through heterotrimeric G proteins

① In the resting state, the GPCR has no ligand bound and the heterotrimeric G protein composed of Gα and Gβγ subunit is inactive with a Gα-bound GDP molecule. ② Upon ligand binding to the GPCR, a conformational change in the receptor induces its close association with the G protein and the activation of a guanine nucleotide exchange factor (GEF), which exchanges the Gα-bound GDP to an energy-rich GTP. ③ Subsequently, the GTP-Gα subunit dissociates from the Gβγ subunit, with both parts independently inducing various effector molecules like ion channels, adenylate cyclases or phospholipases, which generate second messengers for further signal transduction. ④ The intrinsic GTPase activity of the Gα protein catalyzes the hydrolysis of GTP to GDP, thereby terminating the G protein signal. The ligand dissociates from the receptor and inactive GDP-Gα and Gβγ subunits reassociate to get back to the resting state ①.

There are four major subfamilies of heterotrimeric Gα subunits, which specifically trigger different signalling pathways (see also figure 10) (Radeff-Huang, Seasholtz *et al.* 2004; Milligan and Kostenis 2006): **$G\alpha_s$** activates adenylate cyclases (ACs), which induces the generation of cyclic AMP (cAMP) and subsequent activation of cAMP-dependent protein kinase A (PKA) and phosphorylation of cellular target proteins. The cAMP/PKA pathway particularly inhibits cell growth and proliferation, but in only a few cases, it also is able to stimulate cell growth. **$G\alpha_{i/o}$** can also couple to ACs, but in contrast to $G\alpha_s$, $G\alpha_i$ inhibits ACs, thereby decreasing cellular cAMP levels. **$G\alpha_{q/11}$** binds to and activates PLCβ, which in turn catalyzes the conversion of phosphatidylinositol biphosphate (PIP_2) to the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3), resulting in mobilisation of stored Ca^{2+} ions from the endoplasmatic reticulum (ER), altogether finally activating

Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms. The most recently identified class of heterotrimeric G proteins includes those of the **$G\alpha_{12/13}$** family. $G\alpha_{13}$ was originally known as an oncogene and overexpression of GTPase-deficient $G\alpha_{12}$ or $G\alpha_{13}$ has been shown to alter cell shape, gene expression and cell growth, which is due to $G\alpha_{12/13}$-dependent activation of the small G protein Rho. Rho signalling is considered to be important for the control of the actin cytoskeleton, and RhoA also collaborates with the small G protein Ras to drive cell cycle progression.

Recapitulating, the most important physiological functions of GPCR signalling are the regulation of cell proliferation, migration and survival and the induction of changes in cell morphology. These functions, as described for immunoreceptors (see section 1.1.2), can be accomplished by non-transcriptional as well as transcriptional mechanisms, and it is known that also NF-κB activation is regulated by signalling from GPCRs, which makes them an attractive target for studies in the context described here (Ye 2001).

1.5.2 LPA receptors

One example of a prototypic GPCR is the receptor for the bioactive phospholipid lysophosphatidic acid (LPA). It is a member of the *endothelial differentiation gene* (*Edg*) subfamily of GPCRs and up to now, there are four LPA receptors known in mammals. The best characterized homologous receptors are LPA1, LPA2 and LPA3, formerly known as EDG2, EDG4 and EDG7, respectively (Hecht, Weiner *et al.* 1996; An, Bleu *et al.* 1998; Bandoh, Aoki *et al.* 1999; Im, Heise *et al.* 2000) and recently, a fourth LPA receptor (LPA4) has been identified that shares no significant homology with the other LPA receptors and is more related to the purinergic GPCR family (Noguchi, Ishii *et al.* 2003).

Five additional members of the Edg receptor subfamily encode related GPCRs specific for another bioactive lysophospholipid, sphingosine-1-phosphate (S1P) (Hla, Lee *et al.* 2001; Sanchez and Hla 2004), which possess structural homologies and signalling properties like the LPA receptors.

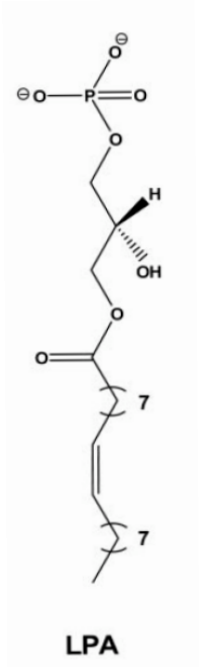


Fig. 9: Structure of the phospholipid L-α-lysophosphatidic acid (LPA)

The phospholipid LPA (see figure 9 for its chemical structure) is mainly produced by activated platelets and growth factor-induced fibroblasts, but also by adipocytes and cancer cells (Eichholtz, Jalink *et al.* 1993; Mills and Moolenaar 2003; Radeff-Huang, Seasholtz *et al.* 2004). LPA is present in the serum at concentrations in the micromolar range, but much higher LPA levels are found in the plasma and malignant ascites fluid in cervical and ovarian cancer (Westermann, Havik *et al.* 1998). LPA acts as a mitogen on multiple target cells, so it induces proliferation and survival of fibroblasts, neuronal cells, lymphocytes and macrophages as well as proliferation and migration of transformed cell types (hepatoma, T lymphoma, carcinoma cells) (van Corven, Groenink *et al.* 1989; Moolenaar and van Corven 1990; van Corven, van Rijswijk *et al.* 1992; Moolenaar 1995) and therefore is important in the initiation and progression of malignant diseases. Furthermore, LPA is involved in wound healing and stimulates rapid processes like smooth muscle contraction or morphological changes in cell shape and increases endothelial permeability (Mills and Moolenaar 2003). Like other GPCR ligands, it activates several Gα subunits and thus, induces a multitude of effector cascades, e.g. activation of G_i to induce Ras/ERK and PI3K/Akt signalling or $G_{12/13}$ to trigger Rho/RhoA pathways, all of them important for cell survival and proliferation (Gohla, Harhammer *et al.* 1998; Kranenburg, Poland *et al.* 1999). LPA has also been demonstrated to activate phospholipase D (PLD), which hydrolyzes phosphatidylcholine, thereby generating phosphatidic acid (PA) and choline. Since PA can be further converted to DAG, this pathway can also activate PKC, and PLD stimulation by LPA has also been shown to be PKC-dependent (van der Bend, de Widt *et al.* 1992). A summary of the major downstream pathways activated by LPA is given in figure 10.

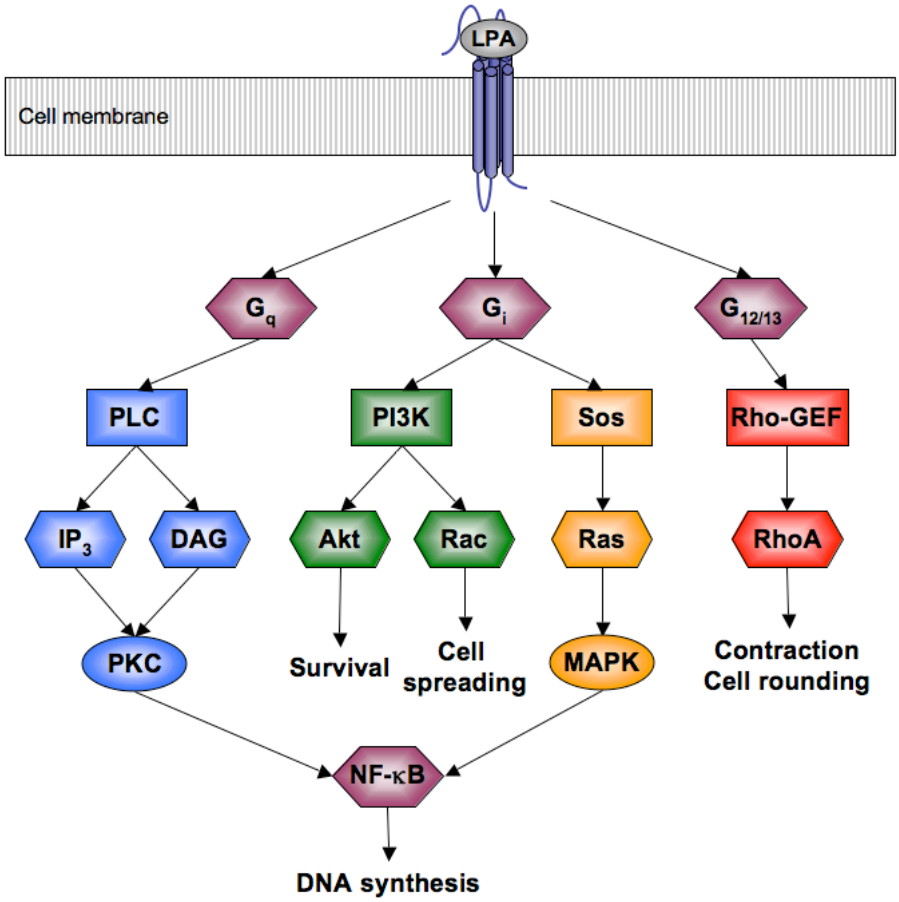


Fig. 10: Important LPA signalling pathways

LPA signals through its own G protein-coupled receptors via at least three distinct classes of heterotrimeric G proteins - G_q, G_i and $G_{12/13}$ - leading to activation of multiple downstream effector pathways. Among the major LPA-induced signalling pathways are: G_q (or/and G_i)-mediated activation of phospholipase C (PLC), which leads to the hydrolysis of inositol-triphosphate (IP_3) with consequent calcium mobilisation and subsequent protein kinase C (PKC) activation; secondly, G_i-mediated activation of the Ras–Erk pathway, leading to DNA synthesis and cell proliferation and G_i-mediated activation of the PI3K–Akt (also known as PKB) survival pathway, which suppresses apoptosis; and finally, the activation of the Rho and Rac GTPases via specific guanine nucleotide exchange factors, e.g. Rho-GEF, which leads to cytoskeletal remodelling (contraction and cell spreading), changes in cell shape and cell migration.

1.5.3 The role of LPA in the immune system

Over the last years, the phospholipid LPA and another important member of this group, sphingosine-1-phosphate (S1P), have been conferred importance in the induction of immune responses. It is known that LPA can act on various cell types including lymphocytes and macrophages by attracting and activating them and it influences their interaction with other cell types (Goetzl, Kong *et al.* 1999; Graler and Goetzl 2002). For instance, LPA stimulates the proliferation and survival of T cells (Xu, Casey *et al.* 1995), activates endothelial cells to recruit macrophages in wound healing (Balazs, Okolicany *et al.* 2001) or induces MAPK activation and DNA synthesis in activated Ig-secreting B cells

(Rosskopf, Daelman *et al.* 1998). LPA has also an emerging role in the initiation and progression of cancer, for it stimulates cell proliferation, migration and survival, also of tumour cells. Furthermore, some LPA receptors and enzymes generating active LPA are aberrantly expressed in several malignant diseases (Mills and Moolenaar 2003).

Apart from these overall functions of LPA in the immune system, recent mechanistical studies have also demonstrated that LPA can activate NF-κB in certain cell lines via the IKK/IκB-α system, this pathway depending on calcium signals, phospholipase and PKC activation (Palmetshofer, Robson *et al.* 1999; Shahrestanifar, Fan *et al.* 1999; Cummings, Zhao *et al.* 2004), but further molecular details downstream of PKC enzymes and the involved signalling adaptors are still to be clarified.

1.6 Questions and aim of the study

In the study in hand, potential roles for the lymphocyte signalling adaptors Bcl10 and Malt1 in immune cells apart from T and B cells and in non-immune cells shall be analysed. Since the TCR and BCR share certain structural homologies with the ITAM-containing high affinity IgE receptor FcεRI (Isakov 1997; Turner and Kinet 1999), the first part (see section 3.1) focuses on the functions of Bcl10 and Malt1 in signalling from FcεRI in murine mast cells, thereby analyzing IgE-induced mast cell responses *in vivo* and *in vitro*.

The second part (see section 3.2) aims at a potential role for Bcl10 and Malt1 in non-immune cells and signal transduction from a G protein-coupled receptor in murine embryonic fibroblasts as an easily accessible model system. Here, *in vitro* signalling pathways from the GPCR for the phospholipid LPA are studied, due to its increasing role in human cancer and the known ability to induce NF-κB in certain settings (Shahrestanifar, Fan *et al.* 1999).

This study aims at the molecular mechanisms of receptor-specific NF-κB activation, the involvement of the Bcl10/Malt1 complex in downstream signalling pathways other than TCR- and BCR-induced signal transduction and the consequences of defective Bcl10/Malt1 signalling concerning physiological outcomes of cell activation.

2 Material and methods

2.1 Equipment and reagents

2.1.1 Equipment

Acrylamide gel electrophoresis chamber	Biorad, Munich
Agarose gel electrophoresis chamber	Biorad, Munich
Centrifuge 5417R	Eppendorf, Hamburg
Centrifuge GS-6K	Beckman, Fullerton, USA
CO_2 incubators	Nunc, Wiesbaden
	Heraeus, Hanau
Cryo freezing container	Nalgene
Developer Hyperprocessor	Protec, Oberestenfeld
Digital scales	Sartorius AG, Göttingen
ELISA washer	Nunc, Wiesbaden
Flow cytometer, FACScalibur	BD Biosciences, Heidelberg
Gel chamber Subcell® GT	Biorad, Munich
GelDoc 2000 (+PC)	Biorad, Munich
Gel dryer 583	Biorad, Munich
Heating block	Kleinfeld Labortechnik, Gehrden
Hoefer SE 600 ruby, standard vertical electrophoresis unit	Amersham Biosciences, Braunschweig
Light microscope, Axiovert 40C	Zeiss, Jena
Magnetic stirrer, heatable	IKA GmbH & Co, Staufen
Micrometer dial thickness gauge	Peacock, Tokyo, Japan
Microspin centrifuge	PeqLab GmbH, Erlangen
	Abimed GmbH, Langenfeld
Nanodrop	PeqLab GmbH, Erlangen
Neubauer counting chamber	Reichert, New York, USA
Nucleofactor II for transfection	Amaxa, Cologne
Pipet boy	Falcon, Heidelberg
	Hirschmann, Eberstadt
Pipettes	Abimed GmbH, Langenfeld
	Gilson, Bad Camberg

PhosphoImager	MWG Biotech, Ebersberg
PCR thermocycler	Biorad, Munich
pH-meter F32	Beckman, Fullerton, USA
Phospo Screen, 20 x 25 cm	Amersham Biosciences, Braunschweig
PowerPac 200/basic	Biorad, Munich
Protean II xi Cell	Biorad, Munich
Rocking platform	Bibby Sterilin, Stone, UK
Rolling shaker	CAT, Staufen
Spectral photometer ND-1000	PeqLab GmbH, Erlangen
Sterile hood, Holten Lamin Air 1.8	Holten, Gydewang, Denmark
Thermomixer	Eppendorf, Hamburg
TransBlot SD Semi-Dry Transfer Cell	Biorad, Munich
Vacuum pump	KNF Neuberger, Freiburg
Varioklav steriliser	H+P Labortechnik, Oberschleißheim
Vortex mixer	Bibby Sterilin, Stone, UK
Water bath	Memmert, Schwabach

2.1.2 Material and molecular biology kits

3 mm Whatman blotting paper	Biorad, Munich
Annexin V-FITC apoptosis detection kit	BD Pharmingen, Heidelberg
Cell scraper	TPP AG, Trasadingen, Switzerland
Cell strainer (70 µM, 100 µM)	BD Biosciences, Heidelberg
DNA extraction kit	Promega GmbH, Mannheim
ECL Western blot detection kit	Amersham Biosciences, Freiburg Pierce, Rockford, USA
ELISA DuoSet (IL-4, IL-6 or TNF-α)	BD Pharmingen, Heidelberg
ELISA DuoSet (MIP-2)	R&D Systems, Wiesbaden
Leukotriene C_4/D_4/E_4 enyzme-linked immunoassay (EIA)	Amersham Biosciences, Freiburg
G25 Microspin column	Amersham Biosciences, Freiburg
Hybond-P PVDF transfer membrane	Amersham Biosciences, Freiburg
Hyperfilm ECL	Amersham Biosciences, Freiburg
MaxiSorp 96-well plates	Nunc, Wiesbaden
Plastic pipets (1, 5, 10, 25, 50 ml)	BD Biosciences, Heidelberg

Reaction tubes (0.5, 1.5, 2.0 ml)	Eppendorf, Hamburg
Reaction tubes (15 ml, 50 ml)	BD Biosciences, Heidelberg
Round bottom FACS tubes	BD Pharmingen, Heidelberg
Sterile needles	BD Biosciences, Heidelberg
Sterile syringes (2 ml, 20 ml)	BD Biosciences, Heidelberg
SS™ II Reverse Transcription System	Invitrogen, Karlsruhe
Tissue culture plates (6, 10, 15 cm; 6-, 12-, 24-, 96-well)	TPP AG, Trasadingen, Switzerland
Tissue culture flasks (15 ml, 75 ml)	TPP AG, Trasadingen, Switzerland

2.1.3 Reagents

2.1.3.1 Chemicals

Acrylamide/bisacrylamide 30% solution	Biorad, München
Agarose	Biozym, Hessisch Oldendorf
Albumin Fraction V, bovine (BSA)	Roth, Karlsruhe
Ammoniumperoxodisulfate (APS)	Merck, Darmstadt
Aqua ad injectabile	Delta Select, Pfulingen
Calcium chloride	Fluka, Deisenhofen
Citric acid, monohydrate	Merck, Darmstadt
Complete Protease Inhibitor Cocktail Tablets	Roche/Boehringer, Mannheim
Cycloheximide	Calbiochem, Bad Soden
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Deisenhofen
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe
Dinitrophenyl-human serum albumin (DNP-HSA)	Sigma-Aldrich, Deisenhofen
Dithiothreitol (DTT)	Roth, Karlsruhe
DNA ladder (100 bp, 1 kb)	Invitrogen, Karlsruhe
dNTP Mix 25 mM	Bioline, Luckenwalde
Ethanol, p.a.	Merck, Darmstadt
Ethidium bromide 0.7 mg/ml	Eurobio, Les Ulis, France
Ethylenediaminetetraacetate (EDTA)	Roth, Karlsruhe
Ethylenglycoldiaminetetraacetate (EGTA)	Sigma-Aldrich, Deisenhofen
Evans blue	Sigma-Aldrich, Deisenhofen
Glycerol	Sigma-Aldrich, Deisenhofen

Glycine	Roth, Karlsruhe
HEPES	Sigma-Aldrich, Deisenhofen
Hydrochloric acid, 37%	Merck, Darmstadt
Imidazol	Sigma-Aldrich, Deisenhofen
Ionomycin (Iono)	Fluka, Deisenhofen
Lipopolysaccharide (LPS)	Sigma-Aldrich, Deisenhofen
Lumigen ^{TM} TMA-6	Amersham Biosciences, Freiburg
L-α-lysophosphatidic acid	Sigma-Aldrich, Deisenhofen
Magnesium chloride	Fluka, Deisenhofen
2-Mercaptoethanol	Sigma-Aldrich, Deisenhofen
Methanol, p.a.	Merck, Darmstadt
4-Nitrophenyl N-acetyl-ß-D-glucosaminide	Sigma-Aldrich, Deisenhofen
Nonidet P-40 (NP-40)	Fluka, Deisenhofen
Phorbol-12-myristate-13-acetate (PMA)	Fluka, Deisenhofen
Phosphate buffered saline (PBS), solide, 10x	Biochrom AG, Berlin
PIERCE ECL Western Blotting Substrate	Pierce, Rockford, Illinois
Ponceau S Solution	Sigma-Aldrich, Deisenhofen
Poly(dIdC)	Sigma-Aldrich, Deisenhofen
Potassium chloride	Merck, Darmstadt
Protein Assay Dye Concentrate	Biorad, München
2-Propanol	Roth, Karlsruhe
Proteinase K	Sigma-Aldrich, Deisenhofen
Puromycin	Calbiochem, Bad Soden
Random primer (3 µg/µl)	Invitrogen, Karlsruhe
Rottlerin (PKC Inhibitor)	Calbiochem, Bad Soden
Skim milk powder	Fluka, Deisenhofen
Sodium dodecyl phosphate (SDS)	Roth, Karlsruhe
Sodium fluoride	Sigma-Aldrich, Deisenhofen
Sodium orthovanadate	Sigma-Aldrich, Deisenhofen
3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB)	Sigma-Aldrich, Deisenhofen
N,N,N',N'-tetramethylethylendiamin (TEMED)	Sigma-Aldrich, Deisenhofen
TransFectin^{TM} lipid reagent	Biorad, Munich
Tris(hydroxymethyl)aminomethan (TRIS)	Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Deisenhofen
TRIzol® Reagent	Invitrogen, Karlsruhe
Tumor necrosis factor (TNF)-α	Sigma-Aldrich, Deisenhofen

Tween-20	Sigma-Aldrich, Deisenhofen
Wortmannin (PI3K inhibitor)	Calbiochem, Bad Soden

2.1.3.2 Cell culture media and supplements

Dulbecco's modified eagle medium (DMEM), cell culture media	Gibco/Invitrogen, Karlsruhe
Fetale Calf Serum (FCS)	Perbio Science, Bonn
L-glutamine, 200 mM	Gibco/Invitrogen, Karlsruhe
2-Mercaptoethanol, 50 mM	Gibco/Invitrogen, Karlsruhe
Penicillin (10 000 U/ml)/streptomycin (10 000 µg/ml)	Gibco/Invitrogen, Karlsruhe
Phosphate buffered saline (PBS), 1ox	Gibco/Invitrogen, Karlsruhe
Recombinant murine interleukin (IL)-3	R&D Systems, Wiesbaden
Recombinant murine interleukin (IL)-6	R&D Systems, Wiesbaden
Recombinant murine stem cell factor (SCF)	R&D Systems, Wiesbaden
RPMI 1640, cell culture media	Gibco/Invitrogen, Karlsruhe
TransFectin^{TM} Lipid Reagent	Biorad, München
Trypan blue, 0.4% Solution	Gibco/Invitrogen, Karlsruhe
Trypsin EDTA	Gibco/Invitrogen, Karlsruhe

2.1.3.3 Cell lines

NIH3T3	Immortalized murine embryonic fibroblast cell line
ΦNX-Eco (Phoenix E)	Retroviral ecotrop packaging cell line (helper-virus-free) 293T (human embryonic kidney carcinoma-) cells, stably transfected with Moloney *GagPol-IRES-Lyt2* construct under the the transcriptional control of the RSV promotor (selection with hygromycin R) and Moloney *ecotropic envelope* gene under the transcriptional control of the CMV promotor (selection with diphtheria toxin)

2.1.3.4 Antibodies

Anti-Actin, rabbit polyclonal IgG	Sigma-Aldrich, Deisenhofen
Anti-Akt, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-Bcl10 (H-197), rabbit polyclonal IgG	Santa Cruz, Heidelberg
Anti-Bcl10, rabbit polyclonal IgG	ProSci Inc., Lörrach
Anti-Bcl-X_L, rabbit poyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-dinitrophenyl IgE, monoclonal (clone SPE-7)	Sigma-Aldrich, Deisenhofen
Anti-dinitrophenyl IgE, monoclonal (clone H1-ε-26)	F.T. Liu, Sacramento, CA and D.H. Katz, San Diego, CA
Anti-FLIP, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-IκB-α, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-Malt1, mouse monoclonal IgG	V. Dixit, San Francisco, CA
Anti-mouse CD117 (c-Kit), PE-conjugated	eBioscience, Kranenburg
Anti-mouse FcεRI α, FITC-conjugated	eBioscience, Kranenburg
Anti-mouse IgG HRP-conjugated, horse	
Anti-p38 MAP kinase, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-p44/42 MAP kinase, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-Akt, rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-IκB-α (Ser32/Ser36), rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-JNK/SAPK (Thr183/Tyr185), rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-p38 (Thr180/Tyr182), rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-p44/42 (Thr202/Tyr204), rabbit polyclonal IgG	Cell Signaling, Frankfurt/Main
Anti-phospho-tyrosine (PY99), mouse polyclonal IgG	Santa Cruz, Heidelberg
Anti-rabbit IgG HRP-conjugated, goat	Cell Signaling, Frankfurt/Main
Anti-SAPK/JNK, rabbit IgG	Cell Signaling, Frankfurt/Main

2.1.3.5 Enzymes

Taq DNA polymerase	PeqLab GmbH, Erlangen
T4 polynucleotide kinase	Invitrogen, Karlsruhe
SuperScript™ II Reverse Transcriptase	Invitrogen, Karlsruhe

2.1.3.6 Primers and oligonucleotides

Name, length, application and sequence of the used primers and oligonucleotides are listed in table 1:

Name	Length (bp)	Application	Sequence (5'→3')
IL-4 forward	21	RT-PCR	gaa tgt acc agg agc cat atc
IL-4 reverse	21	RT-PCR	ctc agt act acg agt aat cca
IL-6 forward	22	RT-PCR	ttc cat cca gtt gcc ttc ttg g
IL-6 reverse	19	RT-PCR	ctt cat gta ctc cag gta g
TNF-α forward	21	RT-PCR	gcg acg tgg aac tgg cag aag
TNF-α reverse	21	RT-PCR	ggt aca acc cat cgg ctg gca
β-actin forward	20	RT-PCR	gca ttg ctg aca gga tgc ag
β-actin reverse	20	RT-PCR	cct gct tgc tga tcc aca tc
NF-κB a	29	EMSA	atc agg gac ttt ccg ctg ggg act ttc cg
NF-κB b	29	EMSA	cgg aaa gtc ccc agc gga aag tcc ctg at
NF-Y a	27	EMSA	aga ccg tac gtg att ggt taa tct ctt
NF-Y b	27	EMSA	aag aga tta acc aat cac gta cgg tct
Bcl10 wt	22	Genotyping	ttg gct ctc tgc tct cct cac t
Bcl10 com	22	Genotyping	cgc tct gag gac tgt ggg act g
Malt1 wt	28	Genotyping	act ttc atc ttg cca gca ctc ttt ctt a
Malt1 com	27	Genotyping	ctg ctg ctg aca tgc tac aat atg ctg
Neo N-73	26	Genotyping	ggg tgg gat tag ata aat gcc tgc tc

Table 1: List of used primer and oligonucleotide sequences

2.1.3.7 Standard solutions and buffers

Antibody incubation and blocking buffer	5% skim milk powder (w/v) in TBST, pH 7.4

Buffer A	10 mM HEPES, pH 7.9
	10 mM KCl
	0.1 mM EDTA
	0.1 mM EGTA
	1 mM DTT (added freshly before use)
	1 tablet EDTA-free protease inhibitor cocktail per 10 ml
Buffer C	20 mM HEPES, pH 7.9
	0.4 M NaCl
	1 mM EDTA
	1 mM EGTA
	1 mM DTT (added freshly before use)
	1 tablet EDTA-free protease inhibitor cocktail per 10 ml
CHAPS lysis buffer	10 mM Tris, pH 7.5
	0.5% CHAPS (w/v)
	1 mM $MgCl_2$
	1 mM EGTA
	10% glycerol
	100 µM $NaVO_3$
	50 mM NaF
	1 tablet EDTA-free protease inhibitor cocktail per 10 ml
DNA loading dye (10x)	50% glycerol (v/v)
	0.2% bromophenol blue (w/v)
	0.2% xylene cyanol (w/v)
	1 mM EDTA, pH 8.0
FACS buffer	PBS
	3% fetale calf serum (v/v)
	0.05% sodium azide (w/v)
PBS	137 mM NaCl, pH 7.2-7.4
	2.7 mM KCl
	8.1 mM Na_2HPO4
	1.5 mM KH_2PO4

PBST	PBS 0.05% Tween-20 (v/v)
SDS-PAGE running buffer	25 mM Tris, pH 8.3 2 M glycine 1% SDS
SDS-PAGE sample buffer (2x)	125 mM Tris/HCl, pH 6.8 20% glycerol (v/v) 4% SDS (w/v) 0.2% bromophenol blue in a.d. (w/v) 10% β-mercaptoethanol (v/v) (added freshly before use)
Stop buffer	1 mM $NaVO_3$ 10 mM NaF in PBS, pH 7.4
Stripping buffer	2.5 mM Tris, pH 6.8 2% SDS (w/v) 0.7% β-mercaptoethanol (v/v) (added freshly before use)
TAE	0.4 M Tris base 1.1% acetic acid (v/v) 0.5 M EDTA
TBE	100 mM Tris base, pH 8.3 83 mM boric acid 1 mM EDTA
TBS	20 mM Tris, pH 7.4 137 mM NaCl
TBST	TBS, pH 7.4 0.025% Tween-20 (v/v)

Transfer buffer	50 mM Tris, pH 8.5
	40 mM glycine
	0.03% SDS (w/v)
	20% methanol (v/v) (added freshly before use)

2.2 Methods

2.2.1 Work with nucleic acids

2.2.1.1 PCR amplification

Polymerase chain reaction (PCR) is mostly used as a tool for the enzymatic amplification of specific nucleotide sequences *in vitro* (Mullis, Faloona *et al.* 1986; Sambrook 2001) and is based on the ability of DNA polymerases to amplify a DNA strand by semiconservative replication and on their thermostability. One amplification cycle starts with ***denaturation*** of the double-stranded matrice DNA (template) at 95°C, followed by ***hybridization*** or ***annealing*** of the DNA oligonucleotides (forward and reverse primers, table 1) with their specific complementary binding sites at their specific annealing temperature. In the ***extension*** or ***polymerisation*** phase, the thermostabile DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase) synthesizes the new DNA double strand from the 3'-end of the primers at 72°C. One PCR run is completed by a 5 min denaturation step at 95°C before the first cycle to make sure that all template molecules are single-stranded and a final 10 min polymerisation step at 72 °C to complete all synthesized DNA strands.
The annealing temperature of the specific primers with a length of approximately 18 bp is chosen on the basis of their melting temperature, which can be calculated with the fast Wallace method according to the following equation (Rychlik, Spencer *et al.* 1990):

$$T_m = 2°C \times n\,(A/T \text{ pairs}) + 4°C \times n\,(G/C \text{ pairs})$$

Generally, an initial annealing temperature of 55°C was used, which is usually adequate for primers with a length of approximately 20 bp. If non-specific products resulted, this temperature was increased, if there was no product, the temperature was decreased.

The following section describes a typical PCR reaction and a PCR cycling programme:

PCR reaction mixture (20 µl):	1.0 µl DNA sample (appr. 20 ng) 1.0 µl forward primer (10 pmol/µl) 1.0 µl reverse primer (10 pmol/µl) 2.0 µl 10x PCR buffer 2.0 µl dNTP mix (0.25 mM each) 0.25 µl *Taq* DNA polymerase ad 20 µl ddH_2O
PCR cycling programme:	95°C 5 min 95°C 30 s (denaturation) 55-62°C 1 min (hybridization) 72°C 1 min/1 kb (polymerisation) } 26-34 cycles 72°C 10 min 4°C final hold of temperature

2.2.1.2 RNA isolation from murine cells

RNA from murine cells is isolated with TRIzol® reagent according to the manufacturer's instructions. All steps are performed under a fume hood with gloves and autoclaved reaction tubes to avoid contamination with RNases. Up to 1 x 10^7 cells are resuspended in 1 ml of TRIzol® Reagent and incubated at RT for 5-10 min to permit complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform are added, vigorously shaken for 15 s and incubated at RT for 2-3 min. Samples are centrifuged for 15 min at 12,000 g and 4°C, which separates the mixture into a lower red, organic phenol-chloroform phase, an interphase and a colourless upper aqueous phase. Since the RNA exclusively remains in the aqueous phase, this upper phase is transferred to a fresh 1.5 ml tube and the RNA is precipitated by addition of 0.5 ml 2-propanol, proper mixing and incubation for 10 min at RT. After centrifugation for 10 min at 12,000 g and 4°C, supernatant is removed and the RNA precipitate is washed with 1 ml 75% (v/v) ethanol by vortexing and centrifugation for 5 min at 7,500 g and 4°C. At the end, the RNA pellet is air-dried for 15 min, dissolved in appr. 20 µl DEPC-treated RNase-free water and RNA concentration measured according to the protocol described in the following section 2.2.1.3.

2.2.1.3 Determination of nucleic acid concentration

The concentration of nucleic acids (DNA or RNA) in aqueous solution can be determined via photometrical measurement at 260 nm. Since the maximal absorption of proteins in aqueous solution is found at 280 nm, it is also possible to determine the purity of the nucleic acid solution with a measurement of the extinction E at 260 nm and 280 nm. The bigger the ratio of E_{260} to E_{280}, the higher is the purity of the nucleic acid solution. Values for E_{260}/E_{280} between 1.7 and 2.0 indicate a pure nucleic acid sample. The extinction E at 260 nm is 1 (E_{260} = 1) for dsDNA at a concentration of 50 µg/ml and for ssRNA at a concentration of 40 µg/ml, which allows the determination of the concentration of the measured DNA or RNA sample. DNA solutions are measured in aqueous solution with distilled water as a negative control, RNA samples are measured in DEPC-treated distilled water with DEPC-treated distilled water as a negative control. All nucleic acid measurements are carried out in quartz glass cuvettes in a total volume of 100 µl.

2.2.1.4 Reverse transcription polymerase chain reaction (RT-PCR)

The reverse transcriptase-polymerase chain reaction (RT-PCR) is a tool for the amplification of specific mRNA fragments after reverse transcription of the isolated cellular RNA to cDNA. The RNA is first reverse transcribed using the SuperScript™ first-strand synthesis system. Therefore, 1 µg RNA is incubated with 500 ng random primers (stock 3 µg/µl) for 10 min at 70°C in DEPC-treated distilled water. Subsequently, dNTPs (final concentration 2.5 µM each), DTT (final concentration 100 mM) and 5x forward reaction buffer are added, incubated for 10 min at 25°C and then for 2 min at 42°C. 1 µl of SuperScript™ II Reverse Transcriptase is added, the reaction incubated for 50 min at 42°C and stopped through heat inactivation for 15 min at 70°C. Total volume of the reaction is 20 µl, all steps are carried out in DEPC-treated distilled water. The obtained cDNA is analysed via PCR for *IL-4*, *IL-6*, *TNF-*α and β*-actin* expression using the primers described in table 1 (section 2.1.3.6).

2.2.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method for the separation, identification, purification and isolation of linear DNA fragments based on the migration of negatively charged nucleic acid molecules to the anode of an electric field (Sambrook 2001). Their speed proportionally depends on the voltage and their mobility mostly is dependent on the molecular size and length. At high agarose concentrations, small fragments are efficiently

separated, whereas low concentrations are used for the separation of high molecular weight DNA fragments. The addition of intercalating fluorescence dyes to the agarose gel, e.g. ethidium bromide, permits the visualization of the DNA fragments after excitation of the dye with ultraviolet (UV) light of 320 nm.

For the analysis of amplified DNA fragments, 0.8 - 2% (w/v) agarose gels in TBE- or TAE buffer with one drop of ethidium bromide per 100 ml are used, the agarose concentration depending on the length of the respective fragment. A 100 bp or 1 kb DNA ladder at a concentration of 0.1 µg/µl is loaded in one lane as DNA standard. Gels are run in 1x TBE or 1x TAE buffer at a voltage of 80-140 V. DNA is visualised under UV light on a GelDoc 2000.

2.2.2 Work with proteins

2.2.2.1 Generation of total cell extracts

Total cell extracts are generated by the lysis of cells with 50-100 µl 0.5% CHAPS buffer for 30 min on ice with repeated shaking, followed by centrifugation for 5 min at 4°C and 14,000 rpm. After removal of cell debris and determination of protein concentration via the Bradford protein assay (see section 2.2.2.2), cell lysates are mixed with the same volume of 2x SDS-PAGE sample buffer and boiled for 5 min and stored at -20°C.

2.2.2.2 Determination of protein concentration (Bradford test)

1 µl of freshly prepared protein extracts (total cell extracts or nuclear extracts) are mixed with 100 µl of diluted protein assay dye concentrate (1:10 with distilled water) in a flat bottom 96-well-plate. The extinction of the blue solution at 595 nm is determined by spectral photometry and protein concentration calculated by the use of a calibrating line obtained from the measurement of different BSA solutions (0, 0.25, 0.5, 1.0, 2.0, 4.0 µg/µl BSA in CHAPS lysis buffer (total cell extracts) or buffer C (nuclear extracts)), respectively. Measurements are carried out in duplicates.

2.2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

4x resolving gel buffer: 1.5 M Tris/HCl, pH 8.8
4x stacking gel buffer: 0.5 M Tris/HCl, pH 6.8

The migration speed of proteins in the electric field depends on their size, form and electric charge. For denaturating SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins are denatured in SDS sample buffer, whereupon the addition of the anionic detergence SDS changes the charge of the proteins. Thus, anionic clusters are formed that have a constant negative charge per mass unit (Laemmli 1970). The addition of the reducing agent dithiothreitol (DTT) causes a reduction of disulfide bridges between the polypeptide chains. In total, this leads to a separation of the proteins during SDS-PAGE exclusively depending on the size of the proteins. In the discontinuous system, proteins first pass the stacking gel with big polyacrylamide pore width, and the composition of the running buffer and the stacking gel causes a concentration of the protein sample in the stacking gel. When proteins migrate into the resolving gel having a smaller pore width and a higher pH value, proteins get separated dependent on their molecular size. The range of separation can be changed by a variation of the resolving gel pore width, thus by a change of the polyacrylamide concentration.

Components	**Stacking gel**	**Resolving gel**		
	4%	**7.5%**	**10%**	**12%**
30% acrylamide/bisacrylamide	0.7 ml	2.5 ml	3.3 ml	4.0 ml
1.5 M Tris/HCl, pH 8.8	_	2.5 ml	2.5 ml	2.5 ml
0.5 M Tris/HCl, pH 6.8	1.2 ml	_	_	_
Aqua bidest.	3.0 ml	4.9 ml	4.1 ml	3.4 ml
10% (w/v) SDS	50 µl	100 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl	5 µl
10% (w/v) APS	25 µl	50 µl	50 µl	50 µl

Table 2: Composition of stacking and resolving gels (each sufficient for 2 gels)

For SDS-PAGE, a polyacrylamide resolving gel at the desired concentration (7.5 - 12%) is poured between two glass plates in a gel chamber array according to the recipe shown in table 2 and overlaid with water to avoid air bubbles. After polymerisation of the resolving gel, the water is removed and the stacking gel (receipt for a 4% polyacrylamide gel shown in table 2) is poured on the resolving gel and a plastic comb for the sample slots is put in. After polymerisation of the stacking gel, the comb is removed and the gel is put into a gel

chamber, which is filled with SDS-PAGE running buffer. Protein samples (prepared as described in section 2.2.2.1) are heated to 56°C for 5 min and then 5-15 µg protein per lane are loaded into the slots of the stacking gel and, through the application of a voltage of 80-140 V, proteins are separated. Electrophoresis is stopped when the blue band of the sample buffer reaches the lower frontier of the resolving gel.

2.2.2.4 Immunoblot (Western blot)

After completion of SDS-PAGE (chapter 2.2.2.3), separated proteins are transferred onto a protein-binding membrane by the semi-dry transfer method. For this purpose, glass plates and stacking gel are removed and the resolving gel is equilibrated in 1x transfer buffer for 5 min at RT. In parallel, the polyvinyldifluoride (PVDF) membrane (5 cm x 8.5 cm) is activated by rinsing in methanol for 1 min and washed in transfer buffer, and two pieces of 3 mm cellulose filter paper (Whatman paper) are also equilibrated in transfer buffer. Subsequently, the membrane is put on one piece of wet filter paper in a semi-dry transfer chamber, the resolving gel is put on the membrane without air bubbles in between and the second piece of filter paper is put on the top and tightly pressed onto the lower layers. Protein transfer is conducted for 90 min at a constant voltage of 25 V (150 mA/gel). After blotting, the membrane is stained with Ponceau S solution to control for proper protein transfer and afterwards washed for 30 min in TBST to remove Ponceau S.

2.2.2.5 Immunochemical detection of transferred proteins

For immunostaining, proteins are marked with a specific antibody and thus, can be detected via a chemiluminescence system (Haas, Becker *et al.* 1986).

For this purpose, the PVDF membrane is washed after transfer (section 2.2.2.4) in TBST and then incubated for approximately 1 h in 5% (w/v) skim milk/TBST, which blocks unspecific protein binding sites on the membrane and thus prevents a non-specific binding of the detection reagents. Since milk contains many protein components in a phosphorylated form that can react with phospho-specific antibodies (e.g. casein), a 5% (w/v) BSA/TBST solution is used as blocking buffer, when phosphorylated proteins shall be detected. After blocking, the membrane is incubated with a specific primary rabbit or mouse antibody against an epitope of the protein of interest at the recommended concentration (in most cases 1:1,000 dilution) in 4 ml of 5% skim milk/TBST or 5% BSA/TBST in a 50 ml falcon tube on a roller shaker at 4°C over night. The next day, the membrane is washed three times for 10 min with 1x TBST to remove unbound antibodies,

then incubated with appropriate anti-rabbit or anti-mouse IgG HRP-coupled secondary antibodies against the Fc part of the primary antibody in 5% skim milk/TBST or 5% BSA/TBST for at least 1 h at room temperature. Then, the membrane is washed again three times for 10 min with 1x TBST and for 1 min with 1x TBS. Subsequently, membranes are incubated for 1-2 min with ECL Western Blotting Substrate (freshly prepared 1:1 mixture of solution A and B), which is cleaved by the horseradish peroxidase (HRP) into a luminescent substance. In a darkroom, a high sensitivity chemiluminescence hyperfilm is exposed to the luminescent membrane in an X-ray cassette for adequate times (1 s – 1 h) and developed in a developer machine.

2.2.2.6 Removal of antibodies from the membrane (stripping)

For the successive detection of several proteins on the same membrane, the bound antibody can be removed by incubation of the membrane in stripping buffer for 20 min at 50°C. After three times of washing for 10 min in TBST, the membrane is blocked again in 5% (w/v) skim milk/TBST and then treated as described in section 2.2.2.5 and incubated with another primary antibody over night.

2.2.2.7 Generation of nuclear extracts

For the generation of nuclear extracts, cells are washed with cold PBS and centrifuged at 300 g and 4°C for 5 min. The cell pellet is resuspended with 800 µl hypotonic buffer A and incubated for 10 min on ice. 50 µl 10% (v/v) NP-40 are added and cell suspension is mixed on a vortex mixer for 1 min or incubated for 5 min on an Eppendorf mixer at 4°C to destroy cell membranes. Lysates are centrifuged at 14,000 rpm and 4°C for 10 min. Obtained nuclear pellets are resuspended with 30-40 µl hypertonic buffer C and shaken at maximum speed for 30 min at 4°C. After centrifugation at 14,000 rpm and 4°C for 10 min, protein concentration of nuclear extracts is determined by Bradford protein assay and 10 µl aliquots are frozen in liquid nitrogen and then stored at -80°C.

2.2.2.8 Preparation of ^{32}P-labelled oligonucleotide probes

To prepare ^{32}P-endlabelled double-stranded oligonucleotide probes for the radioactive detection of specific transcription factors, the following reaction is set up on ice with the oligonucleotides shown in table 1:

2 µl sense oligonucleotide
2 µl T4 kinase
10 µl 5x forward reaction buffer
5 µl γ^{32}P-ATP
31 µl ddH_2O

The reaction is incubated for 1 h at 37°C and then cleaned through a G25 Microspin column. 2 µl of the antisense oligonucleotide strand are added and incubated in boiling water for annealing. After cooling down to room temperature, 1 µl of the probe is measured in 5 ml of scintillation solution in a scintillation counter, then diluted to a final activity of 1 x 10^5 cpm, stored in plexiglass boxes at -20°C and used within 20 days.

2.2.2.9 Electromobility shift assay (EMSA)

4x NF-κB binding buffer: 20 mM HEPES, pH 7.9; 200 mM KCl; 2 mM DTT (added freshly); 40% (v/v) glycerol
4x NF-Y binding buffer: 20 mM HEPES, pH 7.9; 60 mM KCl; 1 mM EDTA; 2 mM DTT; 40% (v/v) glycerol
1 µg/µl poly(dIdC)

To study translocation of distinct transcription factors to the nucleus, 3-4 µg of nuclear protein are incubated with ^{32}P-endlabelled double-stranded oligonucleotide probes (section 2.2.2.8) against NF-κB or NF-Y. The binding reaction is set up on ice as follows:

3-4 µg nuclear protein in buffer C
5 µl 4x NF-κB binding buffer or 4x NF-Y binding buffer
1 µl poly(dIdC)
ad 15 µl aqua bidest.

Samples are mixed with 5 µl of ^{32}P-endlabelled oligonucleotide probe against NF-κB or NF-Y and incubated for 30 min at RT. Then 2.5 µl 10x DNA loading dye are added and samples are applied on a SDS-free, native 5% polyacrylamide gel. PAGE is conducted at 150-200 V for appr. 1.5-2 h until the lower band of the dye reaches the bottom of the gel. Afterwards, the gel is dried for 2 h under vacuum at 80°C in a gel dryer, wrapped in plastic foil and put into a PhosphoImaging cassette. After exposition over night, the screen is analysed with a PhosphoImager and Image Quant software.

2.2.2.10 Measurement of cytokines by ELISA

Blocking buffer: PBS; 1% (w/v) BSA; 5% (w/v) sucrose; pH 7.2-7.4
Reagent buffer: PBS; 1% (w/v) BSA
Phosphate-citrate buffer: 0.05 M Na_2HPO_4; 0.05 M citric acid, pH 5.0
Substrate reagent: 1 tablet TMB in 10 ml phosphate-citrate buffer, pH 5.0; 2 µl 30% H_2O_2

The enzyme-linked immunosorbent assay (ELISA) is a biochemical technique to specifically detect the presence of an antibody, antigen or cytokine in a fluid sample. It uses two antibodies, the first is specific to the antigen and the second reacts to an antigen-antibody and is coupled to an enzyme, which then cleaves a chromogenic substrate to produce a signal. Here, a so-called sandwich-ELISA is used, in which a 96-well microtiter plate is coated with a known quantity of the diluted primary antibody (50 µl/well) at 4°C overnight. The next day, antibody solution is removed, the plate is tap dried and incubated with 200 µl blocking buffer/well for 2 h at RT. Subsequently, the plate is washed three times with washing buffer and 50 µl of samples and cytokine standards diluted in reagent buffer are added per well and incubated overnight at 4°C. After three washing steps to remove unbound antigen/cytokines, detection buffer with secondary antibody and horseradish peroxidase (HRP) enzyme is added (50 µl/well) and incubated for 1-2 h at RT. Then the plate is washed five times to remove unbound HRP enzyme and incubated with 100 µl/well TMB substrate solution (tetramethylbenzidine in phosphate-citrate buffer) until the solution shows a considerable blue colour. Reaction is terminated by the addition of 50 µl 2N H_2SO_4/well and after 5 min, the resulting yellow colour is photometrically measured within 30 min at 450 nm (reference wavelength 570 nm) in a plate reader and results analysed with Magellan software.
With this method, the concentrations of the cytokines TNF-α, IL-4, IL-6 are determined by ELISA DuoSets (BD Pharmingen) on Maxisorp 96-well-plates and required solutions prepared as recommended by the manufacturer.

2.2.3 Work with eukaryotic cells

All cells are cultured in an incubator at 37°C, 5% CO_2 and a relative humidity of 95% and growth speed and phenotype regularly controlled with a light microscope.

2.2.3.1 Cultivation and passaging of adherent cell lines

The adherent cell lines NIH3T3, Phoenix E and murine embryonic fibroblasts (MEFs) are cultured in DMEM supplemented with 10% (v/v) FCS, Pen/Strep and L-glutamine in 10 or 15 cm tissue culture dishes. At 90% confluence, cells are enzymatically detached from the

bottom through trypsin/EDTA solution. For this purpose, medium is removed, cells are carefully washed with PBS and subsequently, a small amount of prewarmed trypsin/EDTA solution is added and the plate is incubated for 5-10 min at 37°C. This incubation time should not be exceeded, because the cells could be damaged by beginning digestion of the cell membrane. After visible detachment of the cells from the bottom, they are mixed with media and cell clods destroyed through pipeting up and down. After centrifugation for 5 min at 300 g to remove of trypsin/EDTA, cells are resuspended in fresh DMEM with 10% FCS and appropriate amounts plated onto new cell culture dishes. Phoenix E cells are very sensitive to cell density and are split every day to the half amount. NIH3T3 cells and MEFs are passaged every 2-3 days in a ration of 1:4.

2.2.3.2 Cultivation and passaging of suspension cells

Suspension cells are grown in 15 or 75 ml tissue culture flasks in RPMI media supplemented with 5-10% (v/v) FCS, Pen/Strep and L-glutamine. Cells are passaged every 2-3 days in a ration of 1:5 via centrifugation of the cell suspension at 300 g for 5 min and distribution of appropriate amounts of cells in fresh RPMI media into new flasks.

2.2.3.3 Freezing of cells

Cells can be frozen for longer storage in liquid nitrogen and rethawed again when required. Therefore, 0.5 ml of a dense cell suspension is taken and mixed with the same volume of precooled 2x freezing media (20% (v/v) DMSO, 20% (v/v) FCS and 60% (v/v) DMEM for adherent cells or 60% (v/v) RPMI for suspension cells) in a cryo vial. These vials are put in a cryo cooler with 2-propanol and frozen at –80°C for 12-24 h. Afterwards, cells can be transferred to liquid nitrogen for longer storage.

2.2.3.4 Thawing of cells

After removal of the cryo vial from the liquid nitrogen tank, it is immediately transferred to a 37°C water bath to thaw the cells. Cells are carefully resuspended in prewarmed culture media and DMSO is removed by centrifugation at 300 g. Now cells can be cultured as usual in appropriate media.

2.2.3.5 Determination of cell number

To determine cell number and to distinguish between living and dead cells, cells are mixed with Trypan blue solution and counted under a light microscope. Living cells are able to exclude the dye and appear bright in the microscopic image, whereas dead cells and cell debris are stained dark blue with Trypan blue.

A small amount of the cell suspension is mixed with a 0.4% (w/v) Trypan blue solution in an appropriate ratio and 10 µl of this mixture are pipetted under the cover slip of a Neubauer counting chamber. Under the light microscope, all living cells in the 25 middle squares are counted and the cell number per ml is determined by multiplication of this counted number with 10^4 and the respective dilution factor.

2.2.4 Mast cell experiments *in vivo* and *in vitro*

2.2.4.1 Bcl10- and Malt1-deficient mice

Bcl10- and Malt1-deficient mice were generated as reported previously by Ruland *et al.* (Ruland, Duncan *et al.* 2001; Ruland, Duncan *et al.* 2003). Mice are housed under specific pathogen-free conditions. The experiments are conducted under federal guidelines for the use and care of laboratory animals and approved by the Government of the District of Upper Bavaria. Mice are of a C57BL/6 and 129/J mixed background. Littermates are used in each experiment.

2.2.4.2 Skin histology

Naïve animals at 10 to 12 weeks of age are sacrificed and skin samples of appr. 1 cm^2 from the ear, neck and groin region are cut off and fixed in 4% (v/v) formalin. Tissue sections (4 µm) are stained with toluidine blue colouring mast cell granules, and toluidine blue-positive mast cells are counted in the dermis of each sample at 40x magnification. A total of 10 fields at 10x magnification (per mouse and location) is used to delineate and integrate the analysed dermal area (KS 300 software, Zeiss).

2.2.4.3 Passive cutaneous anaphylaxis (PCA)

Passive cutaneous anaphylaxis (PCA) as described by Ovary (Ovary 1958) is a useful experimental model to study allergen/IgE interaction leading to inflammatory mediator release from mast cells and the clinical expression of cutaneous anaphylaxis. Although

PCA may be elicited in many animal species, PCA in mice has been widely used for immunopharmacological studies or gene function analysis in allergy. There are two main types of PCA reaction, which can be triggered on mouse ears, on the one hand showing immediate phase reactions and on the other hand late phase anaphylactic responses (LPR) of mast cells. The PCA experiments in mice are based on passive sensitisation with an antigen-specific IgE antibody, either by local or systemic administration, and subsequent challenge with the adequate antigen, this time systemic or locally, dependent on the previous sensitisation.

2.2.4.3.1 Immediate phase PCA

0.5% (w/v) Evans blue in PBS; 1% (w/v) Evans blue
1 M KOH; 0.6 N H_3PO_4/acetone: 1 part 0.3 M H_3PO_4 + 2.6 parts acetone

For immediate phase PCA reactions, 10 to 12 week old naïve mice (littermates) are passively sensitised by intradermal injection of 250 ng anti-DNP IgE mAb (clone H1-ε-26, provided by F.T. Liu, Sacramento, CA and D.H. Katz, San Diego, CA) in 20 µl PBS into the dorsal side of one ear lobe until a bulge is clearly visible under the skin. A subcutaneous injection of 20 µl PBS is given as a negative control into the contra lateral ear. 24 h later, mice are challenged with antigen by intravenous injection of 150 µg of dinitrophenyl-human serum albumin (DNP-HSA, 30–40 moles DNP/mol HSA; Sigma) in 100 µl 0.5% (w/v) Evans blue dye into the tail vein. To determine the amount of extravasated dye, mice are sacrificed after 30-60 min, ears removed and Evans blue dye is extracted by incubation of ears with 140 µl 1 M KOH (potassium hydroxide) at 37°C over night. Reaction is stopped by addition of 860 µl 0.6 N H_3PO_4/acetone (ratio 1:2.6). After vigorous vortexing, tubes are centrifuged for 10 min at 13,000 rpm, supernatants filtered through a 40 µm cell strainer and photometrically quantified at 630 nm (Nagai, Sakurai *et al.* 1995). Values are compared to an Evans blue standard curve generated with Evans blue dilutions from 0 to 40 µg/ml in KOH/H_3PO_4/acetone. Samples and standard curve values are measured in triplicates.

2.2.4.3.2 Late phase PCA

0.2% (w/v) DNFB in acetone/olive oil (4:1)

For late phase PCA reactions, 10 to 12 week old naïve mice (littermates) are passively sensitised by intravenous injection of 2 µg anti-DNP IgE mAb (clone H1-ε-26) in 100 µl

PBS into the tail vein or left unsensitised. 24 h later, the baseline value of ear thickness is measured before antigen challenge using an engineer's micrometer dial thickness gauge. Subsequently, sensitised and non-sensitised mice are challenged by epicutaneous application of 10 µl of 2,4-dinitrofluorobenzene (DNFB) (0.2% w/v) in acetone-olive oil (ratio 4:1) to both sides of both ears. After 1 h, the ear swelling response is measured for the first time and repeated at the time points 2, 4, 6, 8, 12, 24, 48 and 72 h. The increment of ear thickness (post-challenge value – pre-challenge baseline value) is expressed as percentage of the baseline value obtained before antigen challenge according to the following equation:

$$\% \text{ increase in ear thickness} = \frac{\Delta \text{ ear thickness (post challenge - pre challenge)}}{100} \text{ x ear thickness (pre challenge)}$$

2.2.4.4 Generation of bone marrow-derived mast cells (BMMCs) and FACS analysis

30 µg/ml rmIL-3 in PBS; 50 µg/ml rmSCF in PBS

Wt, Bcl10- and Malt1-deficient mice are sacrificed, the femurs taken and rinsed with RPMI 1640 with a sterile needle and a syringe. Bone marrow cells are squeezed through a 70 µm cell strainer with the plunger of the syringe, centrifuged for 5 min at 300 g and 4°C, washed with PBS and counted. 1 x 10^7 bone marrow cells are cultured in suspension in a 10-cm cell culture dish in 10 ml RPMI 1640, supplemented with 20% (v/v) fetal calf serum (FCS), 30 ng/ml rmIL-3, 50 ng/ml rmSCF and 150 µM monothioglycerol. Medium is changed every week and cell density of non-adherent cells adjusted to 1 x 10^6/ml. To quantify BMMC frequencies after 4 weeks, cells are stained with FITC-conjugated anti-FcεRI and PE-conjugated anti-c-Kit (CD117) antibody and analysed by flow cytometry (FACScan, Becton Dickinson).

2.2.4.5 Measurement of calcium flux

Tyrode's buffer: 10 mM HEPES pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% (w/v) BSA

To measure intracellular calcium flux after mast cell activation, BMMCs (2 x 10^6/ml) are loaded with anti-DNP IgE mAb (SPE-7; 5 µg/ml) for 1 h on ice in Tyrode's buffer or are left unsensitised. After washing, cells are permeabilised and incubated with the fluorescence dye Indo-1 (acetoxymethyl ester, 5 µg/ml) for 30 min in a 37°C water bath. Then the baseline fluorescence of BMMCs is analysed by flow cytometry (FL7/FL8) for 1 min, cells

are stimulated with either Tyrode's buffer alone, the antigen DNP-HSA (final concentration 20 ng/ml) or PMA and ionomycin (Iono, final concentration 100 nM each) and the change in fluorescence of activated cells recorded for 10 min.

2.2.4.6 Measurement of mast cell degranulation (β-hexosaminidase assay)

To induce mast cell degranulation, BMMCs (2 x 10^6/ml) are loaded with anti-DNP IgE mAb (SPE-7; 5 µg/ml) for 1 h on ice in Tyrode's buffer. After washing, sensitised cells are stimulated with the indicated concentrations of DNP-HSA in Tyrode's buffer for 60 min at 37°C. Alternatively, cells are left unsensitised and stimulated with phorbol-12-myristate-13-acetate (PMA; 100 nM) and ionomycin (Iono; 100 nM) in Tyrode's buffer at 37°C. After centrifugation for 5 min at 500 g, the enzymatic activities of β-hexosaminidase in supernatants and cell pellets solubilised with 0.5% (v/v) Triton® X-100 in Tyrode's buffer are measured by incubation with a 1.3 M solution of the substrate *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosamine (pNAG) in 0.1 M sodium citrate (pH 4.5) for 90 min at 37°C. The reaction is terminated by the addition of 0.2 M glycine (pH 10.7). Enzymatic cleavage of the substrate pNAG by β-hexosaminidase releases 4-*p*-nitrophenol, whose amount can be photometrically measured at 405 nm (reference wavelength 570 nm) (Nishizumi and Yamamoto 1997). The extent of spontaneous β-hexosaminidase release or the percentage of degranulation is calculated as follows:

$$\% \text{ degranulation} = \frac{E_{405}\ (\text{supernatant})}{E_{405}\ (\text{supernatant}) + E_{405}\ (\text{lysate})}$$

2.2.4.7 Measurement of leukotrienes

BMMCs (2 x 10^6/ml) are loaded with anti-DNP IgE mAb (SPE-7; 0.5 µg/ml) overnight, washed twice and stimulated with increasing doses of DNP-HSA or left unsensitised and stimulated with PMA and Iono (100 nM each) for 30 min at 37°C. Cell supernatants are harvested and leukotrienes LTC_4, LTD_4 and LTE_4 are determined by an enzyme-linked immunoassay (EIA) according to the manufacturer's instructions.

2.2.4.8 Signal transduction

To study FcεRI signalling on a molecular level, BMMCs (2 x 10^6/ml) are preloaded with

anti-DNP IgE mAb (SPE-7; 0.5 µg/ml) and subsequently activated by adding DNP-HSA (20 ng/ml) in a 37°C water bath as indicated. Alternatively, BMMCs are left unsensitised and stimulated with PMA and Iono (100 nM each) for indicated time points or with anti-DNP IgE mAb (5 µg/ml) alone for 24 h at 37°C for Bcl-X_L induction. Reaction is terminated by the addition of precooled stop buffer and immediate centrifugation for 5 min at 500 g and 4°C. Cells are lysed with 0.5% (w/v) CHAPS lysis buffer, protein concentration determined by Bradford assay (section 2.2.2.2) and denatured proteins subjected to immunoblotting as described above (sections 2.2.2.3 - 2.2.2.5).
For electromobility shift assays, BMMCs (1 x 10^7/sample) are stimulated with PMA/Iono (100 nM each) for 1 h in a 37°C water bath and nuclear extracts subjected to electromobility shift assays as previously described (section 2.2.2.6).

2.2.4.9 Mast cell survival assay

BMMCs are cultured as indicated in the presence or absence of growth factors and FCS. After incubation for 24, 48 and 72 h, cells are harvested and cell viability is quantified by flow cytometry after annexin V/propidium iodide staining as recommended by the manufacturer (BD Biosciences) or by microscopical analysis after Trypan blue staining.

2.2.4.10 Statistical analysis

Results are analysed for statistical significance with the unpaired two-tailed student's t-test with Microsoft Excel. Data from *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice or BMMCs are separately compared to the wild type. Differences between groups are considered significant at $P \leq 0.05$.

2.2.5 Experiments with murine embryonic fibroblasts *in vitro*

For all described experiments, murine embryonic fibroblasts (MEFs) are used, either freshly prepared MEFs between passage 3 to 10 or immortalized fibroblasts after recovery of the growth arrest.

2.2.5.1 Retroviral infection

Packaging cells (Phoenix E) are cultured on a 6-well tissue culture plate in DMEM supplemented with 10% (v/v) FCS and transfected with a retroviral vector encoding full

length Bcl10 (pBABE-puro-Bcl10) or an empty retroviral vector (pBABE-puro). For transfection, 1 µg DNA is mixed with 10 µl TransFectin™ Lipid Reagent and 500 µl FCS-free DMEM and added to one well of Phoenix cells on a 6-well plate according to the manufacturer's instructions. 48 h later, virus-containing cell supernatant is collected, polybrene added to a final concentration of 4 µg/ml and MEFs are spin-infected with the virus at 2500 rpm and 30°C for 90 min in a 6-well plate. Subsequently, puromycin is added to a final concentration of 1 µg/ml for cell selection over 10 days.

2.2.5.2 MEF survival assay

MEFs are seeded on 24-well tissue culture plates in DMEM supplemented with 0.2% (v/v) FCS and incubated for 24, 48 and 72 h with or without 10 µM LPA. After the indicated times, adherent and detached cells are harvested, stained with FITC-conjugated annexin V and propidium iodide according to the manufacturer's instructions (BD Pharmingen) and cell viability analysed by FACS analysis.

3 Results

3.1 Function of the Bcl10/Malt1 complex in FcεRI-mediated NF-κB activation

3.1.1 Bcl10 and Malt1 are dispensable for mast cell development and morphology *in vivo*

To study potential roles of Bcl10 and Malt1 in mast cells and FcεRI signalling *in vivo* and *in vitro*, first number, distribution and morphology of skin mast cells from wt mice and mice with a genetic disruption of the *Bcl10* gene (*Bcl10*$^{-/-}$ mice) or *Malt1* gene (*Malt1*$^{-/-}$ mice) were analysed. For this purpose, skin sections from the ear, the neck and the groin from mice of all three genotypes were treated with the metachromatic dye Toluidine blue, which stains the mast cell granules and then, mast cells quantified microscopically (figure 11). The anatomical distribution, morphology and frequency of skin mast cells were comparable between mice of the three genotypes as shown in figure 11a and 11b, indicating that the development of mast cells *in vivo* is not affected by the *Bcl10* or *Malt1* deletion.

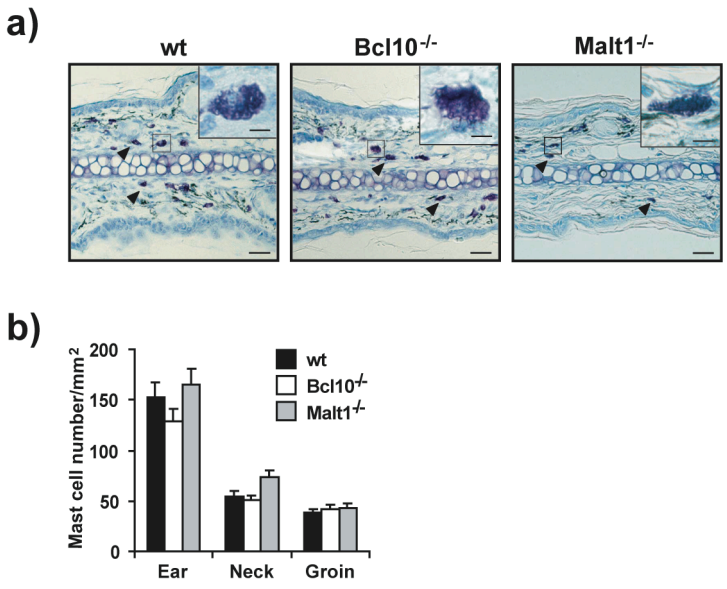


Fig. 11: Mast cell development in the absence of Bcl10 and Malt *in vivo*

a) and b) Skin biopsies from wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice. a) Representative areas from the ear skin show the presence of Toluidine blue-positive mast cells in all three genotypes (black arrowheads) at 20x magnification. Insets show 100x magnification of the mast cells boxed in each section. Scale bars, 10 µm (full pictures) and 2 µm (insets). b) Mast cell frequencies in skin sections from ear, neck and groin (n=3 mice/genotype). There was no significant difference among the three genotypes.

3.1.2 Bcl10 and Malt1 in IgE-induced passive cutaneous anaphylaxis reactions *in vivo*

Most mast cell effector functions depend on the release of proinflammatory mediators, on the one hand the immediate release of preformed substances stored in intracellular granules, on the other hand the delayed *de novo* production of cytokines and chemokines, leading to late phase outcomes like inflammatory processes (Galli, Nakae *et al.* 2005; Metcalfe, Baram *et al.*1997).

This IgE-induced mediator release can be studied *in vivo* in a mouse model through the onset of passive cutaneous anaphylaxis (PCA) reactions on the skin, including a sensitisation phase with IgE antibody and an irritation phase through antigen administration (Inagaki, Goto *et al.*1986; Nagai, Abe *et al.* 2000).

3.1.2.1 Bcl10 and Malt1 do not control early phase PCA reactions

For the analysis of physiological early and late phase mast cell functions *in vivo* in the absence of Bcl10 or Malt1, two types of FcεRI-mediated passive cutaneous anaphylaxis (PCA) experiments were performed separately (figure 12).

In the first set of experiments, the immediate phase PCA reactions were analysed by an Evans blue extravasation test on the ears of wt, Bcl10- or Malt1-deficient mice. One ear was primed by subcutaneous injection of a monoclonal anti-dinitrophenyl (anti-DNP) IgE antibody, the other ear served as an internal negative control and therefore was injected subcutaneously with PBS. 24 h later, all animals were coinjected intravenously (i.v.) with the antigen dintrophenol (DNP) coupled to human serum albumine (HSA) (= DNP-HSA) in a solution of 0.5% (w/v) Evans blue dye. Binding of the antigen to anti-DNP IgE leads to FcεRI crosslinking and rapid mast cell degranulation with release of histamine and serotonin, inducing locally increased blood vessel permeability and subsequent Evans blue extravasation in the sensitised ear. This could be monitored by inspection of the ears already after several minutes (figure 12a), and the extravasated dye was quantified after 60 min by extraction from the ears with potassium hydroxide and photometrical measurement (figure 12b). There were no significant differences in the extravasation kinetics or the total amount of extravasated Evans blue dye in the ears among wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice, indicating that the immediate phase PCA reaction depending on rapid degranulation events is not controlled by Bcl10 or Malt1.

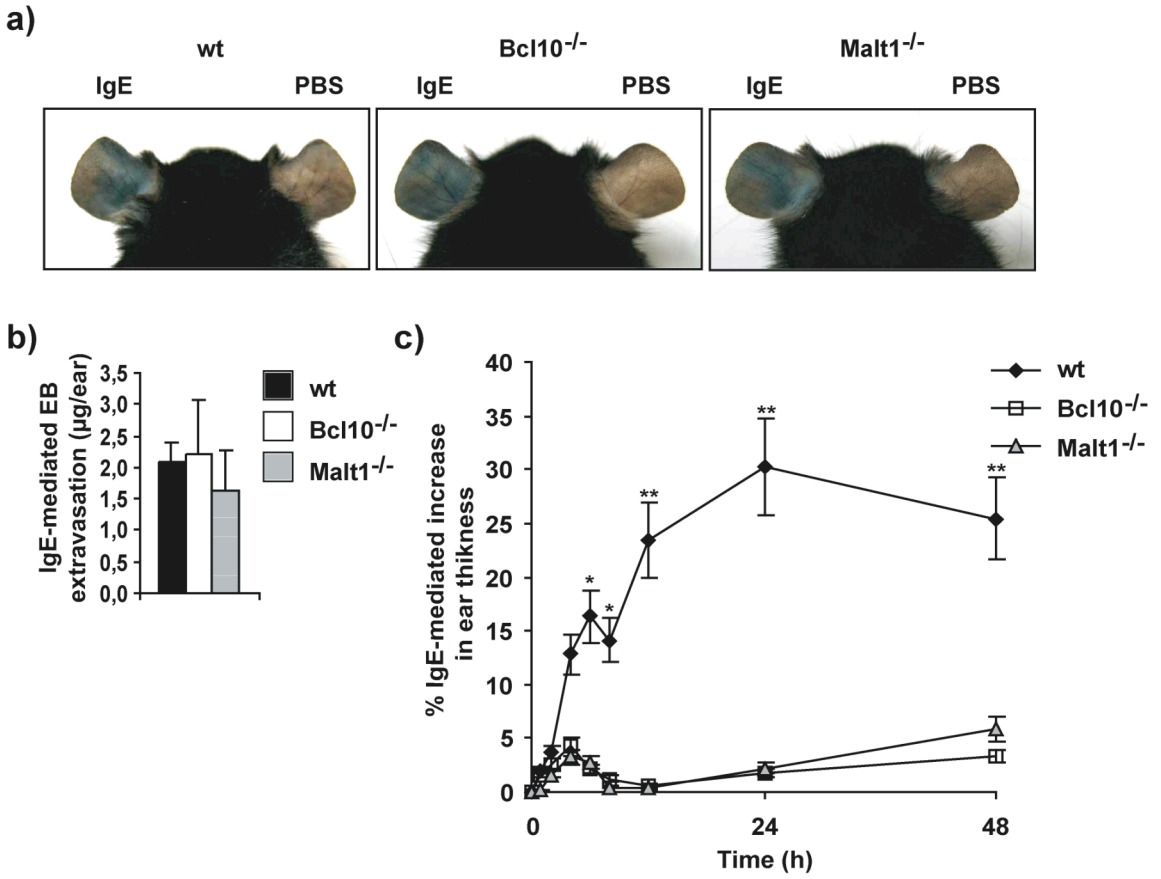


Fig. 12: Normal immediate passive cutaneous anaphylaxis (PCA) reaction, but defective late phase PCA response in Bcl10- and Malt1-deficient mice

a) and b) Immediate phase PCA reaction. Mice were passively sensitised by intradermal injection of anti-DNP IgE into the left ear. The right ear was injected with PBS. Subsequently, mice were challenged by intravenous injection of the antigen DNP-HSA in 0.5% Evans blue in PBS. a) Ears from wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice 30 min after antigen challenge. b) The IgE-mediated Evans blue extravasation was calculated as the difference in the amount of extravasated dye between the IgE-sensitised and the non-sensitised control ear after extraction with KOH. Differences between the three genotypes were not statistically significant. c) Late phase PCA reaction. Wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice were passively sensitised by intravenous injection of anti-DNP IgE. 24 h later, they were challenged by epicutaneous application of 0.2% 2,4-dinitrofluorobenzene to both ears and ear thickness was measured over time. The increase in ear thickness was calculated as described in Material and methods. Results are representative of three independent experiments. Data are presented as mean ± SEM. *, P ≤ 0.05 and **, P ≤ 0.02 between wt and *Bcl10*$^{-/-}$ or wt and *Malt*$^{-/-}$ mice, respectively.

3.1.2.2 Bcl10 and Malt1 are essential for late phase PCA reactions

In the second approach, the late phase PCA was examined, which is mainly dependent on the *de novo* synthesis and release of proinflammatory cytokines. These recruit and activate neutrophils, leading to local inflammation and edema (Wershil, Wang *et al.* 1991; Nakae, Suto *et al.* 2005). For these experiments, wt, Bcl10- and Malt1-deficient mice were i.v. sensitised with monoclonal anti-DNP IgE antibody and 24 h later, the hapten dinitrofluorobenzene (DNFB, 0.2% (w/v) in acetone/olive oil), specifically crosslinking anti-DNP IgE molecules (Ray, Tharp *et al.* 1983), was applied on both sides of both ears. In wt

mice, an increasing ear swelling reaction with a first maximum at 6 h and a total maximum after 24 h could be detected, whereas *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice only displayed a minimal response with an increase in ear thickness not exceeding 20% of that observed in wt animals (figure 12c). These results suggest an essential role for Bcl10 and Malt1 in the elicitation of FcεRI-mediated late phase mast cell functions *in vivo*.

3.1.3 Analysis of FcεRI-mediated mast cell functions *in vitro*

3.1.3.1 Bcl10 and Malt1 are not involved in differentiation and regulation of FcεRI expression in BMMCs

In order to elucidate the basis for the defective late phase PCA reactions in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice, mast cells were generated *in vitro* from the bone marrow of these mice (bone marrow-derived mast cells, BMMCs). The cultivation of bone marrow cells from wt, Bcl10- and Malt1-deficient mice with IL-3 and SCF (figure 13) yielded similar amounts of differentiated BMMCs after 4-6 weeks, as assessed by flow cytometric analysis of the expression of the characteristic mast cell surface receptors FcεRI and c-Kit (figure 13a). Additionally, the expression levels of the FcεRI and c-Kit were not altered in Bcl10- or Malt1-deficient BMMCs, which was an important prerequisite for all further studies (figure 13b).

Since the expression of Bcl10 and Malt1 has never been shown in cell types of the myeloid lineage, a Western blot analysis of total mast cell extracts was conducted to confirm the production of Bcl10 and Malt1 in wt BMMCs (figure 13c). These contained detectable amounts of both Bcl10 and Malt1, and as expected, Bcl10 was not expressed in *Bcl10*$^{-/-}$ mast cells and Malt1 was not produced in *Malt1*$^{-/-}$ mast cells.
Furthermore, the analysis demonstrated a reduction of the Malt1 protein in *Bcl10*$^{-/-}$ mast cells and, *vice versa*, a reduction of Bcl10 in *Malt1*$^{-/-}$ cells compared to wt BMMCs, indicating that the stability and thus, the levels of the two proteins might depend on their interaction. Altogether, this demonstrated a redundancy of Bcl10 and Malt1 for the development of mast cells *in vitro*, coinciding with the histological *in vivo* results (see figure 11).

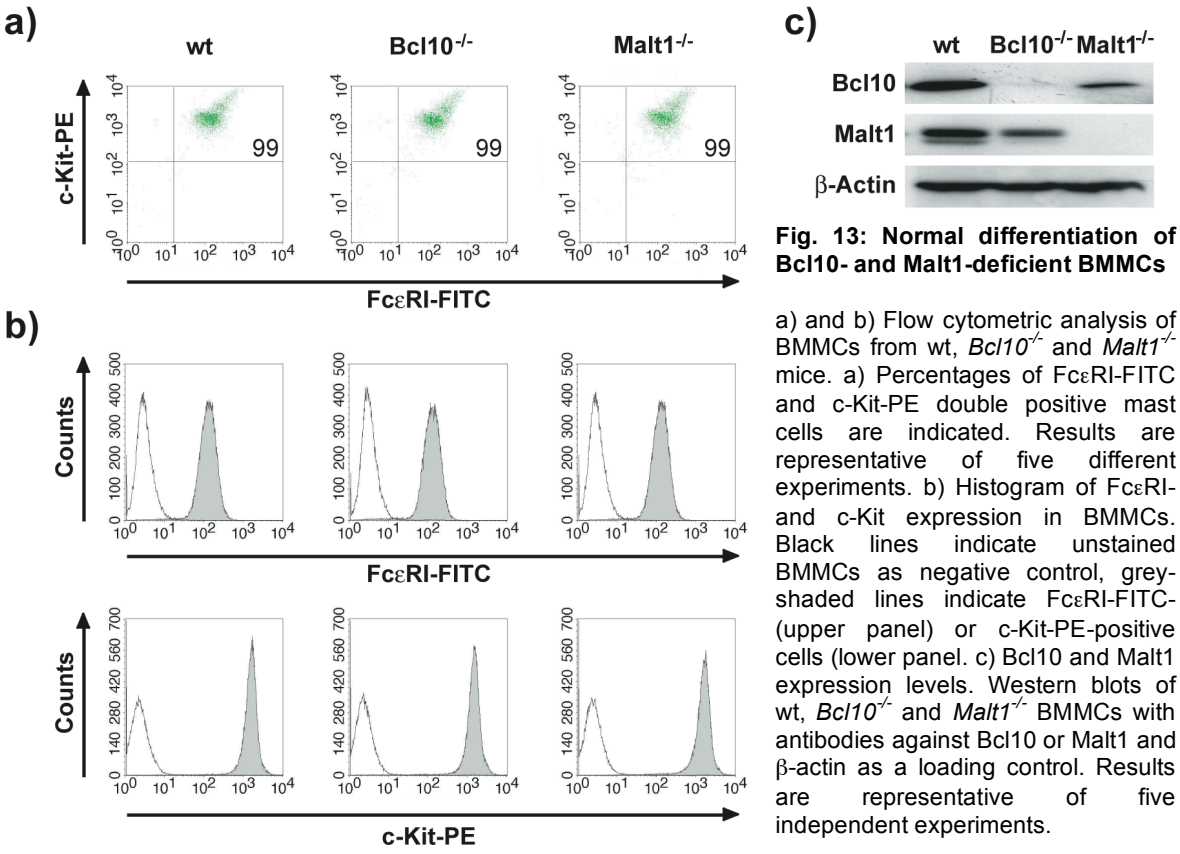


Fig. 13: Normal differentiation of Bcl10- and Malt1-deficient BMMCs

a) and b) Flow cytometric analysis of BMMCs from wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice. a) Percentages of FcεRI-FITC and c-Kit-PE double positive mast cells are indicated. Results are representative of five different experiments. b) Histogram of FcεRI- and c-Kit expression in BMMCs. Black lines indicate unstained BMMCs as negative control, grey-shaded lines indicate FcεRI-FITC- (upper panel) or c-Kit-PE-positive cells (lower panel. c) Bcl10 and Malt1 expression levels. Western blots of wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs with antibodies against Bcl10 or Malt1 and β-actin as a loading control. Results are representative of five independent experiments.

3.1.3.2 Bcl10 and Malt1 are dispensable for FcεRI-induced immediate mast cell responses *in vitro*

The functionality of BMMCs in the absence of Bcl10 or Malt1 was studied by a set of *in vitro* experiments after chemical crosslinking of the FcεRI with monoclonal anti-DNP IgE antibody and the convenient antigen DNP-HSA. All following experiments were conducted with BMMC cultures with a purity of at least 95% of FcεRI- and c-Kit-double positive BMMCs as assessed by FACS analysis as described above.

3.1.3.2.1 Bcl10 and Malt1 are not involved in FcεRI-induced calcium flux

FcεRI crosslinking with IgE and the appropriate antigen is known to induce a series of immediate mast cell responses, among them a rapid mobilisation of free calcium ions from intracellular stores, which subsequently act as important second messengers to induce further reactions like degranulation or leukotriene release (Yamasaki and Saito 2005). This rapid and transient increase in free cytosolic calcium occurs within seconds or minutes after antigen stimulation and is followed by sustained Ca^{2+} concentrations attained by calcium influx. It can be measured by the use of Ca^{2+}-sensitive fluorescence

dyes. Indo-1 is a dye that changes its emission wavelength from 485 nm without Ca^{2+} (detected in FL8) to 408 nm upon Ca^{2+} binding (detected in FL7), which can be measured through an increase in the fluorescence ratio of channels FL7/FL8 upon stimulation.

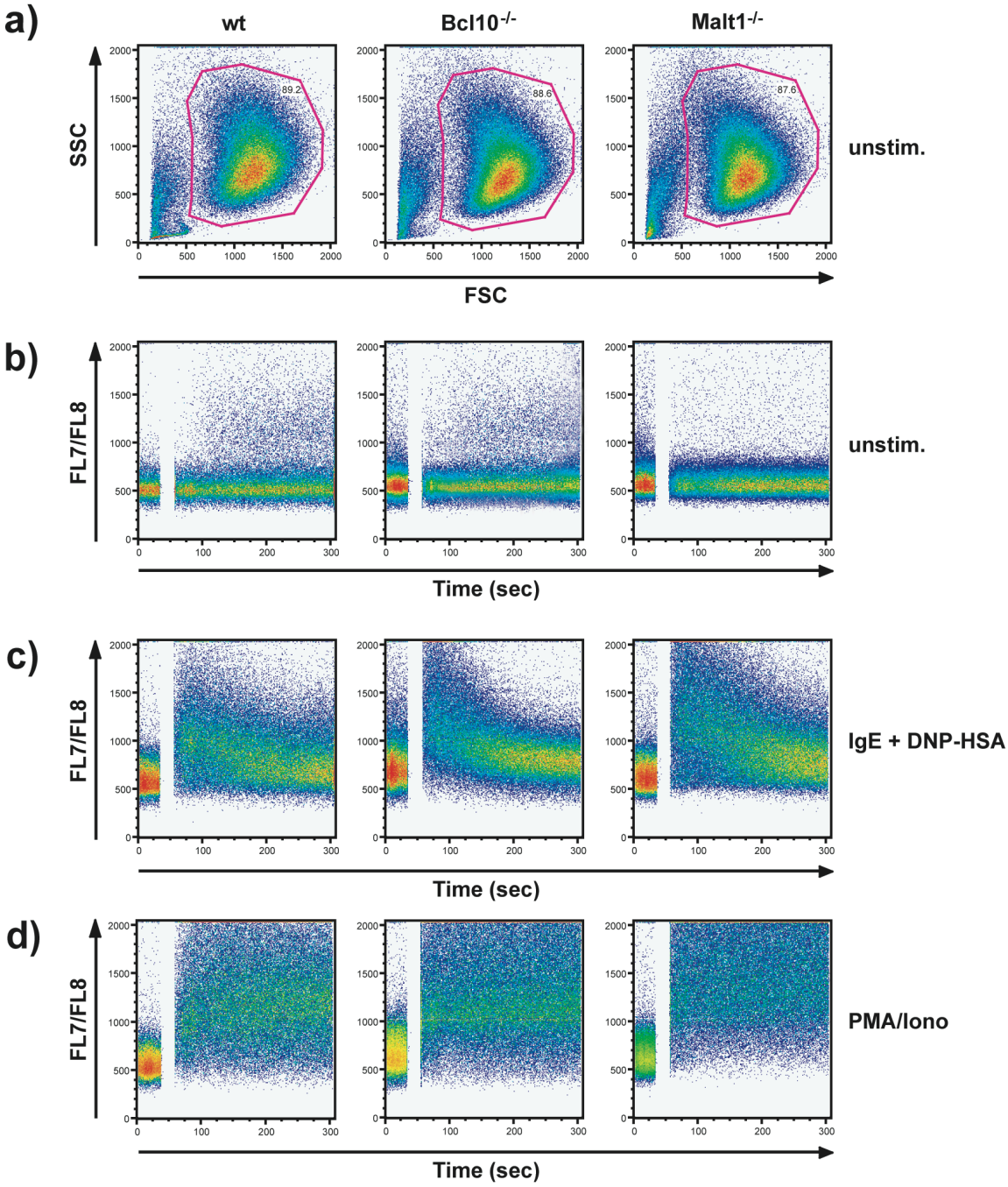


Fig. 14: Regular FcεRI- or PKC-induced calcium mobilization in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs

Wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs (gate in a) were sensitised with 5 µg/ml anti-DNP IgE or left unsensitised. b), c) and d) After sensitisation, BMMCs were washed with Tyrode's buffer, permeabilised and incubated for 30 min with 5 µg/ml Indo-1 at 37°C. Baseline fluorescence of living BMMCs (FL7/FL8) was recorded for 1 min, then (b) Tyrode's buffer alone, (c) DNP-HSA (final concentration 20 ng/ml) or (d) PMA/Iono (final concentration 100 nM each) were added quickly and the change in FL7/FL8 fluorescence recorded for 10 min to visualise changes in free cytosolic Ca^{2+} concentration.

BMMCs of wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice (figure 14a) were sensitised with anti-DNP IgE antibody or left untreated and then incubated with the fluorescence dye Indo-1 for 30 min. The baseline fluorescence was measured (figure 14b, c, d) and after 1 min, mast cells were stimulated with medium alone (14b), DNP-HSA (14c) or the phorbolester phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin (PMA/Iono) (14d), respectively, and the subsequent increase in cytosolic calcium recorded for 10 min in FL7/FL8. Both DNP-HSA and PMA/Iono stimulation could induce a rapid increase of calcium concentration in BMMCs of all three genotypes with similar kinetics and amplitude (figure 14c and 14d), whereas PMA/Iono could induce a more sustained increase of free cytosolic Ca^{2+}. This first set of *in vitro* experiments indicated a dispensable role for Bcl10 and Malt1 in immediate mast cell calcium mobilisation after FcεRI or direct PKC activation.

3.1.3.2.2 Bcl10 and Malt1 are dispensable for mast cell degranulation and leukotriene synthesis

Next, the extent of degranulation was measured by an enzymatic assay detecting the amount of the mast cell-specific granule enzyme β-hexosaminidase in culture supernatants and cell lysates after stimulation with IgE and antigen (Nishizumi and Yamamoto 1997). This yielded an antigen-dose-dependent release of β-hexosaminidase after 30 min in wt as well as in Bcl10- and Malt1-deficient BMMCs (figure 15). Furthermore, direct induction of mast cell degranulation by PKC activation with PMA and Iono showed a comparably stronger response in BMMCs of all three genotypes (figure 15a).

Mast cell activation by the FcεRI or direct PKC stimulation also induces the *de novo* production of lipid mediators, such as the leukotrienes LTC_4, LTD_4 and LTE_4, which can be specifically quantified by an enzyme-linked immunoassay (EIA). As shown for the degranulation, the antigen dose-dependent amount of leukotrienes C_4, D_4 and E_4 in mast cell supernatants after 30 min did not differ significantly between wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs (figure 15b).

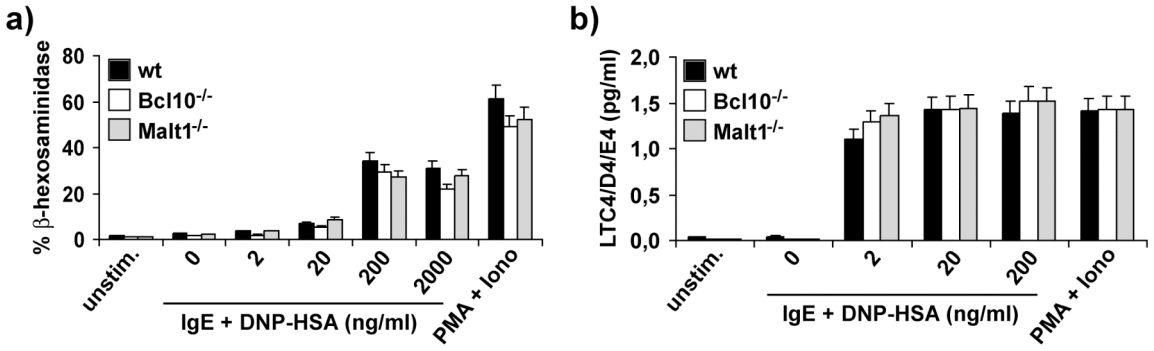


Fig. 15: Normal degranulation and leukotriene synthesis in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs

a) Regular degranulation of *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells. BMMCs from wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice were loaded with anti-DNP IgE and subsequently stimulated for 30 min with the indicated concentrations of DNP-HSA. Alternatively, BMMCs were left unsensitised and stimulated with PMA/Iono (100 nM each) for 30 min. The extent of degranulation was determined by measuring the activity of the granular enzyme β-hexosaminidase in supernatants and cell lysates and calculated as described in chapter 2 (Material and methods). Data are means + SEM from triplicate samples and representative of four independent experiments. b) Regular leukotriene synthesis in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells. Anti-DNP IgE-sensitised BMMCs from all three genotypes were stimulated with DNP-HSA at the indicated concentrations, or BMMCs were left unsensitised and stimulated with PMA/Iono (100 nM each). 30 min later, the supernatants of the stimulated cells were collected and assayed for the concentrations of LTC_4, LTD_4, and LTE_4 by EIA. Data are means + SEM from triplicate samples and representative of four independent experiments.

Together, these results confirm the *in vivo* PCA results and indicate a redundant role for Bcl10 and Malt1 in the elicitation of early phase mast cell functions like calcium mobilisation, degranulation or lipid mediator production.

3.1.3.3 Bcl10 and Malt1 control the FcεRI-induced production of proinflammatory cytokines

Since it is known that the late phase PCA response is particularly dependent on the production of proinflammatory cytokines, e.g. TNF-α or IL-6, from mast cells, which induce neutrophil infiltration and activation (Wershil, Wang *et al.* 1991; Nakae, Suto *et al.* 2005), the *in vitro* release of these cytokines from BMMCs was analysed by ELISA measurement of cell supernatants (figure 16). Considerable amounts of TNF-α and IL-6, two important NF-κB-dependent cytokines, could be detected in supernatants from wt BMMCs already two hours after FcεRI activation with IgE and DNP-HSA, whereas Bcl10- or Malt1-deficient cells produced neither TNF-α nor IL-6, even at later time points up to eight hours and more (figure 16a and 16b). In addition, mRNA transcripts of the respective cytokines after FcεRI stimulation were analysed by semi-quantitative RT-PCR, whereby the quantification of *β-actin* transcripts served as a loading control. Here, a strongly defective transcriptional induction of the *TNF-α* and *IL-6* genes could be shown in *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ mast cells,

while these cytokines could be induced easily by FcεRI stimulation after 30 min in wt BMMCs (figure 16c). Another NF-κB-dependent cytokine, IL-4, was not detectable in cell supernatants by ELISA, but semi-quantitative RT-PCR also showed a reduced *IL-4* mRNA induction in Bcl10- and Malt1-deficient BMMCs compared to wt mast cells (figure 16c, third panel).

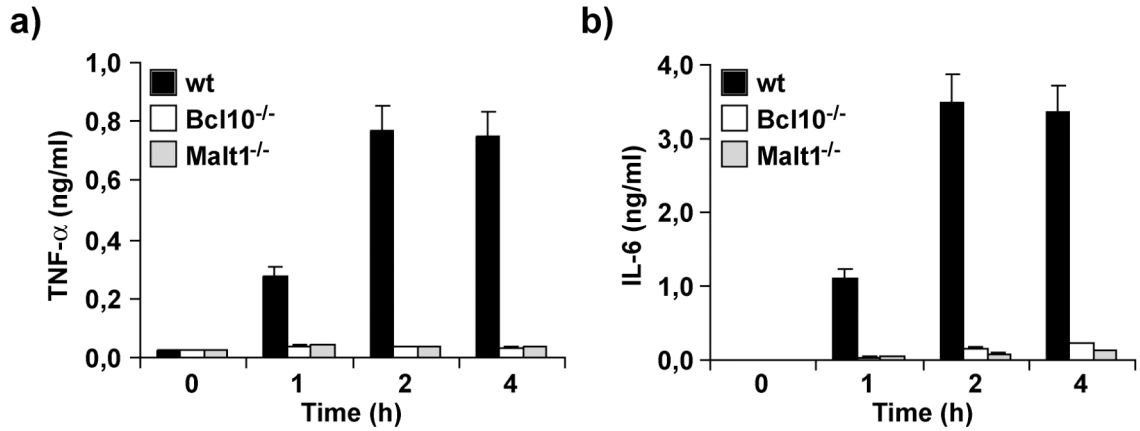
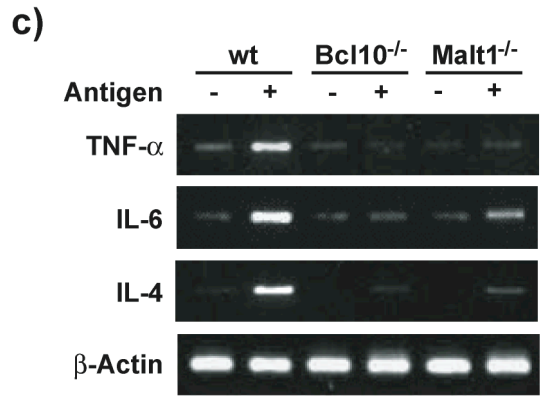


Fig. 16: Defective cytokine production in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs



a) and b) Impaired cytokine secretion in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells. Wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs were loaded with anti-DNP IgE and stimulated with DNP-HSA for the indicated intervals. Supernatants were collected and TNF-α (a) and IL-6 (b) protein concentrations were determined by ELISA. Data are mean + SEM from triplicate samples and representative of at least five independent experiments. *, $P \leq 0.05$ and **, $P \leq 0.01$ between wt and *Bcl10*$^{-/-}$ or wt and *Malt1*$^{-/-}$ BMMCs, respectively. c) Defective expression of cytokine mRNAs in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells. BMMCs were stimulated for 30 min as in a) and b). RNA was purified and *TNF-α*, *IL-6, IL-4* and *β-actin* mRNAs were detected by semi-quantitative RT-PCR using specific primers.

Assigning the defective *in vitro* cytokine production after FcεRI stimulation in Bcl10- or Malt1-deficient BMMCs to *in vivo* mast cells of Bcl10- or Malt1-deficient mice, this could explain the lacking late phase PCA response (see figure 12c), which is particularly due to inflammatory cytokine release from mast cells, and points to an essential role for Bcl10 and Malt1 in FcεRI-mediated cytokine production.

3.1.3.4 IgE-induced mast cell survival does not depend on Bcl10 and Malt1

Another function of FcεRI-induced signals, which has recently been described, is to promote mast cell survival after IgE binding (Asai, Kitaura *et al.* 2001; Kalesnikoff, Huber *et al.* 2001). In lymphocytes, it is already known that Bcl10 and Malt1 not only are crucial for cell activation, but moreover, act as important survival factors (Ruefli-Brasse, French *et al.* 2003; Xue, Morris *et al.* 2003; Patke, Mecklenbrauker *et al.* 2004; Tian, Gonzalez *et al.* 2005), so the role of Bcl10 and Malt1 for FcεRI-mediated mast cell survival was studied next (figure 17). BMMCs from wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mice were incubated for 4 days in media with low concentrations of FCS and without the mast cell growth factors IL-3 and SCF. This induced apoptotic cell death to a comparable extent in mast cells of all three genotypes after as early as 24 h, assessed by flow cytometry analysis after annexin V/propidium iodide staining of apoptotic or dead cells. Addition of IgE antibody alone or with antigen (not shown) decreased the speed of apoptosis and rescued cell death similarly in all three genotypes (figure 17a).

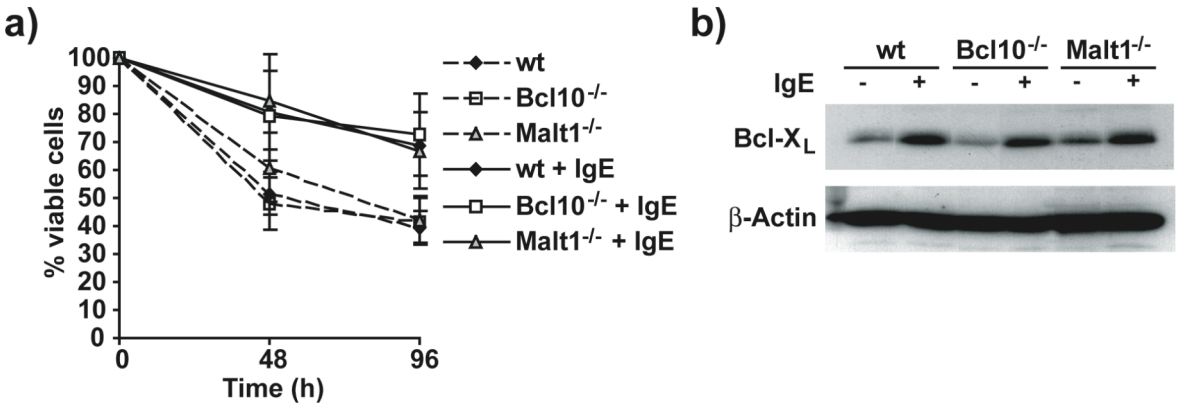


Fig. 17: Regular IgE-induced survival signalling in the absence of Bcl10 or Malt1

a) BMMCs from all three genotypes were deprived of the mast cell survival cytokines IL-3 and SCF and cultured in media with low concentrations of FCS (1%) with or without anti-DNP IgE mAb (5 µg/ml). 48 and 96 h later, apoptotic cell death was determined by flow cytometry after annexin V/propidium iodide staining. The percentages of viable mast cells are shown. Data are mean ± SEM from triplicate samples and are representative of four independent experiments. No significant differences were detected among the three genotypes. b) BMMCs were cultured as in a) and left unstimulated or stimulated for 24 h with anti-DNP IgE (5 µg/ml). Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Bcl-X_L antibody and after stripping reprobed with anti-β-actin antibody to control for equal loading.

Furthermore, to get insight into molecular mechanisms conferring survival advantages to growth factor-deprived cells, the expression level of the prosurvival protein Bcl-X_L was studied following IgE stimulation. Bcl-X_L belongs to the Bcl-2 family of proteins, being

essential regulators of programmed cell-death. Bcl-X_L expression is particularly induced by NF-κB (Chen, Edelstein *et al.* 2000; Khoshnan, Tindell *et al.* 2000; Manicassamy, Gupta *et al.* 2006), which makes it an interesting target to analyse in this context. Immunoblotting of total cell extracts, either of unstimulated BMMCs or BMMCs stimulated for 24 h with monoclonal IgE antibody, showed a similar IgE-induced upregulation of Bcl-X_L in wt, Bcl10- and Malt1-deficient BMMCs (figure 17b). Thus, in contrast to their role in lymphocytes, these results indicate that Bcl10 and Malt1 are dispensable for FcεRI-induced survival signalling.

3.1.4 Molecular mechanisms of FcεRI-mediated cytokine production

3.1.4.1 Bcl10 and Malt1 do not control FcεRI-mediated receptor-proximal signalling and MAPK or Akt activation

To provide further insight into FcεRI signal transduction and to define more precisely the molecular mechanisms responsible for the lacking TNF-α and IL-6 production in the absence of Bcl10 and Malt1, downstream signalling pathways after FcεRI activation were analysed (figure 18).

When the FcεRI is activated via crosslinking of receptor-bound IgE molecules with their specific antigen, activation of receptor-proximal tyrosine kinases occurs within seconds and transduces signals to further downstream effector molecules (Turner and Kinet 1999). This rapid tyrosine phosphorylation of receptor-associated signalling molecules was confirmed by immunoblotting of mast cell extracts with an anti-phospho-tyrosine antibody, and there were no differences in the kinetics or intensity of phosphorylation of effector molecules induced by FcεRI activation between BMMCs of wt, Bcl10- and Malt1-deficient mice, indicating that the Bcl10/Malt1 complex signals further downstream (figure 18a).

The activation of the FcεRI also leads to activation of multiple downstream pathways with different outcomes, such as the activation of the MAP kinases p38, Erk1/2 (extracellular signal-regulated kinase, also named p44/p42) and Jnk, which induce amongst others gene induction via various transcription factors (Turner and Kinet 1999). In lymphocytes, it is known that Bcl10 and Malt1 play a role in antigen-receptor-induced p38 and Jnk

activation (Ruland, Duncan *et al.* 2003; Thome 2004), thus, the FcεRI-induced activation of p38, p44/p42 and Jnk1 was analysed by immunoblotting with phospho-specific antibodies against the respective MAP kinases. There was regular activation in wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs (figure 18b) and additionally, direct PKC activation with PMA/Iono could activate p38, p44/p42 (Erk1/2) and Jnk1 normally in BMMCs lacking Bcl10 or Malt1, compared to the wt (figure 18c).

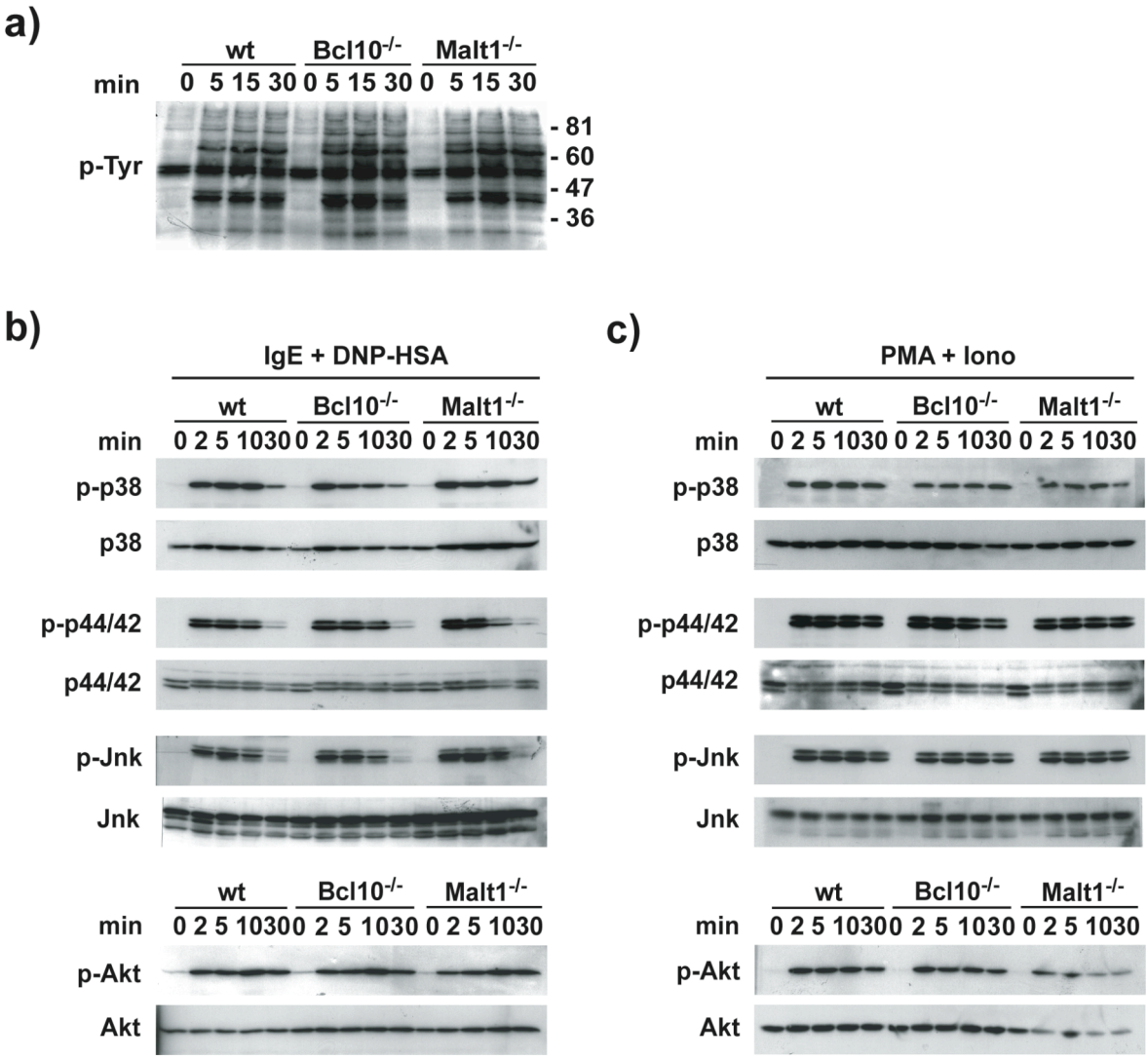


Fig. 18: Normal FcεRI proximal signalling and regular p38, Erk1/2, Jnk and Akt activation in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells

a) and b) Wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs sensitised with anti-DNP IgE were stimulated with DNP-HSA for the indicated time intervals (in minutes) as described in Materials and methods. Total cell lysates were separated by SDS-PAGE (2 x 10^5 cell equivalents). a) Immunoblot with an anti-phospho-tyrosine antibody (PY99). Molecular weight (kilodaltons) markers are shown on the left. b) and c) Immunoblot with anti-phospho-p38, anti-phospho-p44/42, anti-phospho-Jnk and anti-phospho-Akt antibody. After stripping, membranes were reprobed with anti-p38, anti-p44/p42, anti-Jnk and anti-Akt antibody, respectively, to control for equal loading.

Furthermore, the activation of the protein kinase B (Akt), another FcεRI-mediated downstream pathway, is not controlled by Bcl10 or Malt1, for it could be induced similarly by FcεRI crosslinking or direct PKC activation in BMMCs of all three genotypes (figure 18b and 18c, lower panel).

3.1.4.2 Bcl10 and Malt1 are essential regulators of FcεRI-mediated NF-κB activation

As mentioned in section 1.2, NF-κB activation induces the transcription of various proinflammatory cytokines, such as TNF-α or IL-6, also in mast cells (Lorentz, Klopp *et al.* 2003). To see whether the defective cytokine production in the absence of Bcl10 and Malt1 is due to an impaired IKK/NF-κB induction, BMMCs were activated via FcεRI crosslinking and the phosphorylation and subsequent proteasomal degradation of the NF-κB inhibitor IκB-α monitored by Western blotting (figure 19). In wt BMMCs, IκB-α was phosphorylated within 5 min after FcεRI stimulation and degraded nearly completely after 10-30 min. In sharp contrast, there was no IκB-α phosphorylation and degradation detectable in Bcl10- or Malt1-deficient BMMCs (figure 19a). Even after bypassing FcεRI-proximal signalling through direct PKC stimulation with PMA/Iono, phosphorylation-dependent IκB-α degradation could only be observed in wt mast cells (figure 19b).

To confirm this defective NF-κB activation on a nuclear level, electromobility shift assays were performed with nuclear extracts from unstimulated and PMA/Iono-treated wt, *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs, for IgE + antigen could not induce NF-κB to a detectable level even in wt BMMCs. Consistent with the lacking IκB-α degradation in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ BMMCs, there was strong NF-κB induction in wt mast cells, which was completely absent in either Bcl10- or Malt1-deficient cells (figure 19c).

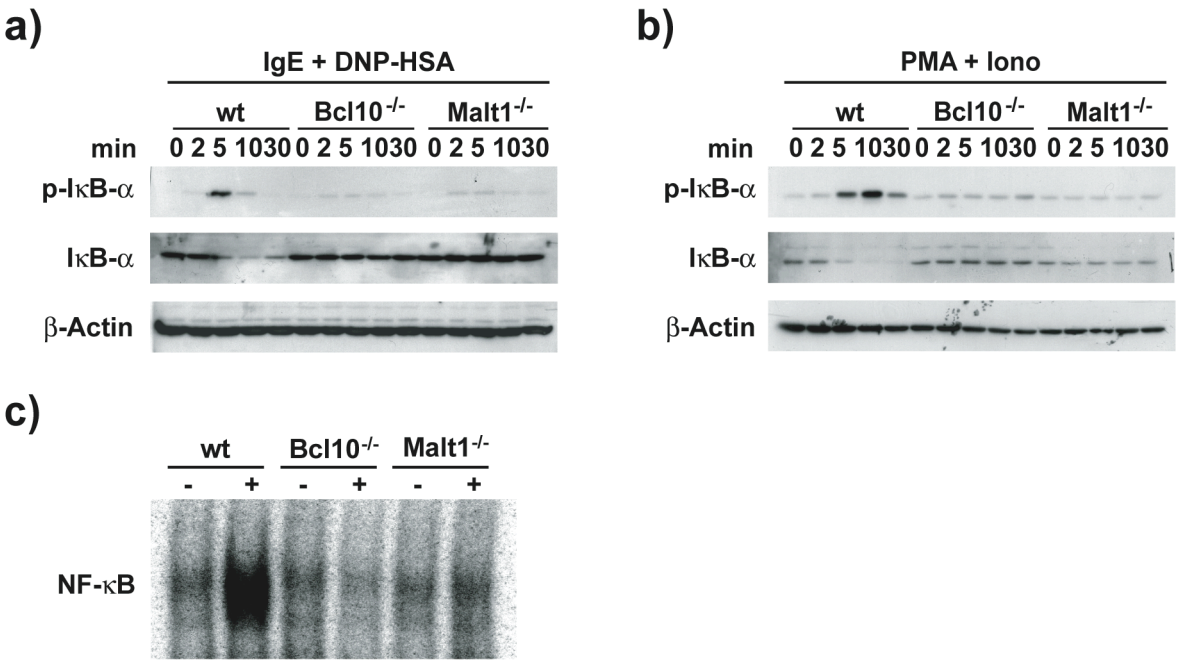


Fig. 19: Bcl10 and Malt1 are required for FcεRI-induced NF-κB signalling in mast cells

a) and b) Defective IκB-α phosphorylation and degradation following FcεRI ligation (a) or PMA/Iono (b) treatment in *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ mast cells. a) BMMCs from all genotypes were sensitised with anti-DNP IgE and stimulated with DNP-HSA for the indicated time intervals (in minutes). b) Alternatively, cells were left unsensitised and stimulated with PMA/Iono (100 nM each). IκB-α phosphorylation and degradation were determined by Western blot. After stripping, membranes were reprobed with anti-β-actin antibody to control for equal loading. c) Defective NF-κB activation in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ mast cells. BMMCs were left untreated or stimulated with PMA/Iono (100 nM each) for 60 min. Subsequently, nuclear extracts were prepared and subjected to electromobility shift assays using a radiolabelled probe containing NF-κB binding site sequences.

3.1.5 Summary and model

As shown in section 3.1, Bcl10 and Malt1 both are essential signal transducers in mast cells, which are recruited after FcεRI ligation and PKC activation and specifically mediate IKK-induced NF-κB activation and cytokine production *in vitro* and late phase PCA reactions *in vivo*. Interestingly, other FcεRI-mediated mast cell functions like degranulation or leukotriene synthesis and early phase PCA reactions are not controlled by Bcl10 and Malt1. This makes the Bcl10/Malt1 complex one of the first identified signalling adaptor selectively controlling one downstream effector arm of FcεRI activation. Taking these results together, a signalling model can be depicted, which is shown in figure 20.

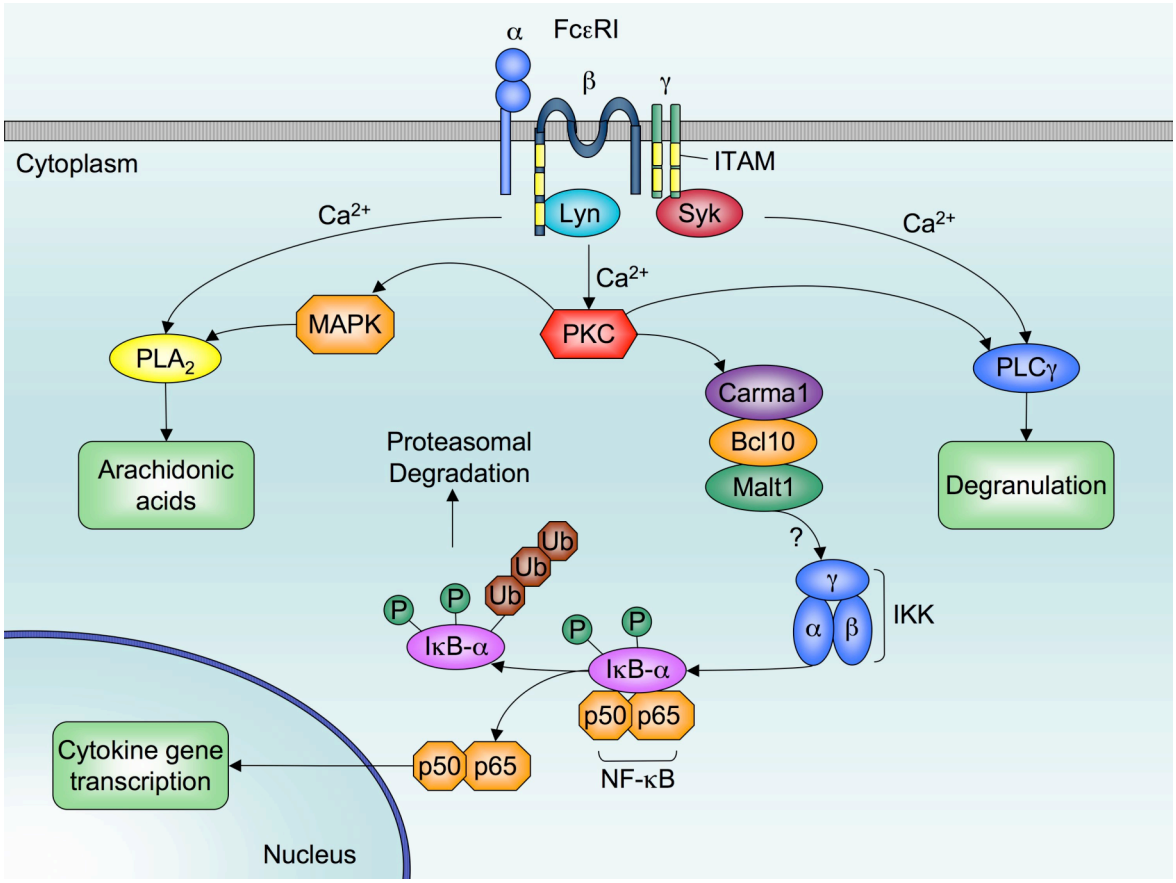


Fig. 20: Model for the role of Bcl10 and Malt1 in FcεRI-mediated signal transduction

After FcεRI crosslinking, Bcl10 and Malt1 operate as specific signal transducers downstream of PKC isoforms to exclusively activate the IκB-dependent NF-κB pathway. Thus, the Bcl10/Malt1 complex selectively controls proinflammatory cytokine production, but is dispensable for degranulation or leukotriene synthesis via phospholipases C and A, respectively.

These results have been previously published in the *Journal of Experimental Medicine* (Klemm, Gutermuth *et al.* 2006) and two short reviews on this subject have been written for *Immunobiology* (Klemm and Ruland 2006) and *Current Immunology Reviews* (Klemm and Ruland 2006).

3.2 Function of the Bcl10/Malt1 complex in LPA signalling in murine embryonic fibroblasts (MEFs)

3.2.1 Bcl10 and Malt1 are dispensable for LPA-induced MAPK and Akt activation in MEFs

To study the potential role of Bcl10 and Malt1 in LPA-induced signalling pathways, MEFs were generated from wt, *Bcl10*$^{+/-}$, *Bcl10*$^{-/-}$, *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ mice and treated with the phospholipid LPA to analyse signalling pathways downstream of its specific GPCR.

Bcl10 and Malt1 are known to control p38 and Jnk activation in lymphocytes (Ruland, Duncan *et al.* 2003; Thome 2004), but, as shown in section 3.1.4.1, are dispensable for FcεRI-mediated p38, Erk and Jnk activation (figure 18). Analysis of MAPK activation in *Bcl10*$^{-/-}$ and *Malt1*$^{-/-}$ MEFs and heterozygous *Bcl10*$^{+/-}$ and *Malt1*$^{+/-}$ as respective positive control demonstrated that Bc10 and Malt1 do not regulate LPA-induced p38, Erk1/2 and Jnk1 activation in fibroblasts (figure 21).

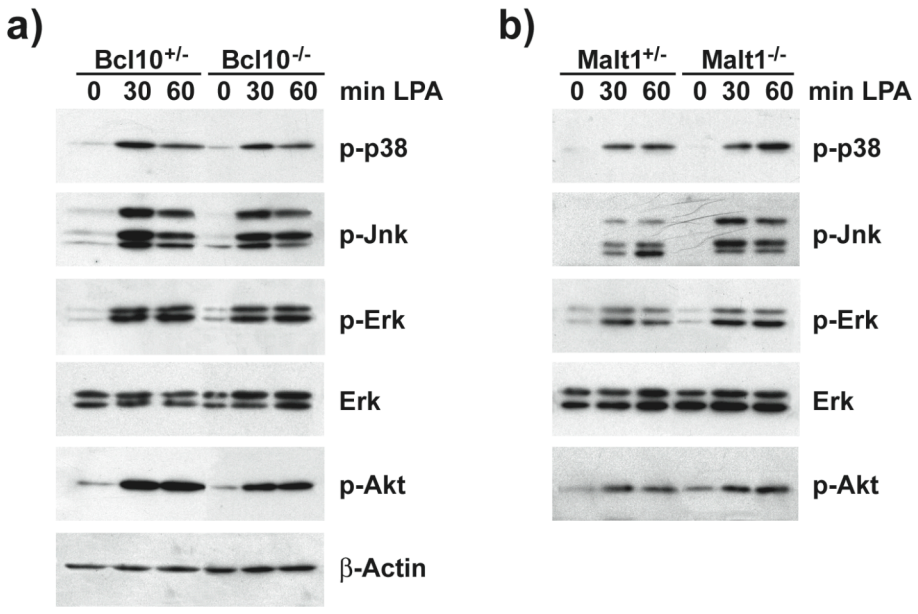


Fig. 21: Normal LPA-induced p38, Erk1/2, Jnk1 and Akt activation in Bcl10- and Malt1-deficient MEFs

a) and b) *Bcl10*$^{+/-}$ or *Bcl10*$^{-/-}$ MEFs (a) and *Malt1*$^{+/-}$ or *Malt1*$^{-/-}$ MEFs (b) were stimulated with 10 µM LPA as indicated. Cell extracts were separated on polyacrylamide gels and immunoblotted with antibodies against phospho-p38, phospho-p44/p42 (phospho-Erk1/2), phospho-Jnk or phospho-Akt. After stripping, membranes were reprobed with antibodies against p44/p42 (Erk1/2) or β-actin to control for equal loading. Results are representative of five independent experiments.

Furthermore, activation of protein kinase B (Akt) could be induced regularly by LPA in Bcl10- or Malt1-deficient MEFs compared to MEFs with a heterozygous *Bcl10* or *Malt1* deletion (figure 21 lower panel).

3.2.2 Bcl10 and Malt1 control LPA-induced NF-κB activation in MEFs

It has been previously shown that LPA stimulation leads to NF-κB activation in fibroblasts (Shahrestanifar, Fan *et al.* 1999; Cummings, Zhao *et al.* 2004). To gain further insight into this signalling pathway downstream of the LPA receptor, LPA-induced IκB-α degradation and NF-κB activation were analysed in MEFs with a hetero- or homozygous *Bcl10* or *Malt1* disruption (figure 22). Since LPA is a relatively weak stimulus for MEFs, cells were previously incubated with cycloheximide in the immunoblot experiments to block *de novo* protein synthesis and thereby also resynthesis of IκB-α to be able to visualize the LPA-induced IκB-α degradation. Western blotting then demonstrated an impaired IκB-α degradation in either Bcl10- or Malt1-deficient MEFs after LPA stimulation (figure 22a and 22c). As a control, MEFs were also treated with TNF-α to induce NF-κB activation via another, in lymphocytes Bcl10/Malt1-independent pathway, and in contrast to LPA, TNF-α could induce potent IκB-α degradation in *Bcl10*$^{+/-}$ and *Bcl10*$^{-/-}$ or *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ MEFs to a comparable extent (figure 22b and 22d).

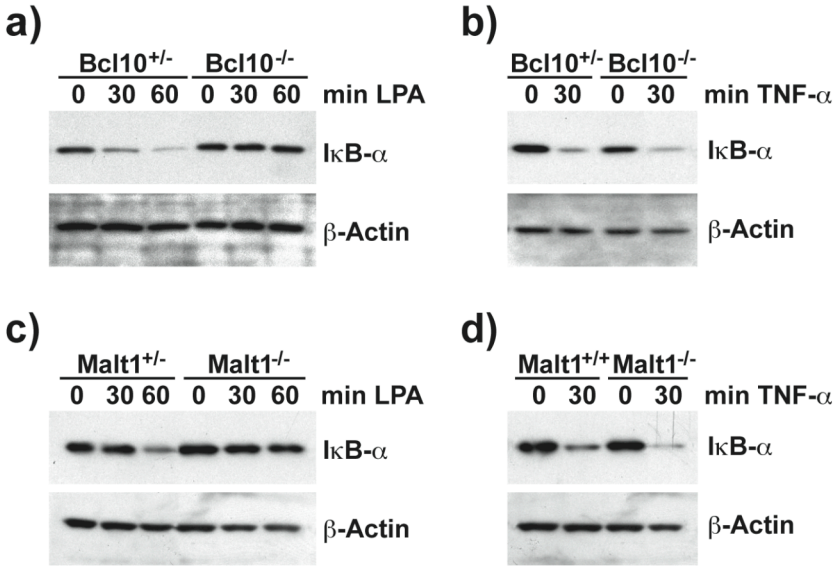


Fig. 22: Bcl10 and Malt1 regulate LPA-induced IκB-α degradation

Bcl10$^{+/-}$ and *Bcl10*$^{-/-}$ MEFs (a and b) or Malt1$^{+/-}$ and Malt1$^{-/-}$ MEFs (c and d) were preincubated for 1 h with 5 µg/ml cycloheximide and then stimulated with 10 µM LPA (a and c) or 10 ng/ml TNF-α (b and d) for the indicated times. Cell extracts were separated on 10% polyacrylamide gels, subjected to immunoblotting with anti-IκB-α antibody and reprobed with anti-β-actin antibody to show equal loading.

To demonstrate NF-κB translocation on the nuclear level, EMSAs were performed with nuclear extracts from MEFs stimulated with LPA or TNF-α (figure 23). Consistent with the results of the anti-IκB-α immunoblots, a potent LPA-induced NF-κB activation was visible in *Bcl10*$^{+/-}$ MEFs, whereas Bcl10-deficient MEFs did not show NF-κB translocation to the nucleus 60 min after LPA stimulation (figure 23a). In contrast, TNF-α was able to strongly induce NF-κB after 30 min in both *Bcl10*$^{+/-}$ and *Bcl10*$^{-/-}$ MEFs (figure 23b).
Accordingly, there was potent LPA-induced NF-κB activation in *Malt1*$^{+/-}$ MEFs, which was absent in *Malt1*$^{-/-}$ cells (figure 23c), while TNF-α could activate NF-κB in *Malt1*$^{-/-}$ MEFs to the same extent as in wt MEFs (figure 23d).

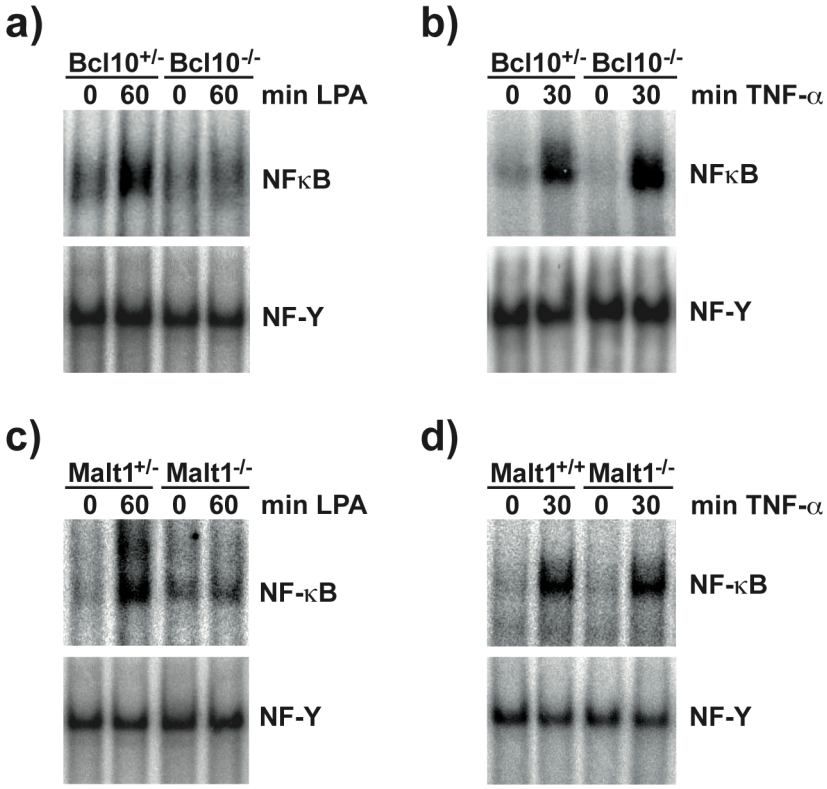


Fig. 23: Defective LPA-induced NF-κB translocation in Bcl10- and Malt1-deficient MEFs

Bcl10$^{+/-}$ and *Bcl10*$^{-/-}$ MEFs (a and b) or *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ MEFs (c and d) were stimulated with 10 µM LPA (a and c) or 10 ng/ml TNF-α (b and d) as described above, nuclear extracts prepared and incubated with radiolabelled oligonucleotide probes specific for NF-κB or NF-Y as a loading control. Results are representative of three independent experiments.

In all EMSAs shown here, the constitutively active transcription factor NF-Y, which is not influenced by LPA or TNF-α stimulation, served as a loading control.

3.2.3 Retroviral reconstitution of Bcl10 rescues NF-κB induction

In the next step, it should be tested whether the defective NF-κB activation in Bcl10-deficient MEFs is a direct consequence of the lack of Bcl10. For this purpose, *Bcl10*$^{-/-}$ MEFs were infected with a retrovirus expressing full-length Bcl10 (pBABE-puro-Bcl10), thereby infection with the empty vector pBABE-puro served as a negative control (figure 24). Western Blotting demonstrated that the *Bcl10*-containing retrovirus was able to reconstitute the *Bcl10* disruption, for full-length Bcl10 protein was produced with endogenous expression levels in *Bcl10*$^{-/-}$ MEFs after 10 days of puromycin-based selection of the infected cells (figure 24a, lane 1 and 3). When the reconstituted cells were stimulated with LPA, the defective IκB-α degradation of *Bcl10*$^{-/-}$ MEFs was restored in cells retrovirally expressing Bcl10 (figure 24b), leading to the conclusion that Bcl10 directly controls IκB-α-dependent NF-κB activation after LPA receptor stimulation.

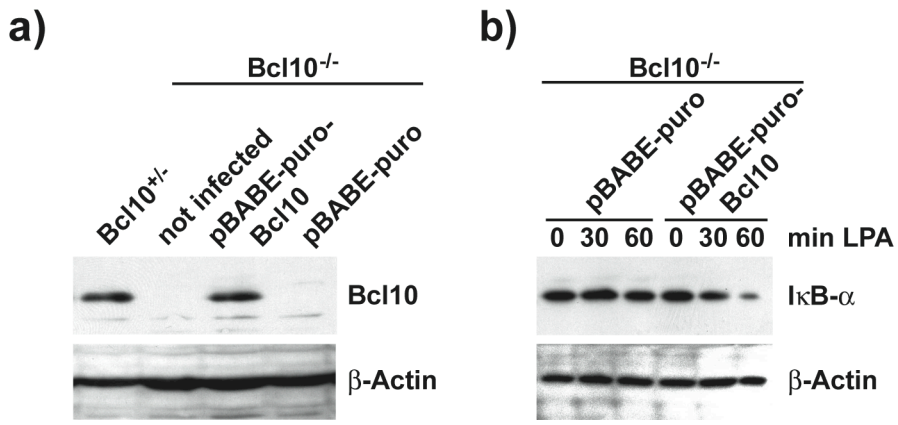


Fig. 24: Retroviral reconstitution of *Bcl10*-deficient MEFs rescues IκB-α degradation in response to LPA stimulation.

a) Bcl10-deficient MEFs were infected with the retroviral vector pBABE-puro encoding full length Bcl10 (pBABE-puro-Bcl10) or an empty vector (pBABE-puro) as a negative control. Cell extracts were prepared and subjected to immunoblotting with anti-Bcl10-antibody and anti-β-actin antibody as a loading control. b) Bcl10-deficient MEFs retrovirally infected with pBABE-puro or pBABE-puro-Bcl10 were stimulated with LPA (10 µM) as described above and cell extracts subjected to immunoblotting using antibodies against IκB-α and β-actin. Experiments were repeated three times.

3.2.4 LPA-induced NF-κB activation is a PKC-dependent, Akt-independent pathway

With the previous results, it could be shown, that Bcl10 and Malt1 are indispensable for LPA-induced, IκB-α-dependent NF-κB activation in fibroblasts, but it was still unclear, which signalling adaptors couple to receptor-proximal signalling events upstream of the Bcl10/Malt1 complex. Recent studies have shown that PKCδ functions in LPA-mediated

NF-κB activation (Shahrestanifar, Fan *et al.* 1999; Cummings, Zhao *et al.* 2004), and furthermore, it is known that Bcl10 operates downstream of PKC isoforms in immune cells (Sun, Arendt *et al.* 2000; Egawa, Albrecht *et al.* 2003; Cho, Woo *et al.* 2004; Shinohara, Yasuda *et al.* 2005).

In addition, the phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway is potentially involved, because the latter has recently been suggested to play a role in Carma1-dependent NF-κB activation in T cells by phosphorylating Bcl10 (Narayan, Holt *et al.* 2006).

To test these hypotheses, MEFs were preincubated with small-molecule inhibitors for PKC or PI3K for 1 h before cell stimulation with LPA (figure 25). Rottlerin generally is a *pan*-PKC inhibitor, but at the low concentrations used in this setting, it inhibits the PKCδ isoform with highest efficacy. Wortmannin blocks PI3K and thus inhibits the phosphorylation and activation of downstream effectors, in particular protein kinase B (Akt).

When MEFs were preincubated with rottlerin, this PKC inhibition, as expected, reduced IκB-α degradation in *Bcl10*$^{+/-}$ MEFs. Interestingly, Bcl10 has also been found to be degraded upon LPA stimulation in *Bcl10*$^{+/-}$ MEFs by anti-Bcl10 immunoblotting, and this Bcl10 degradation could equally be blocked with rottlerin (figure 25a). Consequentially, the defective IκB-α degradation in Bcl10-deficient MEFs was not influenced by rottlerin.
As shown before, LPA stimulation activates Akt both in *Bcl10*$^{+/-}$ and *Bcl10*$^{-/-}$ MEFs (see figure 21), and this Akt phosphorylation can be totally blocked in both genotypes by PI3K inhibition with wortmannin (figure 25b). However, blockage of the PI3K/Akt pathway did not influence LPA-induced IκB-α and Bcl10 degradation in *Bcl10*$^{+/-}$ cells (figure 25b). Thus, the LPA-induced, Bcl10-dependent NF-κB activation pathway is controlled further upstream by a PKC-dependent, but PI3K/Akt-independent mechanism.

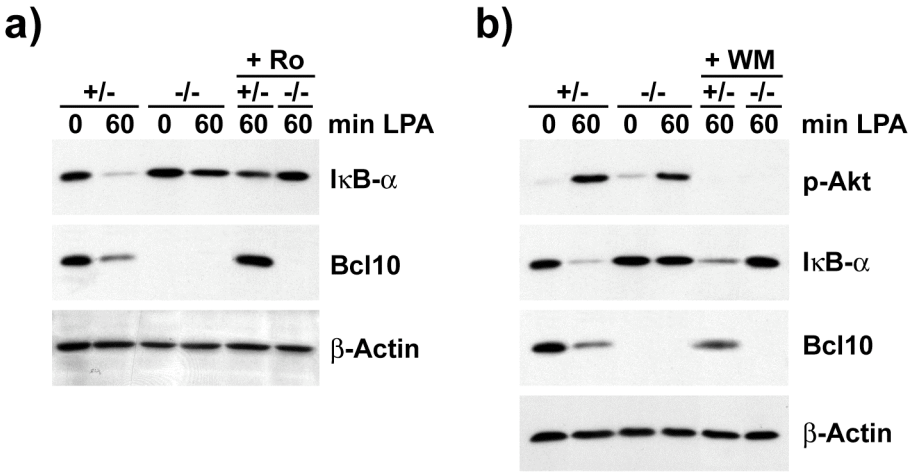


Fig. 25: PKC, not PI3K/Akt, regulates LPA-induced NF-κB activation

a) and b) *Bcl10*$^{+/-}$ or *Bcl10*$^{-/-}$ MEFs were treated for 1 h at 37°C with the PKC inhibitor rottlerin (Ro, 3 µM) (a) or the PI3K inhibitor wortmannin (WM, 1 µM) (b) prior to stimulation with 10 µM LPA for the indicated times. Cell extracts were subjected to immunoblotting with antibodies against IκB-α, Bcl10, phospho-Akt or β-actin as a loading control.

3.2.5 Bcl10/Malt1 signalling regulates LPA-induced production of the NF-κB dependent cytokine IL-6

One of the physiological outcomes of LPA-induced NF-κB activation is the production of proinflammatory cytokines, such as IL-6 or IL-8 (Fang, Yu *et al.* 2004; Oz-Arslan, Ruscher *et al.* 2006). To test whether this function is controlled by the Bcl10/Malt1 complex, the production of the cytokine IL-6 from LPA-stimulated MEFs was tested by ELISA (figure 26). From two to eight hours, *Bcl10*$^{+/-}$ MEFs released considerable amounts of IL-6 in a time-dependent manner after LPA treatment, whereas IL-6 production was strongly reduced in the absence of Bcl10 in response to LPA (figure 26). Thus, this Bcl10-controlled LPA signalling pathway exerts influence on physiological effects of LPA stimulation.

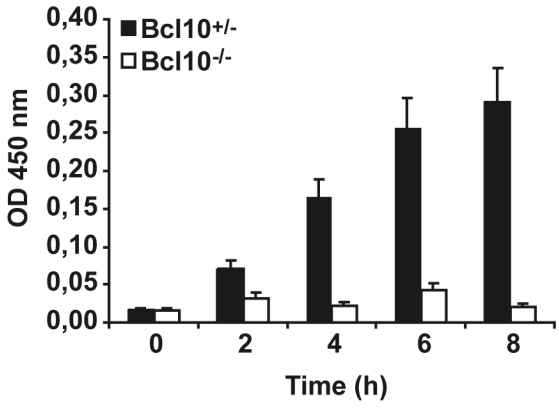


Fig. 26: Impaired cytokine production in Bcl10-deficient MEFs

Bcl10$^{+/-}$ or *Bcl10*$^{-/-}$ MEFs were stimulated with 10 µM LPA for the indicated times. Supernatants were collected and IL-6 concentrations determined by ELISA. Data are mean + SEM and representative of three independent experiments.

3.2.6 Summary and model

The model in figure 27 shows the function of the Bcl10/Malt1 complex in LPA-induced NF-κB activation. LPA acts on its cognate GPCR to induce the activation of distinct G proteins. $G_{12/13}$ activates Rho-mediated pathways leading to e.g. cell contraction, G_i triggers Ras-MAPK activation to induce DNA synthesis and via PI3K, the activation of Akt and Rac, being important for cell survival and migration, respectively. Furthermore, LPA via G_q stimulates PLCβ activation to generate second messengers, which induce PKCδ to transduce signals through the Carma3/Bcl10/Malt1 complex (see Discussion) to IKK, inducing IκB-α phosphorylation and proteasomal degradation. NF-κB translocation then induces transcription of NF-κB-dependent cytokines, e.g. IL-6.

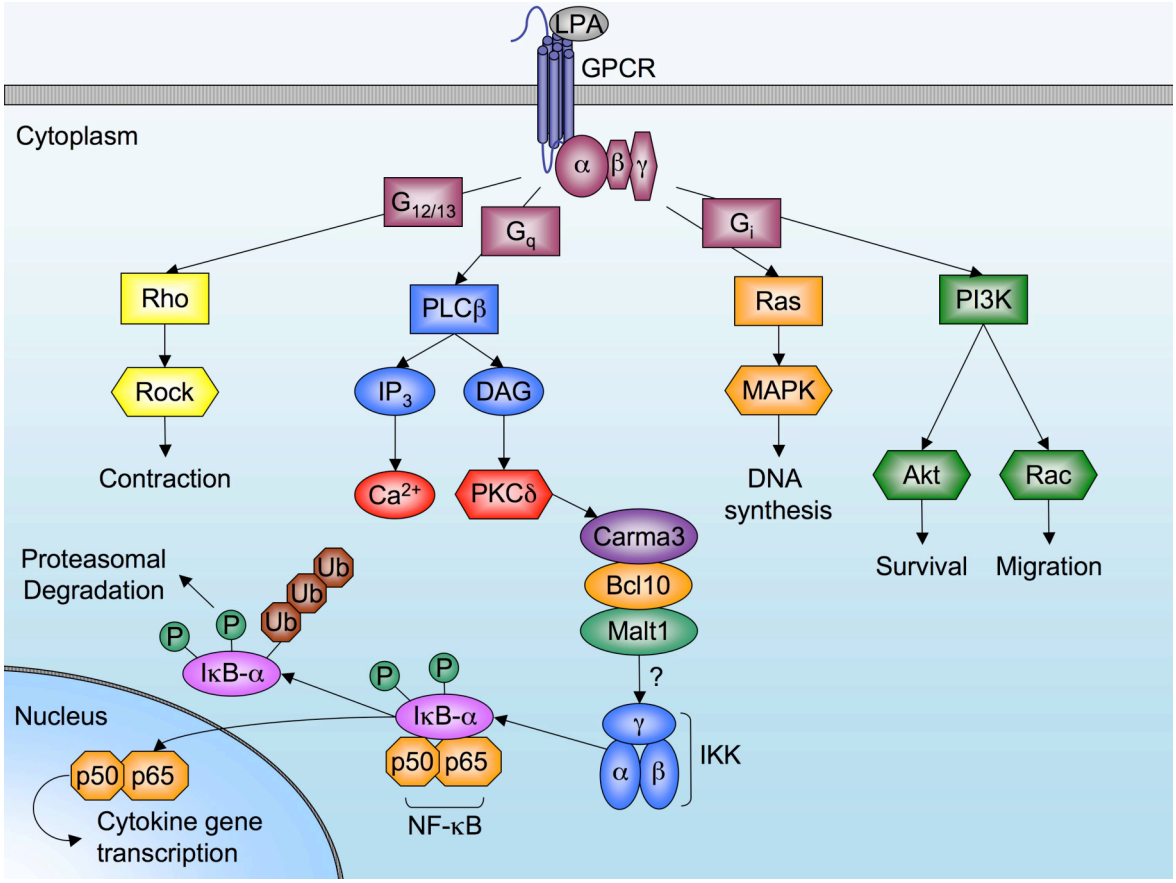


Fig. 27: Model for the role of Bcl10 and Malt1 in LPA-induced signalling and NF-κB activation

LPA binding to its GPCR induces activation of the distinct G proteins. G_q couples to PLCβ, activating PKC isoforms, probably PKCδ in fibroblasts. PKC recruits the Carma/Bcl10/Malt1 complex, which leads to IKK activation with subsequent IκB-α degradation and NF-κB translocation to the nucleus to induce cytokine gene transcription. Other pathways like the activation of MAPKs or Akt are not controlled by Bcl10 or Malt1.

The results described herein have been previously published in the *Proceedings of the National Academy of Sciences of the USA* (Klemm, Zimmermann *et al.* 2007).

4 Discussion

Genetic studies over the last years have identified the Bcl10/Malt1 complex as essential regulator of antigen receptor-induced lymphocyte activation, being indispensable for NF-κB induction as well as activation of the MAP kinases Jnk and p38, which is important for the differentiation, activation and survival of T and B cells (Ruland, Duncan *et al.* 2001; Ruland, Duncan *et al.* 2003; Thome 2004). Beyond this already well-characterized role of Bcl10 and Malt1 in lymphoid cells, a function for the two proteins in non-lymphoid and non-immune cells has not been reported before. In this work, new roles for the Bcl10/Malt1 complex in mast cells representing myeloid cells and FcεRI signalling as well as in fibroblasts as representatives of non-immune cells and GPCR signalling have been revealed.

4.1 The Bcl10/Malt1 complex segregates FcεRI-mediated NF-κB activation and cytokine production from degranulation and leukotriene synthesis in mast cells

Mast cell activation through the high affinity IgE receptor FcεRI leads to a multitude of physiological effector functions like degranulation with rapid release of preformed mast cell mediators, e.g. histamine, serotonin or mast cell-specific proteases, leukotriene synthesis from arachidonic acids and the transcriptional induction and release of proinflammatory cytokines, mainly TNF-α or IL-6, inducing late phase reactions and initiation of inflammatory responses with neutrophil recruitment and activation (Turner and Kinet 1999; Siraganian 2003; Galli, Kalesnikoff *et al.* 2005). These normal mast cell functions can be beneficial or harmful in the setting of innate or allergic immune responses.

In this work, it has been shown that murine mast cells develop normally in the absence of either Bcl10 or Malt1 both *in vivo* and *in vitro*, but Bcl10- or Malt1-deficient mast cells are not able to activate NF-κB after FcεRI crosslinking and consequently, do not produce NF-κB-dependent cytokines, such as TNF-α or IL-6, *in vitro*. *In vivo*, these proinflammatory cytokines are necessary for mast cell-mediated late phase PCA reactions (Wershil, Wang *et al.* 1991; Wang and Thorlacius 2005), which are consequently severely defective in

Bcl10$^{-/-}$ and *Malt1*$^{-/-}$ mice, in contrast to intact IgE-dependent early phase PCA in the absence of either Bcl10 and Malt1.

Recently, Chen *et al.* reported comparable results concerning the role of Bcl10 in FcεRI signalling (Chen, Pappu *et al.* 2007). According to my own results, this group also showed normal mast cell development, calcium flux, arachidonic acid release and MAPK activation, but defective IκB-α degradation, NF-κB activation and IL-6 production in the absence of Bcl10.
However, in contrast to the results presented herein (normal β-hexosaminidase release shown in Fig. 16), Chen *et al.* found impaired *in vitro* degranulation in Bcl10-deficient mast cells indicated by a 30% reduced serotonin release after IgE stimulation, but nevertheless, *Bcl10*$^{-/-}$ BMMCs also exhibited an IgE-dose-dependent increase of released serotonin. However, according to my results, the release of arachidonic acids upon IgE stimulation did not differ between wt and Bcl10-deficient mast cells and IL-6 production was nearly completely absent. Importantly, Chen *et al.* did not stimulate BMMCs with IgE antibody plus antigen, but with monoclonal IgE antibody alone, which could account for this discrepancy in results. Though it is known that monomeric IgE antibodies can induce several mast cell effector functions like cytokine production and in particular promote cell survival (Asai, Kitaura *et al.* 2001; Kalesnikoff, Huber *et al.* 2001; Kitaura, Song *et al.* 2003), those responses differ from IgE + antigen-mediated outcomes in kinetics, quantity and amount of released mediators.
Chen and coworkers also reported an impaired *in vivo* degranulation monitored by a reduction in Evans blue extravasation during IgE-induced early phase PCA, but in these figures, a blue staining of the ear skin or the basolateral side again is visible even in Bcl10-deficient mice, showing that degranulation basically is possible even in the absence of Bcl10. According to Chen *et al.*, Bcl10 additionally controls FcεRI-induced induction of the transcription factor AP-1, which was not included in my work, but they did not elaborate the role of Malt1 in FcεRI signalling in their studies.

The experiments conducted in this work have shown that the Bcl10/Malt1 complex is not involved in FcεRI-proximal signalling events like immediate calcium flux (figure 14) or the phosphorylation of receptor-associated tyrosine kinases (figure 18) leading amongst others to the activation of various PKC enzymes, which are essential for all mast cell outcomes (Leitges, Gimborn *et al.* 2002; Cho, You *et al.* 2004; Abdel-Raheem, Hide *et al.* 2005). Comprehensive studies of the individual roles of all PKC isoforms in mast cells are still lacking, but recent data identified the calcium-sensitive conventional isoforms PKCα

and PKCβ as essential inducers of degranulation and leukotriene and cytokine induction (Kawakami, Kitaura *et al.* 2000; Nechushtan, Leitges *et al.* 2000; Abdel-Raheem, Hide *et al.* 2005). In contrast, the calcium-independent isoform PKCδ seems to function as a negative regulator of degranulation (Leitges, Gimborn *et al.* 2002), but also positively regulates leukotriene production (Cho, Woo *et al.* 2004; Cho, You *et al.* 2004). Furthermore, the T cell-specific isoform PKCθ is expressed in mast cells and additionally involved in FcεRI signalling (Liu, Graham *et al.* 2001).

With this knowledge, it seems probable that multiple PKC isoforms are recruited to the FcεRI after its aggregation, with one isoform specifically activating the Bcl10/Malt1 complex for NF-κB-dependent cytokine transcription and other PKC enzymes controlling degranulation and leukotriene synthesis. Studies with PKC inhibitors or siRNAs that specifically reduce only one isoform could give more insight into detailed mechanisms of PKC-Bcl10/Malt1 interaction in mast cells and could finally identify which PKC isoform selectively activates the Bcl10/Malt1 to induce NF-κB activation and which isoforms control Bcl10/Malt1-independent early phase mast cell functions.

The findings that FcεRI- and PMA/Iono-induced IκB-α phosphorylation, degradation and subsequent NF-κB translocation are completely abolished in Bcl10- or Malt1-deficient mast cells show an essential role for the Bcl10/Malt1 complex downstream of the FcεRI and downstream of PMA-responsive PKC isoforms. However, other FcεRI- or PMA/Iono-mediated downstream pathways like the activation of the MAP kinases p38, p44/42 (Erk1/2) or Jnk and Akt activation still function in the absence of Bc10 or Malt1 and additionally, PKC-dependent mast cell outcomes like degranulation, the release of arachidonic acid metabolites ($LTC_4/D_4/E_4$) or IgE-induced mast cell survival are not controlled by Bcl10 and Malt1. This indicates a selective activation of the Bcl10/Malt1 complex downstream of individual PKC isoforms (see above) in NF-κB-dependent cytokine production.

Concerning FcεRI-mediated activation of Jnk proteins, herein only the phosphorylation and activation of the Jnk1 isoform was analysed with the used antibodies, and there were no defects observed in Jnk1 activation in Bcl10- or Malt1-deficient mast cells. Another recent study, in contrast, showed a role for Bcl10 in TCR-mediated activation of Jnk2, which is severely defective in *Bcl10*$^{-/-}$ T cells (Blonska, Pappu *et al.* 2006). Analysis of Jnk activation in mast cells with Jnk1- or Jnk2-specific antibodies could give further insight into

the detailed regulation of Jnk isoforms after FcεRI crosslinking and clarify whether there is an involvement of the Bcl10/Malt1 complex.

The *in vitro* findings with BMMCs outlined in the model in figure 20 are consistent with the *in vivo* results obtained by early and late phase PCA reactions (figure 12). The early phase PCA reaction, mainly dependent on rapid mast cell degranulation, proceeds normally in Bcl10- and Malt1-deficient mice, which could also be shown by normal FcεRI-proximal signalling, regular degranulation and leukotriene release *in vitro*. In parallel, the severely blunted late phase PCA reaction in *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ mice, which is particularly dependent on the release of proinflammatory cytokines from skin mast cells that recruit and activate leukocytes (Wershil, Wang *et al.* 1991; Wang and Thorlacius 2005), is said to be mainly due to the absent production of the proinflammatory cytokines TNF-α and IL-6 found *in vitro*. However, defects in *in vivo* PCA experiments in Bcl10- and Malt1-deficient mice can - at least partly - be influenced by signalling deficiencies in other cell types besides mast cells, such as basophils, neutrophils or T cells, because all cells lack Bcl10 or Malt1, respectively, in the mutant animals. Possibly, these defects also may contribute to impaired late phase reactions, but with the approach used in this work, it was not possible to separate the single contributions of the different cell types. Detailed histological analysis of leukocyte recruitment during the late phase PCA reaction could probably elucidate further defects in Bcl10- or Malt1-deficient immune cells besides mast cells.

Over the last years, numerous experiments with genetically altered mice have clarified several aspects of the regulation of mast cell activation and have identified a number of positive or negative regulators of FcεRI signalling (Siraganian 2003; Rivera 2002). However, all these findings demonstrated that the absence of one regulator generally affects multiple effector functions downstream of the FcεRI simultaneously. For instance, the Src protein tyrosine kinase Fyn positively regulates degranulation, leukotriene synthesis, p38 and Jnk activation and cytokine production at the same time (Parravicini, Gadina *et al.* 2002; Gomez, Gonzalez-Espinosa *et al.* 2005). In contrast, the kinase Lyn has recently been identified as a negative regulator of mast cell degranulation and anaphylactic responses (Odom, Gomez *et al.* 2004). The Bcl10/Malt1 complex has been shown in this work to selectively control one single downstream pathway of the FcεRI, namely NF-κB-dependent cytokine production, and to be dispensable for other effector functions, which is a new finding in mast cell biology and the specific functions of Bcl10 and Malt1.

Since the FcεRI belongs to the family of multi-subunit immunoreceptors, the described results also give new insights into context-specific regulation of immune responses. The family of ITAM-containing antigen-receptors also comprises the TCR, BCR and further Fc receptors (e.g. Fcγ, Fcα) (see chapter 1.1.2.2, figure 1), and these members display structural homologies and similar antigen-binding properties. However, each of these receptors utilises a unique set of adaptor molecules to transduce signals from the cell membrane to the interior, finally leading to differential cell activation. In case of TCR signalling, the transmembrane adaptor LAT is indispensable, but it does not function in B cells (Finco, Kadlecek *et al.* 1998; Zhang, Sloan-Lancaster *et al.* 1998; Zhang, Sommers *et al.* 1999), and contrarily, B cell activation induced by the BCR critically depends on the tyrosine kinases Syk and Btk, which are redundant in TCR activation (Khan, Alt *et al.* 1995; Turner, Mee *et al.* 1995; Khan 2001). Concerning FcεRI signalling, Syk and Btk are indispensable for antigen-induced mast cell activation and additionally, LAT is an essential adaptor after FcεRI crosslinking (Costello, Turner *et al.* 1996; Hata, Kawakami *et al.* 1998; Saitoh, Arudchandran *et al.* 2000).

This specific engagement of adaptor molecules can also be assigned to the Bcl10/Malt1 complex that is differentially used in signalling pathways downstream of immunoreceptors. Via its CARD domain, Bcl10 binds to a molecule of the Carma family; in case of lymphocytes, this is Carma1 (CARD11/Bimp3) and in myeloid and non-immune cells probably Carma3 (CARD10/Bimp1). Carma1 is constitutively associated with the cell membrane and after antigen-receptor crosslinking activated through phosphorylation by PKCβ in B cells or PKCθ in T cells (Coudronniere, Villalba *et al.* 2000; Gaide, Martinon *et al.* 2001; Gaide, Favier *et al.* 2002; Matsumoto, Wang *et al.* 2005; Shinohara, Yasuda *et al.* 2005; Sommer, Guo *et al.* 2005). PKCs phosphorylate Carma1 at the linker region between its coiled-coil domain and the N-terminal MAGUK region, which dismantles inhibitory interactions and allows the recruitment of Bcl10 and Malt1. Subsequently, Bcl10 is phosphorylated (Gaide, Martinon *et al.* 2001; Rueda, Gaide *et al.* 2007) and this activated Carma/Bcl10 complex then is able to recruit Malt1 and TRAF molecules, e.g. TRAF6, TGFβ-activated kinase (TAK1) or the ubiquitination enzymes Ubc13/Uev1A into the signalling complex, also called “signalosome” (Thome, Gaide *et al.* 2001; Thome 2004). The assembly of this complex and its oligomerisation lead to the activation of multiple downstream pathways like IKK-dependent NF-κB activation or MAPK activation (Wang, Deng *et al.* 2001). Particularly TRAF6 and TAK1 have recently been found to play essential roles in TCR-mediated NF-κB induction downstream of the Carma1/Bcl10/Malt1 complex with TRAF6 activating the IKKγ/NEMO subunit through regulatory lysine 63-

linked poly-ubiquitination and subsequently allowing TAK1 to phosphorylate and activate the catalytic subunit IKKβ (Sun, Deng *et al.* 2004; Zhou, Wertz *et al.* 2004; Sato, Sanjo *et al.* 2005; Shinohara, Yasuda *et al.* 2005). TAK1 also seems to function in B cells (Shinohara, Yasuda *et al.* 2005), but up to now, nothing is known about a role of TRAF6 in BCR signalling.
In mast cells, possible functions of TRAF molecules and TAK1 in FcεRI-induced signalling mechanisms have not been studied so far, and concerning TAK1, even the expression in myeloid cells is unclear. Also expression and functions of members of the Carma family in myeloid cells are not yet well understood. FcεRI-mediated NF-κB activation probably involves IKK activation by regulatory NEMO ubiquitination, but it will be necessary in the future to study mice and mast cells deficient in Carma proteins or TRAFs to elicit a possible involvement of these molecules in FcεRI signal transduction.

In addition to the extensive studies on the molecules involved in NF-κB activation downstream of PKC in the last years, the IKK/NF-κB pathway has also become an ideal drug target. Since it can be blocked by a variety of proteasome inhibitors, anti-oxidants or small peptides, which already are clinically used or at least in preclinical development, it is an attractive pathway in the treatment of chronic inflammatory diseases or certain cancers (Epinat and Gilmore 1999; Karin, Yamamoto *et al.* 2004; Karin 2005; Karin 2006). However, most of these inhibitors completely block the IKK/NF-κB system, thereby totally abolishing NF-κB activation, which can lead to serious side effects, because NF-κB also plays an essential role in normal cell physiology. Thus, it would be important to inhibit NF-κB in a context-dependent manner, e.g. to reduce the production of the proinflammatory cytokine TNF-α, which is crucial for chronic inflammatory diseases like asthma through the induction of tissue remodelling processes, angiogenesis and fibrosis (Wershil, Wang *et al.* 1991; Levine 2003; Nakae, Suto *et al.* 2005). The findings that a genetic deletion of *Bcl10* or *Malt1* selectively abolishes the production of NF-κB-dependent proinflammatory cytokines and subsequent late phase anaphylactic reactions open the possibility that a specific inhibition of the Bcl10/Malt1 complex could be beneficial for the treatment of IgE-mediated chronic allergic diseases. So another important approach in the future will be to identify or design small molecules that selectively inhibit Bcl10/Malt1 signalling without interfering with other Bcl10/Malt1-independent NF-κB pathways to become therapeutics in inflammatory diseases, which minimize toxic side effects.

4.2 The Bcl10/Malt1 complex controls LPA-induced NF-κB activation and cytokine production in murine embryonic fibroblasts

Besides the central function of Bcl10 and Malt1 in the regulation of FcεRI-induced NF-κB activation in mast cells, these studies additionally identified the Bcl10/Malt1 complex as an essential adaptor in the signalling pathway from a specific GPCR to NF-κB in non-immune cells. With MEFs as a model, it could be demonstrated that Bcl10 and Malt1 both are essential for NF-κB activation mediated by the receptor for the phospholipid LPA and for LPA-induced production of proinflammatory cytokines via a PKC-dependent mechanism. In contrast, LPA-induced activation of the MAP kinases p38, p44/42 (Erk1/2) and Jnk and Akt activation are not controlled by the Bcl10/Malt1 complex.

These results are in line with recent findings of Wang *et al.*, who also showed a critical role for Bcl10 in GPCR-induced NF-κB activation in non-lymphoid cells (Wang, You *et al.* 2007). Wang and coworkers stimulated MEFs with either LPA or another GPCR-binding bioactive peptide, endothelin-1, and yielded similar phenotypes to those shown in this work concerning NF-κB induction and the production of the NF-κB-dependent cytokine MIP-2.

A first hint for a role of Bcl10 in non-immune cells was given by the phenotype of 1/3 of *Bcl10*$^{-/-}$ embryos, which have a defect in neural tube closure and develop an exencephalus causing embryonic lethality after day E14.5 (Ruland, Duncan *et al.* 2001). This is probably due to impaired signal transduction in neurons, but the molecular mechanism of Bcl10/Malt1 signal transduction in the nervous system has not been analysed yet. However, it is known that Bcl10 and Malt1 are ubiquitously expressed in murine and human tissues throughout the whole development (Koseki, Inohara *et al.* 1999; Morgan, Yin *et al.* 1999), which warrants the experiments performed here, but most studies in the past focused on their expression in normal and neoplastic lymphoid tissue (Ye, Dogan *et al.* 2000).

The GPCRs are the largest family of cell surface receptors expressed in all mammalian and also non-immune tissues and an example with importance in malignant diseases are the receptors for LPA, belonging to the *Edg* family of GPCRs (Goetzl, Kong *et al.* 1999;

Mills and Moolenaar 2003). LPA induces cell proliferation, migration and survival and previous studies have shown that LPA is furthermore able to induce NF-κB activation via a PKC-dependent mechanism (Palmetshofer, Robson *et al.* 1999; Shahrestanifar, Fan *et al.* 1999; Cummings, Zhao *et al.* 2004; Raj, Sekula *et al.* 2004). Regarding the role of Bcl10/Malt1 in antigen-receptor-induced, PKC-dependent NF-κB activation, an involvement of this complex in LPA/GPCR-mediated NF-κB induction seemed possible. With this study, the molecular mechanism of LPA-induced NF-κB was further clarified to be PKC-Bcl10/Malt1-dependent with PKCδ probably being the responsible PKC isoform consistent with recent findings that PKCδ controls IL-8 production via NF-κB in bronchial epithelial cells and that LPA induces the recruitment of PKCδ to focal adhesions in fibroblasts (Barry and Critchley 1994; Cummings, Zhao *et al.* 2004). Conveniently, the use of the pharmacological PKC inhibitor rottlerin, which particularly blocks PKCδ at the respective dosage, impaired LPA-mediated degradation of IκB-α in *Bcl10*$^{+/-}$ MEFs. In contrast to PKC blockage, inhibition of the PI3K/Akt pathway with the PI3K inhibitor wortmannin did not interfere with LPA-induced IκB-α degradation, indicating an Akt-independent, but PKC-Bcl10/Malt1-dependent way of LPA-mediated NF-κB activation. A more detailed insight into the regulation of LPA signalling and the involved PKC isoforms could be given by the use of siRNAs to specifically reduce the activity of one single PKC enzyme. Nevertheless, since multiple PKC isoforms are expressed in MEFs, it could be possible that there is redundancy at the PKC level.

In immune cells, distinct PKC isoforms control NF-κB activation after antigen-receptor stimulation: PKCθ is activated in T cells by the TCR and PKCβ after BCR stimulation in B cells and probably after FcεRI crosslinking in mast cells (Tan and Parker 2003). As discussed before, PKCθ or PKCβ phosphorylate the Bcl10-binding protein Carma1 in lymphocytes to induce Bcl10 recruitment and subsequent downstream IKK activation (Matsumoto, Wang *et al.* 2005; Sommer, Guo *et al.* 2005). It is likely that Bcl10/Malt1-dependent NF-κB activation by LPA in fibroblasts also involves higher-order signalosomes, which additionally contain a protein of the Carma family, TRAFs, TAK1 and TAK-associated proteins (Tab-1, -2, -3). Since Carma1 specifically is expressed in lymphocytes and Carma2 expression restricted to the placenta (Bertin, Wang *et al.* 2001; Wang, Guo *et al.* 2001), it is supposable that Carma3 accomplishes this function after LPA stimulation in fibroblasts, because Carma3 is broadly expressed in multiple tissues and many cell types respond to LPA. Furthermore, it has been shown that Carma3 can as well be phosphorylated in its linker region by PKCs and that it can functionally replace

Carma1 in lymphocytes (Sommer, Guo *et al.* 2005), but final confirmation can only be given by studies with cell lines deficient in Carma proteins.

Apart from the homologies between antigen-receptor signalling in lymphocytes and mast cells and LPA signalling in fibroblasts, there also are several differences. It has been shown that Bcl10 and Malt1 not only control IKK-mediated NF-κB activation in lymphocytes, but also are essential for activation of the MAPKs p38 and Jnk in T cells (Ruland, Duncan *et al.* 2003; Thome 2004). In contrast, Bcl10 and Malt1 control the activation of neither p38 nor p44/42 nor Jnk1 after LPA receptor stimulation and in this way, display functional similarities to FcεRI signalling, in which the Bcl10/Malt1 complex also seems to be dispensable for MAPK activation.

In addition, current data presented a role for the Carma1/Bcl10 complex in the differential activation of the isoform Jnk2, but not Jnk1, after TCR ligation (Blonska, Pappu *et al.* 2006), in which Bcl10 can inducibly associate with Jnk2 to mediate its activation and to regulate the amount of the transcription factor c-Jun. However, with the antibodies used in my study, the Jnk1 and Jnk2 isoforms could not be distinguished, so conclusions on this subject cannot be made here.

Another recent study suggested that PI3K/Akt signalling mediates NF-κB activation via Carma1 in T cells (Narayan, Holt *et al.* 2006), and Akt also is known to associate with IKK to induce NF-κB after stimulation with the growth factor PDGF (Romashkova and Makarov 1999) and to be essential for TNF-induced NF-κB activation via NIK (NF-κB inducing kinase) and IKK (Ozes, Mayo *et al.* 1999). However, it has been demonstrated in this work by inhibition of PI3K with wortmannin that Akt activation is not necessary for LPA-induced signalling to NF-κB.
In summary, these results again indicate a cell-type- and receptor-specific utilisation of Bcl10, Malt1 and upstream or downstream effectors and emphasise the necessity to analyse detailed functions of the Bcl10/Malt1 complex in a context-dependent manner.

Interestingly, LPA also was able to induce a reduction of Bcl10 levels in *Bcl10*$^{+/-}$ fibroblasts, probably as well due to proteasomal degradation of Bcl10, comparable to the degradation of the NF-κB inhibitor IκB-α. It has been recently described by two independent groups that the IKK complex, namely IKKβ, phosphorylates Bcl10 in the C-terminus after formation of the Carma1/Bcl10/Malt1 complex in TCR signalling. This phosphorylation not only interferes with the stable Bcl10/Malt1 association and

IKKγ/NEMO ubiquitination, but also can induce Bcl10 degradation by the proteasome and thus, induce a down regulation of T cell activation through Bcl10 accumulation in the nucleus (Wegener, Oeckinghaus *et al.* 2006; Lobry, Lopez *et al.* 2007). Studies with phospho-specific anti-Bcl10 antibodies and proteasome inhibitors could elucidate whether the IKKβ-induced phosphorylation and proteasomal degradation of Bcl10 also occurs in non-immune cells and signal transduction from GPCRs and whether changes in cytoplasmatic Bcl10 levels can additionally modulate NF-κB activity.

Previously, abnormalities in Bcl10 and Malt1 signalling have only been considered as important inducers of lymphoma development and progression (Dierlamm, Wlodarska *et al.* 2000; Isaacson and Du 2004), but with the herein described functions in LPA signalling and the known mitogenic activities of LPA in the progression of malignant diseases like hepatoma or carcinoma (Mills and Moolenaar 2003), the Bcl10/Malt1 complex has become an even more attractive target for the treatment of human cancers besides lymphoma (Jost, Peschel *et al.* 2006).

4.3 Outlook

Together with the elucidated role of Bcl10/Malt1 signalling in mast cells and the resulting implications for the treatment of chronic allergic diseases (see chapter 4.1), this work has extended the essential functions of Bcl10 and Malt1 in lymphocytes to a previously unconsidered role in myeloid and non-immune cells, in particular Fc receptor and GPCR signal transduction. Future therapies could be developed to target this complex and specifically inhibit the production of inflammatory cytokines without generally blocking NF-κB activity.

Beyond, it is likely that the Bcl10/Malt1 complex also functions in other cell lineages and receptor types, e.g. in Fcγ signalling in dendritic cells. Lately, another CARD-containing and Bcl10/Malt1-binding protein, CARD9, was surprisingly found to be involved in innate immune responses against fungi (Gross, Gewies *et al.* 2006) or intracellular pathogens (Hsu, Zhang *et al.* 2007). Thus, there are certainly even more uncharacterized functions of the Bcl10/Malt1 complex and other CARD-containing proteins, which deserve further studies in the future.

5 Summary

In this work, the role of the essential lymphocyte adaptor molecules B cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue 1 (Malt1) in mast cells as exemplary myeloid cell line and additionally in non-immune cells has been elucidated.

Mast cells are pivotal effector cells in IgE-mediated allergic inflammatory diseases and central for their activation are signals from the high affinity IgE receptor FcεRI, which induce cell degranulation with the release of preformed mediators and *de novo* synthesis of proinflammatory leukotrienes and cytokines. The differential control of these individual mast cell responses was largely undefined up to now, but in this study, Bcl10 and Malt1 could be identified as novel key regulators of mast cell signalling. Mice deficient for either protein display severely impaired IgE-dependent late phase anaphylactic reactions. Bone marrow-derived mast cells from these animals neither activate the transcription factor nuclear factor κB (NF-κB) nor produce tumour necrosis factor α or interleukin 6 upon FcεRI ligation, even though proximal signalling as well as degranulation and leukotriene secretion are normal. Thus, Bcl10 and Malt1 are essential positive mediators of FcεRI-dependent mast cell activation that selectively uncouple NF-κB-induced proinflammatory cytokine production from degranulation and leukotriene synthesis.

Lysophosphatidic acid (LPA) is a potent bioactive phospholipid stimulating a variety of cellular responses by acting on cognate G protein-coupled receptors (GPCRs). There is increasing evidence that LPA signalling reprograms gene expression and induces cancer, but the GPCR-induced pathways connecting LPA receptor stimulation to downstream transcription factors are not well characterized. By using murine embryonic fibroblasts from Bcl10- or Malt1-deficient mice as a genetic model, it could be shown that Bcl10 and Malt1 are critically required for the degradation of IκB-α and the subsequent NF-κB induction and cytokine production in response to LPA stimulation, but are dispensable for the activation of the Jnk, p38, Erk MAP kinases and Akt signalling pathways.

Taking the results together, this study has revealed previously unrecognized functions for Bcl10 and Malt1 in signalling from the FcεRI in myeloid cells and in signalling from GPCRs in non-immune cells, which makes them an attractive target not only for the treatment of MALT lymphoma, but also for the future therapy of IgE-induced acute and chronic allergic diseases and malignant cancers caused by aberrant GPCR signalling.

6 Zusammenfassung

In dieser Arbeit wurde die Funktion der in Lymphozyten essentiellen Adaptormoleküle *B cell lymphoma 10* (Bcl10) und *Mucosa-associated lymphoid tissue 1* (Malt1) in Mastzellen als exemplarische myeloide Zellinie und des Weiteren in Nicht-Immunzellen analysiert.

Mastzellen sind aussschlaggebende Effektorzellen in IgE-vermittelten, allergischen Krankheiten und für ihre Aktivierung essentiell sind Signale des hoch-affinen IgE-Rezeptors FcεRI, die sowohl die Zelldegranulierung mit der Freisetzung von vorgeformten Mediatoren als auch die *de novo*-Synthese von proinflammatorischen Leukotrienen und Zytokinen induzieren. Die differenzierte Kontrolle dieser individuellen Mastzell-Reaktionen war bisher zum größten Teil noch ungeklärt, jedoch konnten in der vorliegenden Studie Bcl10 und Malt1 als neue Schlüsselregulatoren der Signaltransduktion in Mastzellen identifiziert werden. Mäuse, die für je eines der Proteine defizient sind, zeigen drastisch reduzierte IgE-abhängige, anaphylaktische Langzeitreaktionen. Knochenmarks-Mastzellen dieser Tiere können nach FcεRI-Anregung weder den Transkriptionsfaktor *Nuclear Factor κB* (NF-κB) aktivieren noch die Zytokine Tumornekrosefaktor α (TNF-α) und Interleukin 6 (IL-6) produzieren, wenngleich sowohl die rezeptorproximale Signaltransduktion als auch Degranulierung und Leukotrien-Sektretion nicht gestört sind. Dies macht Bcl10 und Malt1 zu essentiellen positiven Vermittlern FcεRI-abhängiger Mastzell-Aktivierung, die selektiv die Produktion NF-κB-abhängiger Zytokine von der Degranulierung und Leukotrien-Synthese entkoppeln.

Lysophosphatid-Säure (LPA) ist ein wirksames, bioaktives Phospolipid, das durch die Wirkung auf entsprechende G-Protein-gekoppelte Rezeptoren (GPCRs) eine Vielzahl zellulärer Antworten erzeugt. Der Tatsache, dass LPA-Signale die Genexpression umprogrammieren und dadurch Krebs auslösen können, wird immer größere Beachtung geschenkt, jedoch sind die GPCR-induzierten Signalwege, die die LPA-Rezeptoren mit signalabwärts liegenden Transkriptionsfaktoren verbinden bisher nur unzureichend charakterisiert. Durch die Verwendung muriner embryonaler Fibroblasten von Bcl10- oder Malt1-defizienten Mäusen als genetischem Modell konnte hier gezeigt werden, dass Bcl10 und Malt1 für die Degradation von IκB-α und die nachfolgende NF-κB-Aktivierung nach Stimulation durch LPA unverzichtbar sind. Jedoch sind die beiden Proteine für die

Aktivierung der MAP-Kinasen Jnk, p38 und Erk sowie für Akt-induzierte Signalwege entbehrlich.

Betrachtet man diese Ergebnisse zusammenfassend, so hat diese Arbeit bisher unbekannte Funktionen der Bcl10- und Malt1-Signaltransduktion vom FcεRI in myeloiden Zellen und von einem GPCR in Nicht-Immunzellen aufgeklärt, was diese Adaptorproteine zu einem attraktiven Ziel nicht nur für die Behandlung von MALT-Lymphomen macht, sondern sie in Zukunft auch für die Therapie von IgE-induzierten, akuten und chronischen allergischen Erkrankungen sowie malignen Tumorerkrankungen verursacht durch anomale GPCR-Signale interessant erscheinen lässt.

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9 Publications

Parts of this work have been previously published in the following articles:

Klemm S., Gutermuth J., Hültner L., Sparwasser T., Behrendt H., Peschel C., Mak T. W., Jakob T., Ruland J. (2006). "The Bcl10-Malt1 complex segregates FcepsilonRI-mediated nuclear factor-kappaB activation and cytokine production from mast cell degranulation." *J Exp Med* **203**(2): 337-47.

Klemm S. and Ruland J. (2006). "NF-kappaB activation by FcepsilonRI-mediated signal transduction." *Curr Immunol Rev* **2**(4): 357-60.

Klemm S. and Ruland J. (2006). "Inflammatory signal transduction from the FcepsilonRI to NF-kappaB." *Immunobiol* **211**(10): 815-20.

Klemm S., Zimmermann S., Peschel C., Mak T. W., Ruland J. (2007). "Bcl10 and Malt1 control lysophosphatidic acid-induced NF-kappaB activation and cytokine production." *Proc Natl Acad Sci U S A* **104**(1): 134-8.