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B Cell Receptor Signaling in Human B Cells

Doctoral dissertation

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ABSTRACT

B cells play an important role in the early phase of the immune response particularly in polysaccharide-encapsulated bacteria-induced responses, in which B and T cell cooperation is interfered. The mechanisms of these T cell-independent (TI) -antigen-induced B cell responses have been studied mainly in mice, but the responses and the role of BCR-mediated activation in human B cells are not known. The purpose of this study was to analyze the function and regulation of antigen-specific BCR signaling in human B cells.

The role of BCR signaling and a separate second signal was analyzed in an experimental model mimicking TI B cell responses caused by polysaccharide-encapsulated bacteria. It was shown that human macrophage (Mϕ)-derived cytokines, as a second signal, were important enhancers of BCR stimulation-induced class switch recombination and cytokine production in B cells. In addition, it was demonstrated that B cells and Mϕ function in close cooperation in TI responses as soluble mediators from activated B cells significantly enhanced cytokine production in Mϕ.

The regulation of BCR signaling by CD45 isoforms was studied in human GC-derived follicular lymphoma B cell lines. Novel human B cell lines expressing distinct CD45 isoforms (RA and R0) were established, and the CD45 isoform expression was shown to play a role in fine-tuning of the basal, BCR- and cytokine-induced proliferation, and BCR-mediated cytokine production, and BCR-induced intracellular signaling. In addition, CD45R0 was shown to be a positive regulator of BCR-induced cellular events, whereas the CD45RA isoform was shown to function as a negative regulator.

BCR-induced apoptosis is one of the most important ways to eliminate self-reactive B cells during development or GC reaction. The apoptotic process has classically been measured by detecting morphological changes or by biochemical methods such as the detection of DNA degradation. However, these methods have limited sensitivity and ability to detect apoptotic sub-populations. Therefore, a multi-parametric Annexin V-FITC, PI and SYTO 17 staining method for flow cytometric detection of apoptosis was established and evaluated. It was found that this assay increased the sensitivity to detect early apoptotic cells.

As a model for B cell targeted and specific adenoviral gene therapy, a novel fusion gene, hCAR-EGFP, was constructed. It was successfully introduced into hCAR negative human follicular B cell lymphoma cells with a lentiviral gene transfer. In this experimental model it was indirectly shown that adenovirus retargeting made adenovirus resistant cells to sensitive ones, suggesting that adenoviral gene therapy of B cell-specific cancers cells is a feasible method, but further development of appropriately targeted adenovirus vectors is still required to increase the cell-type specificity and efficacy.

National Library of Medicine Classification: QU 375, QW 568, QY 95, QZ 52, WH 200
Medical Subject Headings: Adaptor Proteins, Signal Transducing; Adenoviridae/genetics; Antigens, CD45; Antigens, T-Independent; Apoptosis; Apoptosis Regulatory Proteins; B-Lymphocytes; Cell Line; Cells, Cultured; Cytokines; Flow Cytometry; Gene Therapy; Gene Transfer Techniques; Human; Lymphoma, B-Cell; Lymphoma, Follicular; Receptors, Antigen, B-Cell; Receptors, Virus

To Anna, Roosa and Elsa

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Kuopio, May 2007

Mikko Mättö

ABBREVIATIONS

AAV	adeno associated virus
AdDsRed2	adenovirus vector containing red fluorescent protein DsRed2
AID	activation induced cytidine deaminase
Apaf-1	apoptosis activating factor-1
APC	antigen-presenting cell
BAM32	B lymphocyte adaptor protein of 32 kDa
BCAP	B cell adaptor for phosphatidylinositol 3-kinase
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein 1
Btk	Bruton's tyrosine kinase
β2μG	β2-microglobulin
CLP	common lymphoid progenitor
CSR	class switch recombination
DAG	diacylglyserol
DsRed2	red fluorescent protein from Discosoma sp.
EBF	early B cell factor
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
ΔEGFP	EGFP lacking the ATG start codon
ERK	extracellular signal regulated kinase
FACS	fluorescence activated cell sorter
FDC	follicular dendritic cells
Flt3	fms-like tyrosine kinase 3
FSC	forward scatter
GC	germinal center
GM-CSFR	granulocyte-macrophage colony stimulating factor
GRB2	growth factor receptor bound protein 2
GSK3	glycogen synthase kinase 3
hCAR	human coxsackie adenovirus receptor
ΔhCAR	truncated hCAR
HSC	hematopoietic stem cell
IFN	interferon
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IP3	inositol 1,4,5-trisphosphate
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
JNK	stress-activated Jun amino-terminal kinase
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase

Mϕ	macrophage
Mϕ-SN	macrophage culture supernatant
MHC	major histocompatibility complex
MLP	myeloid-lymphoid progenitor
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid
MW	molecular weight
MZ	marginal zone
NF-κB	Nuclear factor-κB
NO	nitric oxide
Pax5	Paired box gene 5 (BSAP)
PB	peripheral blood
pfu	plaque-forming units
PI	propidium iodide
PIAS	proteins that inhibit activated STATs
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA2	phospholipase A2
PLCγ	phospholipase C-γ
PS	phosphatidylserine
$p27^{kip1}$	cyclin-dependent kinase (CDK) inhibitor
p38	p38 MAP kinase
RAG	recombination-activating gene
RHD	Rel homology domain
SAC	Staphylococcus aureus Cowan I bacteria
SCID	severe combined immunodeficiency disease
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src-homology 2 domain
SLE	systemic lupus erythematosus
SLP-65	Src-homology-2 (SH2) domain-containing leukocyte protein of 65 kDa (BLNK)
SOCS	supressor of cytokine signaling
Src-PTK	Src-family protein tyrosine kinases
SSC	side scatter
STAT	signal transducers and activators of transcription
T-bet	T-box family of transcription factors
TCR	T cell receptor
TD	T cell-dependent
Tg	transgenic
TI	T cell-independent
TI-1	TI type 1
TI-2	TI type 2
TK-GFP	herpes simplex virus type I thymidine kinase and green fluorescent protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
t.u.	transducing units
Vav	a guanine nucleotide exchange factor for Rho/Rac-family GTPases
VP	viral particles
XBP-1	X-box binding protein 1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by Roman numerals (I-IV).

I Mättö M, Pietilä AR, Postila V, Huttunen K, Hirvonen MR, Pelkonen J. Induction of the inflammatory response by B cells. Submitted.

II Mättö M, Nuutinen U, Ropponen A, Myllykangas K, Pelkonen J. CD45RA and R0 isoforms have distinct effects on cytokine- and B cell receptor-mediated signalling in human B cells. Scand J Immunol 2005;61:520-8.

III Eray M, Mättö M, Kaartinen M, Andersson L, Pelkonen J. Flow cytometric analysis of apoptotic subpopulations with a combination of Annexin V-FITC, propidium iodide, and SYTO 17. Cytometry 2001;43:134-142.

IV Mättö M, Nuutinen U, Hakkarainen T, Tallone T, Wahlfors J, Pelkonen J. hCAR-EGFP fusion receptor in human follicular lymphoma B cells - a model for adenoviral gene therapy for B cell malignancies. Int J Mol Med 2006; 17: 1057-62.

 In addition, some unpublished data is presented.

CONTENTS

1. INTRODUCTION

B cells are important members of the adaptive immunity. In general, the role of B cells in immune defense is to recognize microbial pathogens with antigen-specific B cell receptors (BCR), internalize and process them to peptides. Peptides are further presented in MHC (major histocompatibility complex) II class molecules to antigen-specific $CD4^+$ T_{Helper} cells (T_H), an example of a T cell-dependent (TD) immune reaction. Interaction of B and T_H cells further activates the production of antibodies against pathogenic microbes leading to their elimination.

All pathogenic microbes activate TD B cells reactions. However, polysaccharide-encapsulated bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Hemophilus influenzae*, are known to cause severe infections in early childhood due to the fact that polysaccharides do not bind to MHC II class molecules or inhibit antigen presentation, therefore inhibiting the interaction of B cells and T_H cells. Despite defective T cell activation, antigen-specific B cells are known to elicit immune response to polysaccharide-encapsulated bacteria in a T cell-independent (TI) manner. So far, TI B cell responses have been studied mainly in mice, and human TI B cell responses are largely unknown, and need to be studied in more detail.

2. REVIEW OF THE LITERATURE

2.1. B cells

2.1.1. The function of B cells in the immune system

B cells recognize foreign structures with antigen-specific BCR. The major function of B cells in immune defense is to produce antibodies against pathogenic microbes. Antibody-mediated protection against pathogenic microbes is based on the opsonization and neutralization of the pathogen that further activate the complement system and other immune cells, such as macrophages (Mϕ). Finally this leads to the killing of pathogenic microbes. Classically, B cell-mediated immune response to microbial pathogens is thought to require supportive signals from $CD4^+$ T_H cells, and therefore it is termed a TD B cell response. B cells have also been shown to elicit immune reactions against microbes without T cell help, which is then called as a TI response (Mond et al., 1978; Mond et al., 1995; Mond et al., 1995; Rajewsky, 1996; Scher, 1982). TD antigens are structures or organisms that B cells can process to peptides through the endocytic compartment and they present the processed peptides within the groove of a MHC (major histocompatibility complex) class II molecule. It is known that antigen-specific T_H cells recognize peptide-MHC complexes on the surface of B cells with specific T cell receptors (TCR) and become activated (Matsui et al., 1991; Mond et al., 1995). Activated antigen-specific T_H cells proliferate, produce cytokines such as interleukin-4 (IL-4), and up-regulate CD40 ligand (CD40L) that further interact with CD40 receptor (CD40) on the B cell surface. B cells are activated, induced to proliferate and undergo a maturation process, which finally lead to the development of antibody-producing plasma cells (Mond et al., 1995; Snapper and Mond, 1996). In contrast, microbial products or surface structures such as bacterial polysaccharides have been suggested to trigger BCRs in a multivalent fashion. However, these carbohydrate structures are not presented within MHC class II molecules, or they inhibit the binding of peptides to MHC class II molecules (Gonzalez-Fernandez et al., 1997; Harding et al., 1991; Leyva-Cobian et al., 1997; Pillai et al., 2005; Vos et al., 2000). It has also been suggested that

viral glycoproteins are TI antigens because they are known to interfere in the up-regulation and function of the MHC complex, and prevent MHC-mediated T cell activation (Alcami and Koszinowski, 2000; Bachmann et al., 1993; Bachmann and Zinkernagel, 1996). Pure bacterial lipopolysaccharide (LPS) and polysaccharide are considered to be TI type 1 (TI-1) and TI type 2 (TI-2) antigens, respectively. In mice, LPS has been reported to induce a polyclonal B cell activation and effective production of IgM antibodies (Mond et al., 1995; Mond et al., 1995; Scher, 1982; Vos et al., 2000). By contrast, human peripheral blood (PB) B cells are not responsive to pure LPS stimulation which is likely due to the low expression of Toll-like receptor 4 (TLR4), a known LPS receptor (Hornung et al., 2002; Wagner et al., 2004). In mice, TI-2 antigens have been shown to induce B cell activation and Ab production but a soluble second signal is also needed (Mond et al., 1995; Mond et al., 1995; Scher, 1982; van den Eertwegh et al., 1992; Vos et al., 2000). Interestingly, it has been reported that human and mice marginal zone B cells in spleen are required for immune responses to blood-borne TI-2-antigens, such as the polysaccharide-encapsulated bacteria *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Hemophilus influenzae* (Pillai et al., 2005).

In the primary immune reaction, IgM class antibodies are produced. In the secondary immune response, the expression of germline IgM constant region is changed to secondary antibody isotypes by class switch recombination (CSR). It is known that the changed antibody isotype does not affect antigen binding specificity or expressed V(D)J regions in Ig heavy (H) and light (L) chain genes, however, different antibody isotypes have distinct effector functions. In the case of TD antigens, BCR stimulation induced by the antigen, CD40-CD40L interaction and supportive cytokines are known to regulate the CSR and antibody production. By contrast, BCR stimulation with TI antigens alone can also induce CSR but supportive cytokines are also needed as a second signal (Mond et al., 1995; Mond et al., 1995; Stavnezer, 1996). In general, it has been reported that humans vaccinated with Tetanus toxoid, a TD antigen, are induced to produce IgM and IgG1 antibodies (Seppala et al., 1984). By contrast, vaccination of humans with meningococcal polysaccharide, a purified TI antigen, lead to the production of IgM, IgG2 and IgG1 antibodies (Rautonen et al., 1986). However, in vitro stimulation of human B cells with Staphylococcus aureus Cowan I bacteria (SAC) alone induced the

expression of IgG1 germline transcript (Calvert et al., 1990; Kitani and Strober, 1993). By contrast, stimulation with SAC in the presence of IL-4 induced the expression of IgG3 and IgG4 germline transcripts. SAC-induced expression of IgG3 germline transcripts could be prevented when IFN-γ was added into the cell culture medium (Kitani and Strober, 1993).

2.1.2. B cell development in the bone marrow

In mammalian bone marrow, pluripotent hematopoietic stem cells (HSC) differentiate into lymphoid cells including B cells through a complex and highly regulated pathway (Fig. 1). The hallmark of B cell development is the expression of BCR on the surface of B cell and the recombination of IgH and L chain genes. In bone marrow, the expression of BCR requires a genetic recombination of germline V, D and J segments at IgH locus, and V and J segments at IgL locus. V_H to DJ_H rearrangement occurs in the IgH locus of $CD19^+$ pro-B cells, which leads to the expression of pre-BCR components such as Igμ proteins on the cell surface. Pre-BCR signaling induces allelic exclusion at the IgH locus, cellular proliferation and V_L to J_L recombination at the IgL locus. In the final stage in bone marrow, pre-B cells mature into immature B cells, which have VDJ_H and VJ_L rearranged IgH and IgL loci, respectively. Immature B cells express IgM and IgD on the cell surface, and after deletion of self-reactive immature B cells, they migrate from the bone marrow to peripheral organs to mature further (Busslinger, 2004; Ghia et al., 1998; Hagman and Lukin, 2006; Meffre et al., 2000; Spicuglia et al., 2006).

B cell lineage commitment is highly regulated by specific transcription factors. B cell development in mice and humans has certain unique features, which are described below. In the mouse bone marrow, the early B cell development requires the expression of PU.1 (a member of the Ets family transcription factor) (Akashi et al., 2000; DeKoter and Singh, 2000). PU.1 has been shown to regulate the expression of BCR-associated proteins, such as Igα (CD79α, Mb-1), VpreB, Igβ (CD79β, B29) and λ5, and RAG-1 and -2 (recombination-activating gene). In PU.1-deficient mice, transcription factors EBF (early B cell factor) and Pax5 (Paired box gene 5, BSAP) as well as the IL-7 receptor α subunit (IL-7Rα) are absent (DeKoter et al., 2002). The early B cell lineage commitment as well as other lymphoid lineage cells such as T and NK cells are further

regulated by Ikaros, E2A and EBF transcription factors (Hagman and Lukin, 2006; Johnson and Calame, 2003; Medina et al., 2004; Nichogiannopoulou et al., 1999; Wu et al., 1997). Ikaros has been shown to regulate transcription by binding to λ5, TdT and Mcf genes, further enhancing the expression of lymphoid lineage specific genes such as c-kit or Flt3 (fms-like tyrosine kinase 3), and repress the expression of myeloid lineage specific genes such as GM-CSFR (Georgopoulos, 2002; Johnson and Calame, 2003; Kirstetter et al., 2002; Nichogiannopoulou et al., 1999). E2A and EBF have been shown to regulate the transcription of early B cell genes λ5, VpreB, RAG-1, RAG-2, germline Igμ transcripts, Pax5, Ig enhancers, Igα, activation-induced cytidine deaminase (AID) (Hagman and Lukin, 2006; Medina et al., 2004). Pax5 controls B cell lineage commitment downstream of E2A and EBF transcription factors (Nutt et al., 1998). In the absence of Pax5, B cell development was blocked at the early pro-B cell stage (Busslinger, 2004). In addition, IL-7Rα signaling has been shown to regulate the expression of Pax5 (DeKoter et al., 2002). Pax5 induces B cell-specific genes such as BLNK, Igα and CD19, which are important for the pre-BCR signaling. Pax5 has also been reported to control the expression of the Igμ chain by regulating the V_H-DJ_H recombination (Busslinger, 2004; Nutt et al., 1998). In addition, Pax5 has been shown to repress genes unspecific for B cell development such as Notch-1, which is important for T cell development (Busslinger, 2004; Johnson and Calame, 2003; Souabni et al., 2002). In addition, pro-B cell to pre-B cell transition is known to be regulated by the BCR signaling molecule SLP-65 (Src-homology-2 (SH2) domain-containing leukocyte protein of 65 kDa), or by the transcription factors NF-κB (nuclear factor-κB), Sox4 and Lef1 (Flemming et al., 2003; Horwitz et al., 1997; Jumaa et al., 1999; Matthias and Rolink, 2005; Schebesta et al., 2002; Schilham et al., 1996). Aiolos, IRF-4 and -8 (interferon regulatory factors) have been shown to regulate pre-B to immature B cell transition by inducing IgL rearrangement, expression of germline transcripts and down-regulation of surrogate light-chain genes λ5 and VpreB (Busslinger, 2004; Lu et al., 2003; Matthias and Rolink, 2005).

In human bone marrow, B cell development has been shown to be independent of IL-7R signaling or expression of cytokine receptor subunit γ_c. Patients suffering from γ_c deficiency have been found to have normal or elevated amounts of B cells but the

number of T and NK cells were strongly repressed (Buckley et al., 1997; LeBien, 2000). In humans, the mutated Bruton's tyrosine kinase (Btk) gene has been reported to prevent pre-BCR signaling which arrests B cell development in bone marrow to the pre-B cell stage (Noordzij et al., 2002).

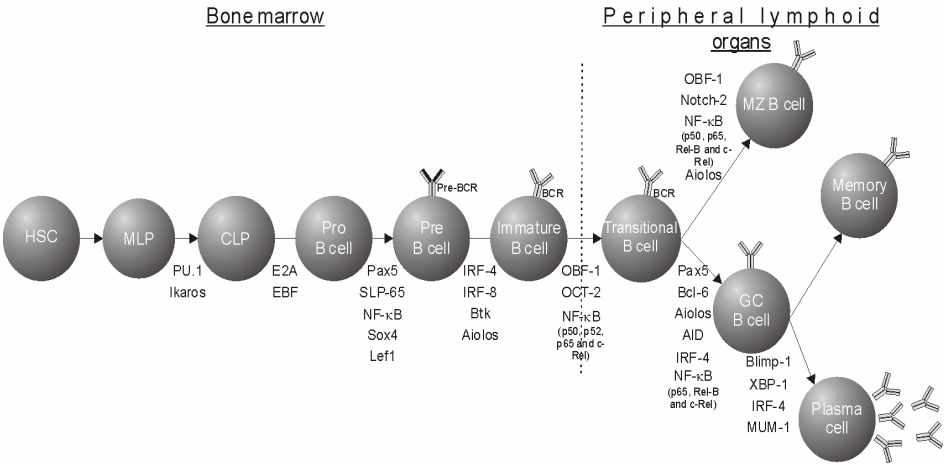

Figure 1. Overview of B cell development. Hematopoietic stem cell (HSC), myeloid-lymphoid progenitor (MLP), common lymphoid progenitor (CLP), germinal center (GC) and marginal zone (MZ).

2.1.3. B cell development in periphery

After maturation in the bone marrow, IgM- and IgD-expressing mature B cells, also called transitional B cells, migrate to the spleen and lymph nodes to mature further (Carsetti et al., 2004; MacLennan et al., 1997). In the spleen or in the other secondary lymphoid organs B cells migrate through the primary follicle to lymphatic vessels and further to the blood circulation to re-circulate between the spleen and lymph nodes, if they do not encounter the appropriate antigen or do not receive correct supportive signals (MacLennan et al., 1997). Antigen-experienced mature B cells interact with T_H cells that are primed with the same antigen. These B cells start to proliferate and form a secondary follicle, also called extra-follicular foci. It has been suggested that during the primary contact with antigen-primed T cells, B cells switch their Ig isotype (McHeyzer-

Williams and McHeyzer-Williams, 2005). Alternatively, antigen-experienced B cells which will not start a germinal center (GC) reaction may also develop into short-lived plasma cells, called non-GC plasma cells, which secrete germline-encoded antibodies. The rapidly proliferating B cells in the secondary follicles, called centroblasts, give rise to a GC. Centroblasts undergo a rapid clonal expansion and form a clearly defined structure, the dark zone. During the GC reaction, centroblasts further initiate a somatic hypermutation process in which the variable region (V) mutations increase the BCR diversity and affinity. After centroblasts move to the edge of dark and light zones, they become centrocytes and move out into the GC light zone area. In this area the centrocytes contact with antigens presented by follicular dendritic cells (FDC), and are selected according to their ability to bind and process the antigen, and further interact with antigen-specific GC T cells. In addition to specific TCR-MHC II contact, centrocytes and GC T cells are known to interact via CD40 and CD40L, which leads to up-regulation of the expression of survival factors such as anti-apoptotic Bcl-2 protein, providing a survival signal for centrocytes with high affinity BCR (MacLennan et al., 1997; McHeyzer-Williams and McHeyzer-Williams, 2005). These positively selected centrocytes exit from GC and further mature either to antigen-specific memory B cells or antibody-producing plasma cells. It has also been reported that high-affinity centrocytes are able to return to the GC dark zone to undergo a new round of clonal expansion and somatic hypermutation of Ig V gene, leading to increased BCR affinity. Finally, those centrocytes that do not have a sufficient BCR affinity for an antigen and do not receive a survival signals will undergo apoptosis (MacLennan et al., 1997; McHeyzer-Williams and McHeyzer-Williams, 2005).

Peripheral maturation of B cells is a complex process that is regulated by multiple factors, including the factors that regulate B cell development in the bone marrow (Fig. 1) (Johnson and Calame, 2003; Matthias and Rolink, 2005). Transcription factors PU.1, E2A, EBF, Ikaros and Pax5 have been shown to participate in B cell development also in the periphery by maintaining B cell lineage specific features that exist from the bone marrow stage up to the plasma cell stage (Johnson and Calame, 2003). It has been reported that initial transition of bone marrow-derived circulating mature B cells into the spleen cells is regulated by transcription factors such as OCT-2, OBF-1 and NF-κB

isoforms p50, p52, p65 and c-Rel (Matthias and Rolink, 2005). The development of marginal zone B cells from transitional B cells has been shown to be regulated by NF-κB isoforms p50, p65, Rel-B and c-Rel, as well as Notch-2 (Matthias and Rolink, 2005). Furthermore, Aiolos has been reported to be an important regulator of both marginal zone B cell and GC development (Busslinger, 2004; Johnson and Calame, 2003; Kirstetter et al., 2002; Matthias and Rolink, 2005). Cells entering a GC reaction have been shown to up-regulate the expression of transcription factors such as Pax5, Bcl-6, Aiolos, AID, IRF-8, p65, Rel-B and c-Rel (Cattoretti et al., 2006; Matthias and Rolink, 2005). By contrast, the cells entering into GCs down-regulate the expression of IRF-4 and MUM1. In addition at the centrocytic stage, AID was shown to be repressed (Cattoretti et al., 2006). In addition, in GC cells the expression of plasmacytic transcription factors Blimp-1 and XBP-1 has been reported to be repressed by Bcl-6 and Pax5, respectively (Matthias and Rolink, 2005). Post-GC and pre-plasma memory B cells have also been identified, but the nature of transcription factors regulating the formation of memory B cells are still unclear. It has been shown that the absence of Blimp-1 abrogates plasma cell differentiation, suggesting a role for Blimp-1 as a regulator of terminal B cell maturation (McHeyzer-Williams and McHeyzer-Williams, 2005). Recently, plasma cell differentiation was induced in the absence of Pax5 leading to the up-regulation of Blimp-1 and XBP-1 genes and down-regulation of Bcl-6 gene (Nera et al., 2006). In contrast, in mouse B cells the loss of Pax5 enhanced the expression of Blimp-1, but this did not reduce the expression of Bcl-6 or up-regulate the expression of XBP-1 (Delogu et al., 2006). IRF-4 was shown to be up-regulated and IRF-8 down-regulated in plasma cells (Cattoretti et al., 2006).

2.2. BCR signaling

2.2.1. BCR-mediated survival signals

BCR-mediated intracellular signaling has multiple outcomes depending on the maturational stage of B cells. In mature B cells, it is known that BCR signaling induces the proliferation and survival of B cells, whereas in immature B cells BCR signaling is known to induce either apoptosis, or inactivation by anergy or receptor editing (Niiro

and Clark, 2003). Functional BCR consists of two transmembrane IgH molecules and two covalently bound IgL molecules. BCR is anchored to the plasma membrane through non-covalently associated transmembrane molecules, Igα (CD79α) and Igβ (CD79β). These proteins form heterodimers, and a functional BCR complex includes two Igα-Igβ heterodimers. The intracellular part of Igα and Igβ contains one immunoreceptor tyrosine-based activation motif (ITAM) which is necessary for the initiation of ligand binding-induced intracellular signaling (Monroe, 2006; Niiro and Clark, 2002).

The change in the phosphorylation status of signaling molecules is the major regulator of activation. Src-family protein tyrosine kinases (Src-PTK) such as Lyn, Fyn and Blk, play a key role in the initiation of BCR signaling. Of these, Lyn is the most abundantly expressed in B cells and it has been shown to be both a positive and a negative regulator of BCR signaling (Hermiston et al., 2003). In unstimulated B cells, Lyn is known to be associated with Igα via its N-terminus (Pleiman et al., 1994). BCR-mediated intracellular signaling is initiated after antigen engagement which further induces the phosphorylation of Igα and Igβ ITAMs by Lyn Src-PTK. Phosphorylation of ITAM tyrosine residues induces the recruitment and activation of SYK, a SH2 domain-containing PTK, and Btk, a TEC-family PTK (Monroe, 2006; Niiro and Clark, 2002). Lyn has been suggested to play a role as a negative regulator of BCR signaling, since after BCR stimulation Lyn is thought to terminate BCR signaling by phosphorylating the ITIMs (immunoreceptor tyrosine-based inhibition motif) of inhibitory receptors such as CD22 and FcγRIIB, which further recruit the phosphatases SHP-1 and SHIP-1 (DeFranco et al., 1998). In summary, Lyn Src, SYK and Btk are the key players in the initiation of BCR signaling, since the deletion of these PTKs prevents BCR signaling (Monroe, 2006; Niiro and Clark, 2002). SYK and Btk have been shown to recruit non-enzymatic linker signaling proteins such as SLP-65 (also know as BLNK), BAM32 (B lymphocyte adaptor protein of 32 kDa), BCAP (B cell adaptor for phosphatidylinositol 3-kinase) and GRB2 (growth factor receptor bound protein 2) (Monroe, 2006; Niiro and Clark, 2002). SLP-65 and BAM32 proteins have been shown to link SYK and Btk to the PLCγ2 pathway leading to increased Ca^{2+} influx (Jumaa et al., 1999; Monroe, 2006; Niiro and Clark, 2002; Niiro and Clark, 2003). GRB2 linker protein has been shown to associate with MAPK (mitogen activated protein kinase)

signaling molecule ERK (extracellular signal regulated kinase) (Monroe, 2006; Niiro and Clark, 2002; Yokozeki et al., 2003).

Downstream from the linker proteins, BCR stimulation is known to induce PI3K, MAPK-ERK and NF-κB pathway signaling, which are known to promote the survival and proliferation of B cells. Activated PI3K further activates the downstream Akt by phosphorylation (Pogue et al., 2000). Akt enhances cell survival by repressing the activation of the pro-apoptotic molecule Bad. Additionally, Akt phosphorylates and inhibits the constitutively active GSK3 (glycogen synthase kinase 3) which is known to phosphorylate and destabilize cell cycle proteins MYC and cyclin D (Brazil and Hemmings, 2001; Niiro and Clark, 2002).

The activation of ERK has also been connected to B cell survival. The MAPK-Ras-Raf 1 pathway is the major regulator of ERK activity, but the PLCγ2 pathway has also been shown to participate in the regulation (Hashimoto et al., 1998; Niiro and Clark, 2002). BCR stimulation-induced expression of cyclin D2 and cell-cycle progression are regulated by the Raf 1-ERK pathway (Piatelli et al., 2002). In addition, the sustained activation of ERK after BCR stimulation has been reported to activate transcription factors such as CREP and Elk-1, which are important regulators of cell proliferation (Koncz et al., 2002). In B cells, Raf 1 has also been suggested to be a survival factor that translocates to the mitochondrial compartment and regulates the functions of mitochondria (Wang et al., 1996).

BCR stimulation-induced activation of the NF-κB signaling pathway has been demonstrated to be important throughout the B cell lifespan (Niiro and Clark, 2002). p65, Rel-B, c-Rel, p50 and p52 are known to be members of the highly conserved NF-κB family of transcription factors. All the NF-κB family members contain the Rel homology domain (RHD) that mediates the dimerization, DNA-binding, nuclear translocation and interaction with IκB (inhibitor of κB) family members (Gerondakis et al., 1998; Ghosh et al., 1998). In unstimulated cells, NF-κB dimers are localized in the cytoplasm since the nuclear localization is sequestered by IκB family proteins (Burstein and Duckett, 2003). After BCR stimulation, the PKCβ (protein kinase C-β isoform) kinase activates the IκB kinase (IKK) complex which phosphorylates IκB. Phosphorylated IκB is further ubiquinated and proteosomally degraded (Burstein and

Duckett, 2003; Su et al., 2002). Upon IκB degradation, NF-κB homo- or heterodimers translocate into the nucleus and induce gene transcription by DNA binding (Burstein and Duckett, 2003; Gerondakis et al., 1998). In B cells, different pairs of NF-κB protein subunits have been reported to function at different stages of B cell development. NF-κB family proteins are known to induce the expression of anti-apoptotic proteins Bcl-2, Bcl-X_L and A1, which further repress the expression of pro-apoptotic molecules Bik, Bim, Bax, Bad and Bam. In addition, NF-κB has been shown to participate in the regulation of the cell cycle by inducing the expression of cyclin D2 (Niiro and Clark, 2002). Experiments with the B cells from knock-out mice have demonstrated that NF-κB subunits c-Rel, p50 and p65 regulate proliferation (Gerondakis et al., 1998).

2.2.2. BCR-induced apoptotic cell death

During B cell development in the bone marrow and GCs, BCR specificity to self-proteins might be produced. To delete self-reactive BCR specificities, BCR-mediated apoptotic cell death is induced. Although BCR-induced apoptotic cell death has been studied widely, the exact mechanism is still unknown (Eeva and Pelkonen, 2004).

Multiple intracellular signaling molecules have been connected to BCR-induced apoptosis. The PLCγ signaling pathway has been reported to induce apoptosis in certain B cell lines. Activated PLCγ induces the cleavage of PIP2 (phosphatidylinositol 4,5-bisphosphate) to downstream products IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglyserol) (Campbell, 1999). Released IP3 binds to specific receptors in the endoplasmic reticulum and further induces the release of calcium from intracellular calcium stores. Elevated cytosolic calcium concentration activates calcineurin, a protein phosphatase, which is reported to activate caspase-2, NF-ATc2 or MAPK p38 and JNK (stress-activated Jun amino-terminal kinase) (Chen et al., 1999; Graves et al., 1996; Kondo et al., 2003). In a B lymphoma cell line, B104, delayed and prolonged activation of JNK and p38 has been shown to induce apoptosis after BCR stimulation (Graves et al., 1996). In addition, BCR-induced activation of p38 has been suggested to participate in a positive feedback loop that activates caspases and amplifies the apoptotic response (Graves et al., 1998). The participation of the ERK signaling molecule in the apoptotic process is controversial. BCR stimulation-induced early and transient activation of ERK

has been proposed to induce apoptosis. As a consequence of early ERK activation, phospholipase A2 (PLA2) is activated, leading to the impaired function of mitochondria, and ultimately to apoptosis (Gauld et al., 2002; Katz et al., 2004). It has also been suggested that BCR-induced down-regulation of survival factor PI3K induces apoptosis by increasing the activation of cyclin-dependent kinase (CDK) inhibitor, $p27^{kip1}$, and depressing the activity of c-myc (Carey and Scott, 2001; Eeva and Pelkonen, 2004). In WEHI-231 B cells, BCR-induced accumulation and stabilization of IκB proteins in the cytosol leads to decreased DNA binding of NF-κB. Decreased DNA-binding of NF-κB has been reported to induce apoptosis by increasing the activity of pro-apoptotic p53 protein (Eeva and Pelkonen, 2004; Ku et al., 2000). It is widely accepted that in BCR-induced apoptosis the decreased mitochondrial membrane potential leading to mitochondrial dysfunction eventually induces cell death. Disintegration of the mitochondrial membrane releases cytochrome c to the cytoplasm, further forming a complex with caspase-9, Apaf-1 (apoptosis activating factor-1) and dATP. This complex then further recruits other effector caspases such as caspase-3,-6 and -7. Finally, cell death is induced after activation of effector caspases that cleave and destroy cell architecture (Eeva and Pelkonen, 2004).

Alternatively, the association of BCR complex with lipid rafts in distinct B cell maturational stages has been suggested to regulate the induction of apoptosis. In pre-B cells, lipid rafts have been shown to associate constitutively with pre-BCR complex, and survival is induced after BCR signaling. By contrast, in immature B cells lipid rafts have not been reported to associate with BCR, and antigen ligation-induced signaling activates apoptotic cell death (Pierce, 2002).

2.3. CD45

2.3.1. Structure and functions of CD45

CD45 is a transmembrane receptor that is expressed in high levels on the surface of lymphocytes (Thomas, 1989). CD45 has three separate parts. Of these, the extracellular part is known to contain three segments encoded by alternatively spliced exons 4-6 (also termed as A, B and C) that form different isoforms, and a cysteine rich domain, and

three fibronectin-like domains. The other parts of the CD45 receptor, the transmembrane segment and two cytoplasmic protein tyrosine phosphatase domains (D1 and D2), are identical in all CD45 isoforms (Tchilian and Beverley, 2006). The phosphatase D1 is known to be catalytically active, that regulates the BCR signaling, whereas the phosphatase D2 has been proposed to control the stability and activity of D1 phosphatase. Thus D2 regulates the overall functions of CD45 (Hermiston et al., 2003).

As mentioned above, different CD45 isotypes are formed after conditional mRNA splicing of the alternative exons 4-6. The high molecular weight (MW) isoforms contain the exon A (CD45RA cells), but the low MW isoform, CD45R0, does not contain these exons at all (Tchilian and Beverley, 2006). Conditional splicing of mRNA leading to a differential expression of exons 4-6 has been proposed to be regulated by serine- and arginine-rich (SR) family splicing factors or consensus sequence motifs in exons 4-6 such as ESS (exonic splicing silencer sequences), ESE (enhancer sequences) or ARS (activation responsive sequences) (Tchilian and Beverley, 2006; ten Dam et al., 2000). In T cells, the memory cells are known to express the CD45R0 isoform, whereas the CD45RA isoform is expressed on naïve cells (Hermiston et al., 2003). Human B cells in PB or tonsils express the CD45RA isoform (ABC), but the role of other possible CD45 isoforms is still largely unknown (Jensen et al., 1989; ten Dam et al., 2000; Yu et al., 2000; Yu et al., 2002).

The complete lack of CD45 receptor or abnormal expression of CD45 exons 4 or 6 have been connected to human diseases such as SCID (severe combined immunodeficiency disease), B cell lymphoma, infantile cholestasis, malnutrition, SLE (systemic lupus erythematosus), rheumatoid arthritis and Alzheimer's disease (Kung et al., 2000; Tchilian and Beverley, 2006). Overall, CD45 has been reported to regulate lymphocyte function, survival and disease by controlling the threshold of immune receptor signaling (TCR and BCR) or by regulating the production of cytokines (Tchilian and Beverley, 2006).

2.3.2. Role of CD45 in the B cells

In mice, expression of the CD45 has been reported to be important for BCR-mediated proliferation, the deletion of autoreactive B cells and persistence of GC reaction (Huntington et al., 2006). In humans, the lack of CD45 expression has also been shown to regulate B cell maturation and GC reaction (Kung et al., 2000).

The CD45 receptor is known to regulate the threshold of BCR signaling and BCR-mediated proliferation (Hermiston et al., 2003; Huntington et al., 2006). CD45 controls the phosphorylation and kinase activity of Lyn (Benatar et al., 1996; Dornan et al., 2002; Hermiston et al., 2003; Huntington et al., 2006; Pao et al., 1997; Shrivastava et al., 2004). CD45 activates Lyn by dephosphorylation of the activating tyrosine residue 508 (Tyr508) which leads to refolding and autophosphorylation of Lyn at tyrosine residue 397. In addition, CD45 inactivates Lyn by dephosphorylating Tyr397, which results in the refolding of Lyn to an inactive resting form (Huntington and Tarlinton, 2004; Xu et al., 1997). Recent findings suggest that CD45 regulates the activation of Lyn by controlling the equilibrium between active and inactive forms of Lyn. In unstimulated B cells, this results in basal phosphorylation of tyrosines in the ITAMs of Igα/β, CD19, and in ITIMs of CD22 and CD72 (Greer and Justement, 1999; Huntington and Tarlinton, 2004). After antigen binding to BCR, CD45 is known to be excluded from the BCR-containing lipid rafts, which further increases the activity of Lyn and leads to increased phosphorylation of ITAMs in Igα/β and CD19, increasing the recruitment of SYK and PI3K, and leading to downstream signaling (Huntington and Tarlinton, 2004; Pierce, 2002; Shrivastava et al., 2004).

In addition to Src regulation, CD45 has also been shown to regulate the phosphorylation and activity of BCR-induced mitogenic signaling pathways, since in the absence of CD45, the BCR-induced activation of ERK, Akt and NF-κB was defective (Healy et al., 1997; Huntington et al., 2006). In addition, CD45 has been reported to control the activation of JNK and p38 (Ogimoto et al., 2001).

CD45 isoform-specific effects on antigen receptor signaling has been studied mostly in T cells (Hermiston et al., 2003). In B cells, the CD45 receptor and its different isoforms have been reported to regulate the signal transduction of cytokine receptors

(Irie-Sasaki et al., 2001; Li et al., 2005). It has been reported that selective translocation of the IL-6Rα chain with the CD45RB isoform to the same plasma membrane lipid raft might regulate cytokine signaling (Li et al., 2005). Alternatively, the CD45RA isoform can dephosphorylate JAK1 (Janus kinase) and JAK3, thus regulating cytokine receptor signaling (Irie-Sasaki et al., 2001; Yamada et al., 2002; Zhou et al., 2003). In T cells, CD45 isoform-specific effects on cytokine production have been described. After simultaneous TCR and CD28 stimulation, higher production of IFN-γ was induced in $CD45RA^{Hi}$ T cells than in $CD45R0^{Hi}$ T cells (Dawes et al., 2006). In addition, it has been proposed that the CD45R0 isoform preferentially homodimerized, further decreasing the total phosphatase activity of CD45 (Xu and Weiss, 2002). It has also been suggested that CD45RABC, as a larger isoform might sterically interfere the MHC-peptides presentation to TCR. This may result in attenuation of the T cell-mediated immune responses (McNeill et al., 2004). In B cells, MHC class II molecule signal transduction has been shown to be regulated by CD45 (Greer et al., 1998). Finally, it has been suggested that CD45 isoforms might have specific exogenous ligands, but so far no such ligands have been described (McNeill et al., 2004).

2.4. Cytokines

2.4.1. Cytokine signaling

Cytokines are important signal transmitters in the human immune system. The development and function of lymphocytes are known to be regulated by cytokines. $CD4^+$ T_H cell-derived cytokines are divided into T_H1 and T_H2 subclasses. T_H1 cytokines IL-2, IFN-γ (interferon-gamma), IL-12, IL-18 and TNF-β (tumor necrosis factor-β), are known to be produced in response to infection with intracellular microbes and in autoimmunity. By contrast, the production of T_H2 cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13 is known to be induced after infection with extracellular bacteria and parasites, but they are also produced in allergic reactions. T_H cytokines such as IL-4, IL-6 and IFN-γ are important regulators of B cell functions such as the proliferation, affinity maturation and the production of antibodies (Romagnani, 2004).

Hematopoietically important cytokines can also be divided into type I and type II cytokines based on the receptor structure. Type I cytokines are further divided into γ_C, β_C or gp130 subunit-containing receptor subfamilies. Receptors of IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 share the γ_C subunit. By contrast, the gp130 subfamily includes cytokines such as IL-6 and IL-12, whereas IL-3, IL-5 and GM-CSF belong to the subfamily of β_C cytokine receptors. In addition, type II cytokines such as IFN-α, -β, -γ and IL-10 share a similar receptor structure (Leonard and O'Shea, 1998). The activation of cytokine receptor signaling is initiated by cytokine-binding, which induces dimerization or oligomerization of the receptor. Cytokine binding to its receptor is known to activate receptor-associated JAKs such as JAK1-3 and TYK2 by tyrosine transphosphorylation. Activated JAKs phosphorylate intracellular parts of the receptors and recruit STATs (signal transducers and activators of transcription) to the receptors. STATs (1-6) bind to phosphorylated receptor tails with their SH2 domains. Tyrosine residues in these receptor-associated STATs are further phosphorylated by JAKs, leading to dimerization of activated STATs. Finally, dimerized STATs are known to enter the nucleus and activate the transcription of specific target genes (Ihle, 2001; Levy and Darnell, 2002). Cytokine receptor signaling has also been shown to be regulated by SOCS (supressor of cytokine signaling) family proteins SOCS-1, -3 and CIS-1. It has been reported that SOCS inhibit cytokine receptor signaling using different mechanisms. SOCS-1 and SOCS-3 bind directly to the kinase domain of JAK, and inhibit its kinase activity and cytokine signaling. By contrast, CIS-1 is known to bind to the intracellular tail of the cytokine receptors, thus inhibiting the recruitment and activation of STAT5. In addition to the SOCS family of inhibitors, it has been reported that cytokine receptor signaling is regulated by common cellular phosphatases such as SHP-1 and SHIP-1 which associate with CD22 and FcγRIIB (Yasukawa et al., 2000). Additionally, as mentioned above CD45 has also been connected to the regulation of cytokine receptor signaling (Irie-Sasaki et al., 2001; Yamada et al., 2002; Zhou et al., 2003). Cytokine signaling has also been reported to be controlled in the nucleus by different mechanisms. The binding of STAT dimers to DNA and activation of transcription has been shown to be blocked by nuclear phosphatases dephosphorylating

the STAT dimers, or by PIAS (proteins that inhibit activated STATs), or by the short transcriptionally inactive forms of STATs (Levy and Darnell, 2002).

2.4.2. IFN-γ

Activated $CD4^+$ T_H cells are thought to be the major source of the cytokines that direct B cell functions. However, B cells are also known to produce cytokines. In the mouse model, it has been reported that B cells could develop into effector B cells producing polarized cytokines such as IFN-γ and IL-4, which can then regulate T cell responses (Harris et al., 2000; Harris et al., 2005). In humans, it has been reported that normal B cells produce several cytokines such as IL-6 and TNF-α (Boussiotis et al., 1994; Pistoia, 1997; Rieckmann et al., 1997). It has also been reported that human B cells produce IFN-γ in the presence of SAC and IL-12, but effector B cell development or function, as in the mouse, has not been described (Airoldi et al., 2000; Li et al., 1996).

IFN-γ is one of the most important cytokines in T_H1 type responses. In T_H1 cells, the production of IFN-γ is regulated by TCR-induced transcription factors NF-κB and AP-1. In addition, IFN-γ production is regulated by IL-12 and IFN-γ via activation of STAT4 and STAT1, respectively (Barbulescu et al., 1998; Harris et al., 2005; Sica et al., 1997). These transcription factors are known to have binding sites in the promoter and intron regions of the IFN-γ gene (Barbulescu et al., 1998; Sica et al., 1997; Xu et al., 1996). In T_H1 cells, IFN-γ expression is enhanced by STAT1-induced T-bet (T-box family of transcription factors) (Harris et al., 2005; Szabo et al., 2000; Szabo et al., 2002). It was recently reported that the production of IFN-γ in mouse B cells was regulated mostly by IFN-γR signaling and T-bet (Harris et al., 2005). By contrast, human B cells have been reported to produce IFN-γ after simultaneous BCR and IL-12 stimulation, but the exact mechanism regulating BCR-induced IFN-γ production is not fully characterized (Airoldi et al., 2000; Li et al., 1996).

2.5. Gene therapy in B cells

The treatment of B cell malignancies in adults could be more efficient (Diehl et al., 2003; Fisher et al., 2004; Hoelzer et al., 2002; Pui et al., 2004), and new therapeutic strategies need to be developed. It is known that adenovirus, Epstein-Barr virus (EBV),

adeno associated virus (AAV) and lentivirus vectors have been successfully used in gene therapy (Bovia et al., 2003; Cantwell et al., 1996; Hellebrand et al., 2006; Von Seggern et al., 2000; Wendtner et al., 2002; White et al., 2002). B cell-specific viral gene therapy, even if it could not completely replace chemotherapy, could play supportive role in reducing residual disease-induced relapses, and increasing the efficacy of B cell cancer therapy.

Adenovirus vectors are one of the most interesting tools in cancer therapy, because of the many advantageous features of this vector type, such as high titer recombinant virus production, capability to transduce postmitotic cells, large DNA packaging capacity and broad target cell tropism (Kovesdi et al., 1997). In addition, adenoviral DNA is transiently and extrachromosomally expressed, which is a desired feature in most forms of cancer gene therapy (Kovesdi et al., 1997). However, human B and T cells are known to be resistant to adenoviral gene therapy because they do not express the attachment receptor hCAR (human coxsackie adenovirus receptor) (Rebel et al., 2000). Therefore, the development of new retargeted adenoviral vectors is required for B cell-specific gene therapy experiments, both *in vitro* or *in vivo*. Recently, normal human PB B cells expressing CD46 were successfully transduced with Ad5/F35 chimeric adenovirus vector, in which the classical adenovirus fiber (Ad5) was replaced with the fiber of human B adenovirus serotype 35 (Jung et al., 2005). In addition, adenovirus retargeting towards B cells could be achieved by a fusion protein containing an antibody that recognizes specific molecules on the B cell surface, and adenovirus capsid-targeted protein, as described previously (Hakkarainen et al., 2003). It has also been suggested that the use of strictly B cell-specific promoter-enhancer combination (VH promoter-3' enhancer) (Pettersson et al., 1990), might increase the cell type-specificity and decrease severe side-effects, such as by-stander cell death or tissue destruction.

3. AIMS OF THE STUDY

The aims of the study were

1. To investigate the production of cytokines and antibodies from B cells in BCR stimulation-induced response in the absence of T cell help
2. To investigate the cooperation of B cells and macrophages in the early phases of inflammatory response
3. To establish B cell lines expressing distinct CD45 isoforms and describe the role of various CD45 isoforms in BCR and cytokine signaling.
4. To study BCR-induced apoptosis and establish a multiparametric apoptosis detection system for flow cytometry
5. To construct and use a novel adenovirus receptor construct in experimental B cell cancer gene therapy

4. MATERIALS AND METHODS

4.1. B cell purification and B cell lines HF28 and HF-1 (I-IV)

PBMCs were purified from freshly collected blood of healthy donors (Blood Transfusion Service of the Finnish Red Cross, Kuopio, Finland) by centrifugation with Lymphoprep™ (Nycomed Pharma AS, Norway). B cells were separated by using CD19 (pan-B) Dynabeads M-450 (Dynal AS, Norway) and the purity was analyzed with flow cytometry (see below).

Human follicular lymphoma (FL) B cell lines HF28 and HF-1 were established as described earlier (Eray et al., 1994; Eray et al., 2003; Knuutila et al., 1994).

4.2. FACS analyses (I-IV)

In study I, B cell purity (98%) was analyzed with a Coulter EPICS Elite (Beckman Coulter, USA) flow cytometer (FACS, fluorescence activated cell sorter). Cells were stained with 0.5 μg of the following anti-human monoclonal antibodies (mAb) in various combinations: CD3 (FITC), CD19 (PE) and CD45 (PC5) (BD Biosciences, USA).

In study II, HF28RA and R0 cells were cloned from original HF28 cell line cells after staining of cells with anti-human CD45RA FITC and CD45R0 APC mAb (BD Biosciences, USA) and using an EPICS Elite ESP flow cytometer equipped with an Autoclone unit (Beckman Coulter, USA).

In study III, the detection of apoptotic cell subpopulations was performed by staining the cells simultaneously with Annexin V-FITC (Annexin V), Propidium Iodide (PI), and SYTO 17 and further analyzing the samples with an EPICS Elite ESP flow cytometer (Beckman Coulter, USA). For each staining, a total number of 1.5×10^5 cells from each sample were washed once with ice-cold 10 mM HEPES buffer and further stained by combining SYTO 17 (Molecular Probes, USA) with the Annexin V-FITC apoptosis detection kit (Genzyme, USA). The staining solution was prepared as follows. Annexin V-FITC (0.5 mg/ml) and PI (5 mg/ml) were added to the binding buffer supplied by the manufacturer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8

mM $CaCl_2$). SYTO 17, a 633-nm excitable short stokes shift dye, was diluted to a final concentration of 50 nM in the binding buffer, which already contained both Annexin V-FITC and PI. The cells were resuspended in 100 µl of the staining solution and stained for 20 min at RT, in the dark. Finally, the cells were diluted in 10 µM HEPES buffer to a final volume of 600 µl, and immediately analyzed by flow cytometry.

In study IV, expression of the fluorescent proteins hCAR-EGFP, DsRed2 (red fluorescent protein from Discosoma sp.) and TK-GFP (herpes simplex virus type I thymidine kinase and green fluorescent protein) was detected by using FACScan and FACSCalibur (BD Biosciences, USA) flow cytometers. Cells expressing hCAR-EGFP fusion gene were further enriched and purified by cell sorting using an EPICS Elite ESP flow cytometer (Beckman Coulter, USA). The cell surface expression of hCAR was detected with hCAR specific anti-hCAR mAb (RmcB, mouse anti-human mAb) (Hsu et al., 1988) and Tri-Color conjugated goat anti-mouse Ab (Caltag Laboratories, USA).

4.3. Production of culture supernatants from stimulated macrophages (I)

Macrophage culture supernatants (Mϕ-SN) were obtained by stimulating the human Mϕ cell line, CRL-9855 (ATCC, USA), with IFN-γ (200 U/ml) (BD Biosciences, USA) for 24 h. The IFN-γ was washed away and the cells were further stimulated with E. Coli (Serotype 0111:B4) (Sigma, Germany) LPS (1 µg/ml) for 24 h. The supernatants were collected and used in B cell stimulations.

4.4. B cell cultures (I-IV)

In studies I-IV, primary human PB B cells or FL cell line cells were cultured in RPMI-1640 (GibcoBRL Life Technologies Ltd., Scotland) containing FCS (5%), Hepes (10 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1.0 mM) (GibcoBRL), 2-mercaptoethanol (20 µM) (Fluka Chemie, Switzerland), streptomycin (200 µg/ml) (Sigma, Germany), penicillin (240 IU/ml) (Orion, Finland) on 24-well culture plates (10^6 cells/ml) (Corning Inc., USA) at 37 °C in a humidified 5% CO_2 atmosphere.

In study I, human PB B cells were stimulated by BCR triggering, Mϕ-SN (30 % final volume) or a combination of recombinant cytokines IL-12 (10 ng/ml) (BD Biosciences,

USA) and IL-18 (10 ng/ml) (R&D Systems Inc., USA) or a combination of all these stimuli. In studies I-IV, the BCRs were triggered by incubating cells with (5 μg/ml) anti-human kappa light chain constant region (κ^+) mAb (Seppala et al., 1984) for 30 min on ice. The cells were washed and BCRs were further cross-linked with (1.8 μg/ml) human absorbed rabbit anti-mouse Ab (DAKO A/S, Denmark). The supernatants were collected, B cells were washed, pelleted and stored at -80°C for mRNA isolation.

In study II, HF28RA and R0 cell lines were stimulated with cytokines (at a concentration of 10 ng/ml): IL-2 (CLB, the Netherlands); IL-4, IL-6, IL-10, IL-12, IL-15, and IFN-γ (BD Pharmingen, USA); IL-13 (Immunogenex, USA); and TNF-α (Genzyme, USA). In addition, these cell lines were stimulated with anti-human CD45 mAb (panCD45) (CALTAG Laboratories, USA) using a concentration of 1 μg/ml.

In both the proliferation and Western blot studies of HF28RA and R0 cells, a specific inhibitor of the PI3K pathway LY294002 (LY) (Calbiochem, USA) was used at a concentration of 10 μM. A specific inhibitor of the ERK pathway, PD98059 (PD) (Calbiochem, USA) was used in the proliferation assays at a concentration of 15 μM.

In study III, HF-1 cells were induced to apoptosis with BCR triggering by incubating the cells with (5 μg/ml) anti-human kappa light chain constant region (κ^+) mAb (Seppala et al., 1984) at a final concentration of 5 μg/ml, or with 0.2 mM of Ca^{2+} Ionophore A23187 (Sigma Immunochemicals, USA), or with 30 ng/ml CD40 mAb (Immunotech, France), or with the hybridoma culture supernatant containing mAb against class II HLA-DR molecule at a final dilution of 1:40 (a kind gift from A. Ziegler to L.C. Andersson). The selected Ab concentrations of CD40 and HLA-DR gave comparable homotypic aggregation of HF-1 cells.

4.5. mRNA isolation, cDNA synthesis and PCR analyses (I-II and IV)

In studies I-II and IV, total cellular mRNA was isolated from 10^6 cells from each sample using MasterPure™ Kit (Epicentre, USA). DNase I and Proteinase K treatment was included in the total mRNA isolation procedures. cDNA was synthesized according to standard protocols as previously described (Eray et al., 2003), and stored at -20°C.

In study I, the expression of cytokine-specific mRNAs was analyzed from cDNA samples prepared from 4 h B cell cultures. Nested PCR amplifications were performed using UNO-Thermoblock (Biometra GmbH, Germany). First, cDNA samples (0.4 µl / reaction) were amplified for 15 cycles with the first cytokine-specific primer pair (a sense primer (S) and an external anti-sense primer (AS(2)) using the following cycling parameters: +94°C for 3 min, and amplification of 30 s at +94°C, 30 s at +57°C and 1 min at +72°C, program ended with 10 min at +72°C. Then 5 µl of this amplification product was carefully transferred into another tube, and PCR amplification was carried out for 35 cycles with the second cytokine-specific primer pair (the same sense primer and an internal anti-sense primer (AS)). For TNF-α RT-PCR the primers used in the first round were S(1) and AS, and in the second round S(2) and AS. Specific RT-PCR products were separated with 1.3% agarose gel and analyzed. The β2-microglobulin (β2µG) (S and AS), IL-4 (S and AS), IL-6 (S and AS) and IFN-γ (S and AS) primers used have been described previously (Bouaboula et al., 1992). The sequences for the other primers listed (IL-4 AS(2), IL-6 AS(2), IL-10 S, IL-10 AS, IL-10 AS(2), IL-13 S, IL-13 AS, IL-13 AS(2), IL-15 S, IL-15 AS, IL-15 AS(2), IFN-γ AS(2), TNF-α S, TNF-α AS TNF-α AS(2)), were as published earlier (Eray et al., 2003).

In study I, cDNA samples for secretory-IgG mRNA from human PB B cells were amplified for 35 cycles with the same cycling parameters as described above. The following primers were used for the analysis of secretory-IgG mRNA: IgG-C3 5'-GAG GTG CAT AAT GCC AAG AC-3'; IgG-Se 3'-GCT GTC GCA CTC ATT TAC CC-5'.

In studies II and IV, the expression of CD45- and TK-specific mRNAs was detected after 35 cycles with RT-PCR using the same cycling parameters as described above. The primers used have been described previously (Kanegane et al., 1991; Palu et al., 1999).

In study I, the expression of specific IFN-γ, β2µG and T-bet mRNA in HF28R0 cell line was analyzed with quantitative RT-PCR using a QuantiTect SYBR Green PCR kit (Qiagen GmbH, Germany) according to the manufacturer's instructions, and a Rotor-Gene 3000 PCR cycler (Corbett Research, Australia). IFN-γ and β2µG quantitation using the two standard curve method was performed according to instructions published

earlier (Schwarz et al., 2002). T-bet mRNA expression was analyzed using the 'Comparative Quantitation' method described earlier (Warton et al., 2004), and supplied by the Corbett Research for the Rotor-Gene. IFN-γ, β2μG and T-bet cDNA samples (0.5 μl / reaction), IFN-γ and β2μG standards were amplified for 35 cycles using the following cycling parameters: +95°C for 15 min, amplification of 30 s at +95°C, 30 s at +60°C and 1 min at +72°C, program ended with Melt Curve analysis in which the temperature was increased stepwise from +45°C to +99°C within 5 min. The specific primers used for the detection of IFN-γ, β2μG and T-bet mRNAs have been described earlier (Bouaboula et al., 1992; Durali et al., 2003; Kitani and Strober, 1993).

4.6. IgG detection with ELISA and quantitation of secretory-IgG (I)

ELISA plates (Nunc, Denmark) were coated with (38 ng/ml) rabbit anti-human IgG (DAKO A/S, Denmark) for 24 h at +4°C. Pooled healthy human blood donor serum from the Blood Transfusion Service of the Finnish Red Cross (Kuopio, Finland) was used as a standard. The amount of IgG in standards was between 0.1-108 ng/ml. After blocking, standards, controls and 1:20 diluted samples were added to the wells and IgG was detected with (1.3 μg/ml) rabbit anti-human IgG HRP Ab (DAKO A/S, Denmark). Substrate solution was added and the reaction was stopped with 50 μl of H_2SO_4 (2 M) (Fluka Chemie, Switzerland). The absorbance was measured at 450 nm with Multiskan PLUS ELISA reader (ThermoLabsystems, Finland).

Detection of secretory-IgG and β2μG RT-PCR products was designed to detect the secretory region in the Cγ3 exon 4. RT-PCR products were separated by agarose gel electrophoresis, transferred to a PVDF-membrane and cross-linked with UV light (Stratagene, USA). After blocking, the filters were hybridized with digoxigenin-labeled oligonucleotides (either IgG-Se-specific: 5'-GCA TCA CGG AGC ATG AGA AGA TGT-3' or β2μG-specific: 5'-ATG TCT CGA TCC CAC TTA ACT ATC TT-3'), followed by incubation with anti-DIG-HRP mAb (1/10,000 dilution) (Jackson ImmunoResearch Laboratories Inc., USA). ECL Plus Reagent (ECL, enhanced chemiluminescence) (Amersham Biosciences, Sweden) was added and chemiluminescence was detected. The hybridization signal was analyzed from

digitalized Hyperfilm MP X-ray films (Amersham Biosciences, Sweden) using ImageQuant software (Molecular Dynamics Inc., USA), and the ratio of IgG-Se and β2μG signals was calculated.

4.7. Production of B cell-derived culture supernatants and Mϕ stimulation (I)

B cell-derived culture medium was produced by stimulating HF28R0 cells for 2 h in the presence of IL-10 (10 ng/ml) and anti-human κ^+ mAb (5 μg/ml). After 2 h the cells were washed and further incubated in fresh RPMI-1640 medium until 24 h of total incubation time (stimulated B cell culture supernatant). B cell-derived control supernatant was produced by culturing the cells in RPMI-1640 medium for 24 h (control B cell culture supernatant). B cell-derived supernatants were collected and used in Mϕ stimulation.

To study the cooperation between B cells and Mϕs, cells of human Mϕ cell line, CRL-9855, were primed for 24 h either with RPMI-1640 or in the presence of IFN-γ (200 U/ml) (BD Biosciences, USA) or B cell-derived control medium or culture supernatant from IL-10 and BCR stimulated B cells. Priming agents were washed away and the cells were further stimulated for 24 h in the presence of LPS (1 μg/ml) from E. Coli (Serotype 0111:B4) (Sigma, Germany). The supernatants (Mϕ-SN) were collected from the Mϕ culture and the production of TNF-α and IL-6 was analyzed with the ELISA as described earlier (Huttunen et al., 2003; Penttinen et al., 2005).

4.8. Transcription factor analysis with electrophoretic mobility shift assay (I)

Nuclear proteins were extracted using the modified protocol (Huttunen et al., 2004) of the one described by Dignam et al. (Dignam et al., 1983). The nuclear protein concentrations were analyzed with Lowry's method (DC Protein Assay, BioRad Laboratories, USA).

Consensus double-stranded oligonucleotides containing the binding sites for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Santa Cruz Biotechnology, USA) and AP-1 (5'-CGC TTG ATG ACT CAG CCG GAA-3'; Santa Cruz Biotechnology, USA) were labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech Benelux, The Netherlands) using T4-polynucleotide kinase (MBI Fermentas, USA). Labeled

probes were separated using Probe Quant™G-50 micro columns (Amersham Pharmacia Biotech Benelux, the Netherlands) before EMSA (electrophoretic mobility shift assay) analyses.

Nuclear proteins (3 µg in each reaction) were incubated for 20 min with [γ-^{32}P]ATP labeled probes in binding buffer (10% glycerol, 1 mM DTT, 1 mM EDTA, 25 mM HEPES, 100 mM NaCl) and 1.5 µg poly(dI-dC) (Amersham Biosciences, Sweden) in a final reaction volume of 20 µl. The protein-DNA complexes were separated (25 mA, 90-120 min) on a high ionic strength gel (6% acrylamide) in running buffer (50 mM Tris, 380 mM glycine, 1 mM EDTA). After electrophoresis, the gel was dried (1h, +60°C) and exposed to autoradiography film for 1-2 days at -80°C.

4.9. BCR signaling, immunoprecipitation and Western blot analysis (II)

Anti-human κ^+ mAb (Sarnesto et al., 1983) was used for BCR stimulation at a concentration of 5 µg/ml. In BCR signaling experiments, 2 x 10^6 cells were used per sample. Cells were suspended in 1 ml of culture media. In all experiments, 2 x 10^6 untreated cells were used as controls. Termination of signaling was attained by centrifuging the samples at 15 000 x g for 5 s at +4°C and removing culture media and placing samples on ice. Immediately after this, the samples were either transferred to -80°C for storage, or 50 µl of ice-cold lysis buffer (20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 3% NP-40, 100 mM NaCl, 0.1 mM sodium orthovanadate, aprotinin 10 µg/ml, leupeptin 10 µg/ml, 1 mM phenylmethylsulfonyl fluoride (PMSF)) was added. Samples were incubated on ice with the lysis buffer for 30 min and centrifuged at 15 000 x g for 10 min at +4°C. The supernatant was removed and the protein content was determined using the Lowry method (Bio-Rad Laboratories, USA). The samples were diluted in an equal volumes of SDS-buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glyserol, 5% 2-mercaptoethanol, 0.5 mM orthovanadate) which had been boiled for 3 min. The lysis procedure was completed with a 2 min additional boiling.

For phosphothreonine/-serine immunoprecipitation lysates, 8 x 10^6 cells (lyzed with 500 µl of lysis buffer) were first incubated for 1 h with 2 µl of rabbit anti-phosphothreonine (Zymed Laboratories, USA) and rabbit anti-phosphoserine (Zymed

Laboratories) Ab. Then 30 µl of GammaBind® G Sepharose® (Amersham Biosciences, Sweden) was added and the samples were incubated on a rocking platform for 1 h 30 min at +4°C. The samples were centrifuged for 20 s at +4°C and the supernatant was removed. The precipitates were then washed three times with 0.1% lysis buffer and once with PBS. Finally, the precipitates were suspended in 50 µl of SDS-buffer and boiled for 3 min.

In 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 20 µl of cell lysate or immunoprecipitate/lane was used. Prestained SDS-PAGE Standard (Bio-Rad Laboratories) was used as a molecular weight marker. From the gel, proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were blocked in an orbital shaker over night in PBS containing 3% BSA and 0.1% Tween-20. The next day, incubation for 2 h with the primary Ab (listed below) was followed with 2 h incubation with the HRP-conjugated secondary Ab. The detection was done using the ECL reagents (Amersham Biosciences) and autoradiography film (Amersham Biosciences).

In Western blot studies, the primary mAb and the used dilutions were as follows: phospho-Tyrosine (PY99) mAb (1/6000), phospho-JNK mAb (1/2000), phospho-Akt 1 (Ser 473) rabbit polyclonal Ab (1/500), PKC rabbit polyclonal Ab (1/3000) (Santa Cruz Biotechnology, USA), phospho-p38 (1/1000) rabbit polyclonal Ab, phospho-p42/p44 (p-ERK1/2) MAP kinase mAb (1/2000) (Cell Signaling Technology, USA). HRP-conjugated secondary Ab, goat anti-mouse and goat anti-rabbit (Zymed Laboratories, USA) were diluted at 1/20000 before use.

4.10. Proliferation assays (II and III)

The proliferation of cells was studied by (^{3}H) thymidine incorporation. Thymidine incorporation was carried out using 6 x 10^4 cells/well in 200 µl of medium containing appropriate stimuli (triplicate wells/stimuli). After 20 h of incubation, 3 µCi of (^{3}H) thymidine (specific activity 2.0 Ci/mmol) (Amersham Biosciences, Sweden), was added to each well for 4 h. Cells were harvested and the radioactivity was detected by beta scintillation counting using a Microbeta® counter (Wallac, Finland).

4.11. Cloning methods (IV)

Truncated hCAR (ΔhCAR) without the cytoplasmic domain was amplified from a plasmid, pBluescriptSK(+)-hCAR(1-262), by PCR with primers containing BamHI and Sal I overhangs. The primers for hCAR PCR were hCAR sense (S) 5'-AAA GGA TCC GGC AGC CAC CAT GGC GCT CC-3' and hCAR anti-sense (AS) 5'-AAA GTC GAC CTT TTT ACG ACA GCA AAA GAT G-3'. The EGFP lacking the ATG start codon (ΔEGFP) was amplified by PCR from a plasmid, pEGFP-C3, using primers containing SalI and HindIII overhangs. The primers for EGFP PCR were EGFP (S) 5'-AAA GTC GAC GTG AGC AAG GGC GAG GAG CTG TT-3' and EGFP (AS) 5'-AAA AAG CTT CTA CTT GTA CAG CTC GTC CAT GCC G-3'. ΔhCAR was further subcloned into a BamHI-Sal I-digested pGEM3Z -plasmid, and fusion gene hCAR-EGFP was created by subcloning ΔEGFP into a SalI-HindIII-digested hCAR-pGEM3Z. The plasmid containing hCAR-EGFP was subcloned into the BamHI-HindIII-digested pSCT1, derived from the eukaryotic expression vector pSTC (Wieland et al., 1991). The hCAR-EGFP was further cloned into the BamHI-KpnI-digested pHRCMV plasmid (Naldini et al., 1996). Finally, the lentiviral vector containing the fusion gene hCAR-EGFP was produced following the protocol described earlier (Pellinen et al., 2004). The titer of the hCAR-EGFP lentivirus produced was 5 x 10^6 t.u. (transducing units)/ml as measured by FACS analysis (Pellinen et al., 2004).

First-generation E1/E3-deleted serotype 5 AdDsRed2 vector (adenovirus vector containing red fluorescent protein DsRed2) was created by inserting a BglII-NotI-digested DsRed2 fragment from pDsRed2-N1 (BD Biosciences Clontech, USA) into pShuttleCMV (AdEasy-kit^{TM}, Qbiogene, France) to obtain pShuttleDsRed2. PmeI-linearized pShuttleDsRed2 and circular pAdEasy-1 were cotransfected into *E. coli* strain BJ5183 cells followed by homologous recombination to obtain pAdDsRed2 (Adenoviral plasmid). PacI-linearized pAdDsRed2 was transfected into 293 cells, CRL-1573 (ATCC, USA) with Superfect (Qiagen, Germany) following the manufacturer's instructions. Adenovirus colonies were picked and the virus was propagated in 293 cells and purified using standard techniques. Viral particles (VP) were determined with spectrophotometry and plaque-forming units (pfu) with plaque assay. AdTK-GFP vector

(adenovirus vector containing TK-GFP fusion gene) was constructed as described earlier (Hakkarainen et al., 2005). The titers of the viruses used were: AdDsRed2, 5.0 x 10^{11} VP/ml; 3.28 x 10^{10} pfu/ml, and AdTK-GFP, 4.2 x 10^{11} VP/ml; 2.2 x 10^{10} pfu/ml.

4.12. Transductions with lenti- and adenovirus vectors (IV)

The HF28RA cells were transduced with hCAR-EGFP lentivirus in a 6-well plates using 100,000 cells/ml/well, in the presence of polybrene 8 µg/ml (Sigma-Aldrich, Germany) and with 1.5 t.u./cell. Adenoviral transductions with AdDsRed2 or AdTK-GFP vector (both with 1, 10 and 100 pfu/cell), were done according to the following protocol: 1 x 10^6 cells were added onto a 24-well plate in 300 µl of serum free growth medium containing adenovirus and incubated at 37°C for 3 h. After transduction, 1 ml of complete growth medium was added to the wells and incubation was continued at 37°C for 24-48 h before the analysis of DsRed2 or TK-GFP expression.

4.13. Cell viability testing with MTT-assay (IV)

Cell viability was analyzed with MTT-assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid) according to the manufacturer's instructions (Cell proliferation Kit II, Roche Diagnostics, USA). Briefly, the cells were plated onto a 96-well plate at the density of 40,000 cells/well followed by transduction with AdTK-GFP for 6 h at 37°C using 1, 10 and 100 pfu/cell. After transduction, fresh growth media was added. Ganciclovir (GCV) containing media was added 24 h post- transduction and cells were grown in the presence of GCV (10 µg/ml) for 5 days.

4.14. May-Grünwald-Giemsa staining (III)

Cellular responses were also measured after treatments with morphological examination. In May-Grünwald-Giemsa (MGG) staining, cytospin-centrifuged samples were air-dried, fixed with methanol for 5 min., stained and visualized with a microscope.

5. RESULTS AND DISCUSSION

5.1. Cooperation of B cells and Mϕ in TI-like microbial inflammatory response (I)

T cell-mediated responses to polysaccharide-encapsulated bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Hemophilus influenzae* can be severely delayed, since either polysaccharides cannot efficiently be processed or they can inhibit the binding of bacterial peptides to MHC class II molecules (Gonzalez-Fernandez et al., 1997; Harding et al., 1991; Leyva-Cobian et al., 1997; Vos et al., 2000). In spite of a defective T cell response, immune responses to polysaccharide-encapsulated bacteria can be elicited by human PB B cells (Rijkers and Mosier, 1985). However, the mechanisms regulating this phenomenon in human B cells are not completely understood. In this study, the function of human PB B cells in a simulated bacterial polysaccharide response in the absence of additional help was analyzed *in vitro*. Multivalent bacterial polysaccharide stimulation of B cells was mimicked by cross-linking BCRs with anti-Ig Ab. Using this approach, a large fraction of B cells (κ^+ B cells) could be specifically stimulated. This approach has a marked advantage over the other commonly used stimuli such as SAC or DEAE, which may directly stimulate TLRs or other receptors, inducing a second stimulatory signal in B cells. In the first phase of studies, concerning B cell activation and the production of Ab and cytokines after simulated bacterial polysaccharide challenge were studied. Furthermore, the role of a separate second signal and cooperation of human PB B cells with human Mϕs was analyzed.

5.1.1. Production of IgG antibodies from human PB B cells

Initially, it was analyzed whether a second soluble signal, in addition to BCR stimulation, is required for IgG production in human PB B cells. As the second signal, Mϕ-derived culture supernatant (Mϕ-SN) containing unknown growth factors or IL-12 + IL-18, typical human Mϕ-derived cytokines, were used (Miettinen et al., 1998; Wittmann et al., 1999). In the presence of anti-Ig and Mϕ-SN for 3 days, B cells produced 4.3 times more IgG than unstimulated control cells. In contrast, in the

presence of IL-12 + IL-18 alone or together with anti-Ig stimulation, IgG secretion increased only 2.4- and 2.6-fold as compared with unstimulated B cells, respectively. These results show that Mϕ-derived soluble support was important for IgG production from B cells. Furthermore, Mϕ-SN contained other important cytokines or soluble mediators than simply IL-12 and IL-18 since these two cytokines had clearly a weaker stimulatory effects on B cell IgG production than Mϕ-SN.

A novel approach was to measure the secretion of IgG indirectly by analyzing the expression of mRNA coding for the secretory form of IgG, IgG-Se mRNA. These results also showed that BCR stimulation-induced IgG-Se expression was more efficiently enhanced by Mϕ-SN than by IL-12 + IL-18 (2.5 and 1.2 times more than unstimulated control cells, respectively). This observation was in good agreement with IgG production.

5.1.2. Induction of IFN-γ mRNA expression in human B cells

The expression of B cell-derived cytokines was also analyzed, because these secreted cytokines might also have a regulatory role in the inflammatory response induced by polysaccharide-encapsulated bacteria. Human PB B cells expressed constitutively IL-6, IL-10, IL-15 and TNF-α mRNAs. Furthermore, the effect of BCR stimulation on the production of cytokines was studied. BCR stimulation alone enhanced the production of IFN-γ (in some individuals) and decreased the production of IL-6 (Mättö M. et al., unpublished observation). In contrast, it has been shown that human tonsillar B cells were not able to produce IFN-γ after SAC or BCR stimulation alone (Airoldi et al., 2000; Li et al., 1996). These differences could be due to the fact that BCR cross-linking is sufficient to induce the production of IFN-γ in PB B cells but not in tonsillar B cells, which appear to require another stimulatory signal. It is also possible that SAC binds both to BCR and TLR (Bourke et al., 2003; Silverman and Goodyear, 2002; Zarember and Godowski, 2002). It could be speculated that the TLR component of the SAC response could inhibit the production of IFN-γ induced by BCR stimulation.

IFN-γ is an important cytokine in the regulation of anti-microbial defense systems, so the effect of Mϕ-SN or IL-12 + IL-18 on BCR stimulation-induced IFN-γ production

was studied further. It was shown that BCR or Mϕ-SN stimulation alone or combined were not able to induce detectable expression of IFN-γ mRNA. By contrast, IL-12 + IL-18 alone or together with BCR stimulation induced significantly higher expression of IFN-γ mRNA as compared to unstimulated control cells. These results confirm our previous conclusions that Mϕ supernatants also contain other soluble factors apart from IL-12 and IL-18, and that induction of IgG and IFN-γ production requires distinct soluble factors.

To further study the regulation of IFN-γ production in human B cells, a cell line model was established. The effect of typical Mϕ-derived cytokines (Miettinen et al., 1998; Wittmann et al., 1999), such as IL-12, IL-18 and IL-10, on IFN-γ mRNA production was analyzed in human FL B cell line HF28R0. IL-10 stimulation significantly increased the expression of IFN-γ mRNA as compared to unstimulated control (~13-fold increase) or BCR stimulated cells (~20-fold increase).

Transcription factors regulating IFN-γ mRNA expression in the human B cell line model were studied. In mice, T-bet is known to be essential for IFN-γ production in T and B cells (Harris et al., 2005; Szabo et al., 2000; Szabo et al., 2002). T-bet has also been shown to be constitutively expressed in human PB B cells (Durali et al., 2003), so its role was studied in HF28R0 cells. T-bet mRNA expression was increased 1.6-fold after IL-10 stimulation compared with unstimulated control cells (Fig. 2, Mättö et al., unpublished observation). These results show that there is a positive correlation between T-bet and IFN-γ expression in various stimulations although it did not reach statistical significance. It seems that in human B cells the regulation of IFN-γ production requires other factors than T-bet but T-bet is likely to play an important role.

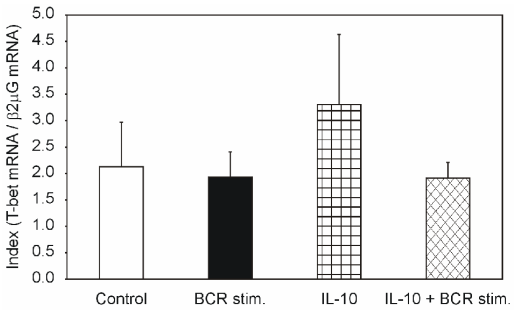

Figure 2. T-bet mRNA expression in human B cells. Specific T-bet mRNA expression in HF28R0 cells was analyzed using SYBR Green based quantitative RT-PCR. HF28R0 cells were cultured for 4 h in RPMI-1640 medium (control), or with BCR stimulation (BCR stim.), or in the presence of IL-10 (10 ng/ml) alone (IL-10) or together with BCR stimulation (IL-10 + BCR stim.). Quantitation results of T-bet were normalized with mRNA expression of the house keeping gene β2μG. Data are shown as a mean ± SEM of three independent experiments using three replicates in the quantitative PCR analysis.

The transcription factors NF-κB and AP-1 have been shown to participate in the regulation of IFN-γ mRNA expression in T cells (Barbulescu et al., 1998; Sica et al., 1997). The role of NF-κB and AP-1 transcription factors in the regulation of IFN-γ mRNA expression in B cells was analyzed with EMSA method (Fig. 3, Mättö et al., unpublished observation). Interestingly, the binding of NF-κB to a consensus NF-κB binding site correlated with IFN-γ expression in such a way that IL-10 induced the highest binding which was down-regulated by BCR stimulation. In contrast, both IL-10 and BCR stimulation induced a strong synergistic binding of AP-1 to its consensus sequence. In T cells, the IFN-γ promoter and intron regions have been reported to bind transcription factors NF-κB, AP-1 and STATs (Barbulescu et al., 1998; Sica et al., 1997; Xu et al., 1996); however, in B cells these regions do not seem to contain any functional promoter or enhancer elements (Mättö et al., unpublished observation). This finding may be due to the fact that in B cells the distal part of the IFN-γ locus is involved in the regulation of IFN-γ gene expression (Lee et al., 2004; Shnyreva et al., 2004). Furthermore, it has been reported that in T cells the expression of T_H2 cytokines IL-4 and IL-13 is regulated as a single transcriptional locus (Agarwal and Rao, 1998; Li-Weber and Krammer, 2003; Takemoto et al., 2000). Therefore, the expression of IFN-γ- clustered genes IL-26 and IL-22 was analyzed. The expression of IL-26 and IL-22 mRNA was not up-regulated after IL-10 stimulation, suggesting that IFN-γ mRNA

expression is not regulated as a single transcriptional locus (Mättö et al., unpublished observation, data not shown).

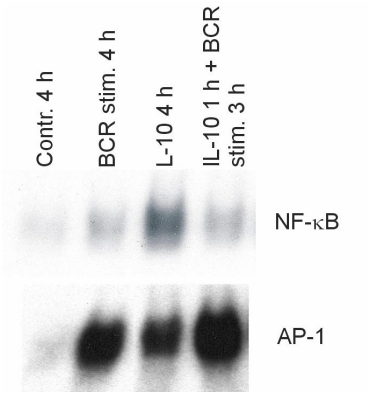

Figure 3. Induction of NF-κB and AP-1 binding after B cell stimulations. Formation of NF-kB and AP-1 complexes was analyzed with EMSA method. HF28R0 cells were cultured for a total of 4 h in various stimuli; RPMI-1640 medium (Contr. 4 h), or with BCR stimulation (BCR stim. 4 h), or in the presence of IL-10 (10 ng/ml) alone (IL-10 4 h) or together with BCR stimulation (IL-10 1 h + BCR stim. 3 h).

5.1.3. Mϕ cytokine production is enhanced by B cell-derived growth factors

The ability of human B cells to activate human Mϕ inflammatory cytokine production was also analyzed. The cytokine production from human Mϕ cell line cells was studied after the cells were primed with different soluble mediators for 24 h, followed by 24 h stimulation with LPS. It was shown that B cell-derived soluble support was able to induce TNF-α and IL-6 production from a human Mϕ cell line. The production of TNF-α was significantly higher after induction with stimulated B cell culture supernatant than after control B cell culture supernatant (~4-fold). The production of IL-6 was also markedly induced by B cell culture supernatants, but this activity was not dependent on B cell activation substance. As a whole, the human Mϕ cell line, CRL-9855, used in these experiments was a good model cell system for normal Mϕs, since they produced IL-6 and TNF-α, and also expressed IL-15 specific mRNA after LPS (+ IFN-γ) stimulation (Eissner et al., 2000) (IL-15 data not shown). Classically, T cell-derived

IFN-γ has been known to be an important activator of Mϕ in bacterial responses. The novel finding in this study, however, was that B cell-derived culture supernatant from activated B cells was more potent in inducing Mϕ TNF-α and IL-6 production than IFN-γ. In addition, the production of nitric oxide (NO) from the Mϕ cell line used was also detected, indicating an increase in antimicrobial activity (MacMicking et al., 1997). However, NO production was not regulated by B cell-derived cytokines or classical activators IFN-γ or LPS (data not shown).

In conclusion, our data suggests that B cells are important players in close cooperation with Mϕ in the initiation and regulation of inflammatory response when T cell help is not available. Overall, this study shows that human B cells are active cytokine producers which could effectively modify host immune responses, as has been shown to occur in mouse B cells (Harris et al., 2000; Harris et al., 2005).

5.2. CD45 isoforms on B cells (II)

Human B cells in PB or tonsils express mostly CD45RA isoform (Jensen et al., 1989; Yu et al., 2000; Yu et al., 2002). In this study we were able to establish B cell lines expressing either the CD45R0 or CD45RA isoform. Furthermore, the function and importance of distinct CD45 isoforms in particular the CD45R0 isoform-specific regulatory mechanisms in BCR signaling are presently not fully characterized.

5.2.1. Human B cell line cloning and expression of CD45 isoforms

As the role and significance of CD45 isoforms, especially CD45R0, for BCR and cytokine signaling in human B cell is not clear, two FL B cell lines expressing either CD45RA (HF28RA) or CD45R0 (HF28R0) were established using flow cytometric cell sorting from the original HF28 cells, which has been shown to express both CD45RA and CD45R0 isoforms on the cell surface (Eray et al., 2003). The established cell lines were interesting, because human PB and tonsillar B cells or malignant B cell lines have been reported to express predominantly the CD45RA isoform (Jensen et al., 1989; Yu et al., 2000; Yu et al., 2002). The CD45R0 isoform was shown to be expressed only in a minor population of normal human PB B cells and plasmacytoma cells (Jensen et al., 1991; Yu et al., 2000).

5.2.2. Association of CD45 isoforms to cytokine production

The expression of cytokines was measured from the established cell lines. In particular, we were interested in studying whether CD45 isoforms have effect on the endogenous expression of IL-4, IL-6, IL-10, IL-13 and IFN-γ mRNA. Constitutive expression of IFN-γ, IL-6 and IL-13 mRNA and low expression levels of IL-10 mRNA were detected in HF28R0 cells. In HF28RA cells, the endogenous expression of all tested cytokine mRNAs, excluding IL-10, was parallel and comparable to that in HF28R0 cells. The results of this study, the constitutive expression of analyzed cytokines in human B cells does not seem to be regulated by distinct CD45 isoforms.

The influence of CD45 isoforms on BCR stimulation-induced cytokine expression was studied. In HF28R0 cells, expression of IL-4 and IL-10 mRNAs were enhanced after BCR stimulation, but expression of IFN-γ, IL-6 and IL-13 mRNA remained at the same level as in unstimulated control cells. By contrast, in HF28RA cells the expression of all detected cytokines was weakly down-regulated after BCR stimulation. The connection of CD45 isoforms and cytokine production has been studied in transgenic (Tg) mice, where T cells expressed either CD45RA or CD45R0 isoforms. However, B cell-specific CD45 expression was not seen in these mice. Simultaneous stimulation of T cells with CD3 and CD28 mAbs induced a higher production of IFN-γ in $CD45RA^{Hi}$ T cells as compared to $CD45R0^{Hi}$ T cells (Dawes et al., 2006). Based on this data, distinct CD45 isoforms may play a role in the regulation of BCR-induced cytokine production, but other factors are clearly needed.

5.2.3. The effect of CD45 isoforms on cellular proliferation and BCR-dependent intracellular signaling

The function of CD45 isoforms in human B cells was further studied, involving further investigation of the basal and BCR stimulation induced cellular proliferation of HF28 cells. The basal proliferation of HF28RA cells was significantly higher (~2-fold; $p<0.0005$) than that of HF28R0 cells. The proliferation of HF28R0 cells was significantly enhanced after BCR stimulation (~1.4-fold; $p<0.0005$). By contrast, the proliferation of HF28RA cells was reduced after BCR stimulation. In these cell lines,

the surface expression of the BCR was at the same level (Mättö M. et al., unpublished observation), so it is evident that there are CD45 isoform-specific mechanisms regulating cellular proliferation. In addition, BCR triggering induced a similar and rapid signaling molecule phosphorylation in both cell lines, supporting the idea of qualitative differences in BCR-mediated signaling. The complete absence of CD45 has been shown to lead to dramatically reduced BCR-induced cell proliferation (Huntington et al., 2006), whereas the result in the present study show that the CD45 isoforms had a weaker effect on BCR-induced proliferation. Therefore, CD45 isoforms seems to play a role in fine-tuning of the basal and BCR-induced cell proliferation.

Since there is evidence that CD45 isoforms regulate cytokine signaling (Li et al., 2005; Yamada et al., 2002), the effect of selected cytokines on the proliferation of HF28RA and R0 cells was studied in more detail. In the presence of IL-2, IL-4, IL-10, IL-12, IL-13 and TNF-α, the proliferation of HF28RA cells was significantly decreased ($p<0.0005$, $p<0.02$ for IL-4). By contrast, the proliferation of HF28R0 cells was significantly increased ($p<0.0005$) after stimulation with the same set of cytokines. The results support an idea that cytokine receptor signaling is regulated in a CD45 isoform-specific way, leading to an altered cellular proliferation. The detected differences in cellular proliferation were not due to differences in the cytokine receptor expression profile (Mättö M. et al., unpublished observation). In human B cells, selective translocation of the IL-6Rα chain with the CD45RB isoform to the same plasma membrane lipid raft was shown to regulate cytokine signaling (Li et al., 2005). It has also been reported that the CD45 receptor (Irie-Sasaki et al., 2001; Zhou et al., 2003) or CD45RA isoform (Yamada et al., 2002) dephosphorylate JAK1 and JAK3 suggesting another possible mechanism to connect CD45 with cytokine signaling and cell proliferation.

Interestingly, when the cells were stimulated via BCRs in the presence of cytokines, the cell proliferation was markedly altered. In HF28RA cells, BCR stimulation reversed IL-2 ($p<0.005$), IL-12 ($p<0.0005$), IL-13 ($p<0.005$), IFN-γ ($p<0.0005$) and TNF-α-induced ($p<0.05$) down-regulation of cell proliferation. By contrast, BCR stimulation of HF28R0 cells down-regulated IL-12 ($p<0.005$), IL-15 ($p<0.005$) and TNF-α-induced ($p<0.0005$) cellular proliferation.

The MAPK-ERK and PI3K signaling pathways are known to regulate cellular proliferation (Fruman et al., 1999; Fruman et al., 2002; Richards et al., 2001). In human B cells, the influence of CD45 isoforms on the MAPK, PI3K or PLCγ signaling pathways or cellular proliferation regulated by these pathways has not been studied. The molecular mechanisms connecting the ERK signaling pathway and CD45 isoforms, may be connected with the phosphorylation status of ERK1/2, since in HF28R0 cells BCR-dependent phosphorylation of ERK1/2 peaked clearly faster than in HF28RA cells. Furthermore, specific inhibition of ERK signaling with PD98059 showed that BCR-dependent proliferation of HF28R0 cells was markedly enhanced, whereas in HF28RA cells BCR-mediated inhibition of proliferation was abolished. The proliferation studies in the presence of a specific PI3K inhibitor, LY294002 suggest that the basal proliferation in these B cell lines is most likely regulated by the PI3K pathway. By contrast, BCR-induced proliferation of HF28R0 cells was not inhibited by the PI3K inhibitor, suggesting that other pathways, such as the ERK, might be responsible for BCR-mediated proliferation. In addition, based on BCR-induced phosphorylation studies, the other MAPK pathway components, JNK and p38, or the PLCγ pathway molecule, PKC, did not participate in the regulation of the cellular events in these cells.

5.3. Apoptotic cell death in B cells (III)

Binding of the specific antigen to BCR induces intracellular signaling cascades that further lead to distinct responses such as proliferation, anergy or cell death by apoptosis (Donjerkovic and Scott, 2000). Induction of apoptosis through BCR is one of the important ways to regulate B cell fate and eliminate self-reactive B cells during development or GC reaction (Billian et al., 1997). Classically, apoptosis has been measured by detecting morphological changes in cells or by biochemical methods to detect DNA degradation. However, these methods have a limited sensitivity and ability to detect apoptotic sub-populations. Multicolor Annexin V-FITC (Annexin V), PI and SYTO 17 staining method for flow cytometric apoptosis detection was established in order to increase the sensitivity to identify early apoptotic cells. In addition, this flow cytometric detection approach enables the detection of the apoptotic process in distinct cell populations.

5.3.1. Flow cytometric detection of apoptosis by multicolor staining

The formation of apoptotic subpopulations in HF-1 cells was analyzed by combining three stains with different staining properties, Annexin V, PI, and SYTO 17. In apoptotic assays, the detection of phosphatidylserine (PS) exposure to outer plasma membrane by Annexin V (Koopman et al., 1994) and plasma membrane integrity by PI staining (Zamai et al., 1996) has been widely used. In this study, the combination of scatter parameters and SYTO 17 staining was used for the first time with Annexin V and PI.

It has previously been shown that HF-1 cells died by apoptosis after BCR stimulation or treatment with Ca^{2+} Ionophore A23187 (Eray et al., 1994; Knuutila et al., 1994). In the present study, these stimulations were used to induce apoptosis. Intact cells had the staining profile of Annexin V^{dim} PI^{dim} SYTO 17^{bright} (Fig. 4, Live cells). The first sign of change in the staining profile after BCR stimulation lead to apoptosis which was characterized by gradually decreased intensity of SYTO 17 staining and decreased cell size as detected by forward scatter (FSC), which are typical apoptotic changes as described earlier (Frey, 1995; Darzynkiewicz et al., 1992). Interestingly, decreased intensity of SYTO 17 staining was detected before any changes in the Annexin V or PI staining were observed. Multiple mechanisms have been suggested for the detection of apoptotic cells by SYTO dyes, e.g. the detection of changes in chromatin structure (Frey, 1995; Sparrow and Tippett, 2005) or detection of DNA condensation during apoptosis and thereby induced self-quenching of the SYTO dye (Frey, 1995; van Zandvoort et al., 2002) or detection of mitochondrial DNA or changed mitochondrial membrane potential (Sparrow and Tippett, 2005; van Zandvoort et al., 2002). The ongoing apoptotic process changed the staining profile and the cells became Annexin V^{dim}, but PI^{bright}, SYTO 17^{int} further leading to the Annexin V^{int} PI^{bright} SYTO 17^{int} staining profile. Previously, PI has been successfully used to detect live (PI^{neg}), apoptotic (PI^{dim}), and necrotic cells (PI^{bright}) (Zamai et al., 1996). Furthermore, necrotic and apoptotic cells undergoing a secondary necrosis can be identified by PI staining (Ormerod, 1998). It has been shown that SYTO 16^{int} 7-AAD^{neg} cells are apoptotic and SYTO 16^{neg} 7-AAD^{high} cells are necrotic (Sparrow and Tippett, 2005). Moreover, in

BCR-induced apoptosis, the exposure of PS to the external surfaces as detected by Annexin V positivity did not occur before the plasma membrane became leaky as detected by PI positivity (Fig. 4, region no. 1). However, PI positive cells were apoptotic rather than necrotic according to the FSC/SSC scattering, the appearance of apoptotic bodies in morphological studies, and the DNA laddering (Eray et al., 1994; Knuutila et al., 1994). In contrast, in some models Annexin V staining proved to be the most sensitive marker for apoptotic changes (Walsh et al., 1998). It has also been reported that Annexin V positivity did not fully correlate with apoptotic changes shown with other dyes (Frey, 1995). However, apoptotic processes in different model systems have clearly different staining patterns with cytofluorometric dyes, as reported earlier (Frey, 1995). The staining profile at the stage of final plasma membrane disintegration was changed to Annexin $V^{int/bright}$ PI^{int} SYTO 17^{dim} (Fig. 4, region no. 2). The staining intensity of PI^{int} has been shown to take place in the late stage of apoptosis (Hamel et al., 1996). Finally, the very late apoptotic or dead cells lost their staining intensity completely (Fig. 4, Dead cells).

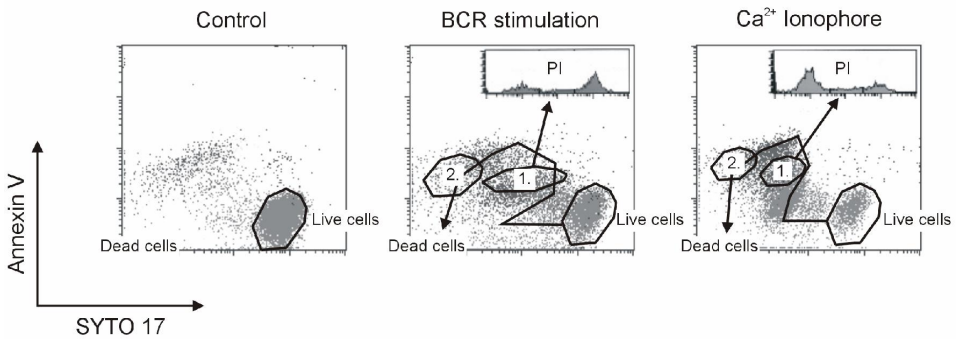

Figure 4. Flow cytometric analysis of HF-1 cells after Annexin V, PI and SYTO 17 staining. Cell subsets were analyzed with FACS after culturing the cells for 24 h in RPMI-1640 medium (Control), or with BCR stimulation, or in the presence of Ca^{2+} Ionophore A23187. Discrimination between live and dead cells was done according to staining with Annexin V, PI and SYTO 17 and FSC/SSC scattering. Annexin V^{dim} PI^{dim} SYTO 17^{bright} (Live cells), and Annexin V^{dim} PI^{dim} SYTO 17^{dim} (Dead cells).

In contrast to BCR-induced apoptosis, the induction of apoptosis after Ca^{2+} Ionophore A23187 treatment lead to a moderate change in cellular staining pattern (Fig.

4). Initially, SYTO 17 staining intensity was decreased whereas the intensity of Annexin V staining was increased before PI. In contrast to BCR stimulation, when the cells were Annexin V^{int} they were still mostly PI^{dim} (Fig. 4, region no. 1). The final stages were comparable to BCR stimulation.

In conclusion, the use of a combination of scatter parameters, SYTO 17, Annexin V and PI staining may be more reliable in detecting early cellular apoptosis as compared to classical biochemical or morphological methods. The sensitivity; reliability to discriminate apoptotic and necrotic cells; quick and easy staining procedure; the ability to use unfixed cells, provides more reliable results since the cells are closer to their natural stage. Our data is consistent with previous data (Zamai et al., 1996). In addition, cells stained and sorted with this combination could be further analyzed using other biochemical methods as suggested previously (Frey, 1995).

5.4. Gene therapy in B cell malignancies (IV)

It is known that $CD19^+$ B cells in bone marrow or $CD4^+$ and $CD8^+$ T cells are resistant to adenoviral gene therapy because the primary attachment receptor, hCAR (human coxsackie adenovirus receptor), is not expressed on the surface of these cells (Rebel et al., 2000). Efficient adenovirus-mediated gene delivery to B cells requires modifications in adenovirus vectors, so that B cell-specific molecules on the cell surface can be recognized. Since we had no access to adenovirus modification technology, B cell targeting was simulated by modifying these cells to express hCAR to make them more permissive to standard adenovirus vectors. The introduction of hCAR-EGFP fusion gene to HF28RA cells was carried out with lentivirus gene transfer, since these cells were resistant to non-viral gene delivery methods such as polycation-, liposome- or electroporation-mediated techniques. In addition, lentivirus vectors have unique features, such as wide target cell specificity (Pellinen et al., 2004) and the ability to cause a long-term gene expression by integrating the transgene into the target cell genome.

5.4.1. Lentiviral transduction and expression of hCAR-EGFP fusion gene in human B cells

HF28RA cells were transduced with a lentivirus vector containing hCAR-EGFP fusion gene. The hCAR-EGFP^{bright} positive cells were enriched by cell sorting based on their EGFP expression. Analysis of hCAR-EGFP surface expression showed that 27% of HF28RA hCAR-enriched cells were double-positive for hCAR-EGFP and anti-hCAR mAb, further ensuring that the EGFP-tailed hCAR was properly expressed on the surface of these cells. Previously, truncated hCAR was successfully expressed on transgenic mouse B cells (Tallone et al., 2001). However, the established hCAR-EGFP fusion gene construct was novel. In the present work, it was also found that the lentivirus vector used with a CMV promoter lead to a long-term, stable and efficient expression of the hCAR-EGFP fusion gene.

5.4.2. Function of the hCAR-EGFP protein

The functionality of the hCAR-EGFP fusion protein was studied by transducing the cells with AdDsRed2 (adenovirus vector expressing DsRed2 protein). In flow cytometric analysis, HF28RA hCAR cells expressed significantly higher levels of DsRed2 after AdDsRed2 transduction with 1 (~4.5 times more, $p<0.05$) or 10 pfu/cell (~2.8 times more, $p<0.05$) than parental HF28RA cells with the respective pfu/cell values. The expression of DsRed2 in each cell would be higher if B cells expressed higher levels of $\alpha_V\beta_3$ integrins, which are known to internalize the adenoviruses attached to hCAR (Neering et al., 1996; Wickham et al., 1993).

The applicability of hCAR-EGFP in gene therapy was further analyzed by transducing HF28RA hCAR cells with the AdTK-GFP vector (adenovirus vector expressing TK-GFP fusion protein) that contains the HSV-TK suicide gene. An efficient transduction of cells was confirmed by analyzing the expression of the HSV-TK gene by RT-PCR in DNase I treated mRNA samples from transduced cells. HF28RA hCAR and parental HF28RA cells were transduced with AdTK-GFP vector, followed by a 5-day culture in the presence of GCV. In HF28RA hCAR cells, both viral doses (1 and 10 pfu/cell) yielded significantly ($p<0.05$) decreased cell viability after GCV treatment as

compared to parental HF28RA cells. The viability of parental HF28RA cells was unaffected under conditions that destroyed about one third of the hCAR expressing cells.

Adenoviruses have been widely used in gene therapy because of the ability to obtain high titer virus preparations, broad target cell tropism, capability to transduce postmitotic cells and large DNA packaging capacity. In addition, adenoviral DNA is transiently and extrachromosomally expressed, which is a desired feature in most forms of cancer gene therapy (Kovesdi et al., 1997). Human PB leukocytes and spleen and thymus cells do not express hCAR, which makes them nearly resistant to adenovirus-mediated transduction (Colin et al., 2004; Tomko et al., 1997). Similarly, the unmodified FL B cells used in this study were resistant to adenovirus transduction due to the lack of endogenous hCAR on the plasma membrane. In our indirect targeting model, we were able to show that originally hCAR negative non-adherent cancer cells could be efficiently transduced and treated with adenovirus-mediated suicide gene therapy.

The development of new retargeted virus vectors is required for B cell-specific gene therapy experiments, *in vitro* or *in vivo*. Recently, it was reported that normal human PB B cells among other immune cell types expressing CD46 receptor were successfully transduced with Ad5/F35 chimeric adenovirus vector, in which the classical adenovirus fiber (Ad5) was replaced with the CD46 interacting fiber of human B adenovirus serotype 35 (Jung et al., 2005). The B cell-specificity could also be increased by a fusion protein containing a B cell-specific Ab and adenovirus capsid targeted protein, as previously described with ovarian cancer cells (Hakkarainen et al., 2003). B cell-specific surface molecules could be BCR complex molecules e.g. Igα and Igβ, since they are predominantly B cell-specific and abundantly expressed on the B cell surface throughout the B cell development. Additionally, the use of a strictly B cell-specific promoter-enhancer combination (VH promoter-3' enhancer) (Pettersson et al., 1990), would decrease severe side-effects such as by-stander cell death or tissue destruction.

6. CONCLUSIONS

The aim of the present study was to analyze the function and regulation of antigen-specific BCR signaling in human B cells.

In the first study the role of BCR signaling and a separate second signal was analyzed in an experimental model mimicking polysaccharide-encapsulated bacteria-induced TI responses of B cells (a model presented in Fig. 5). It was shown that Mϕ-derived cytokines, as a second signal, were important enhancers of BCR stimulation-induced class switch recombination and cytokine production in B cells. In addition, it was demonstrated that B cells and Mϕs function in close cooperation in TI responses as soluble mediators from activated B cells significantly enhanced cytokine production from Mϕ.

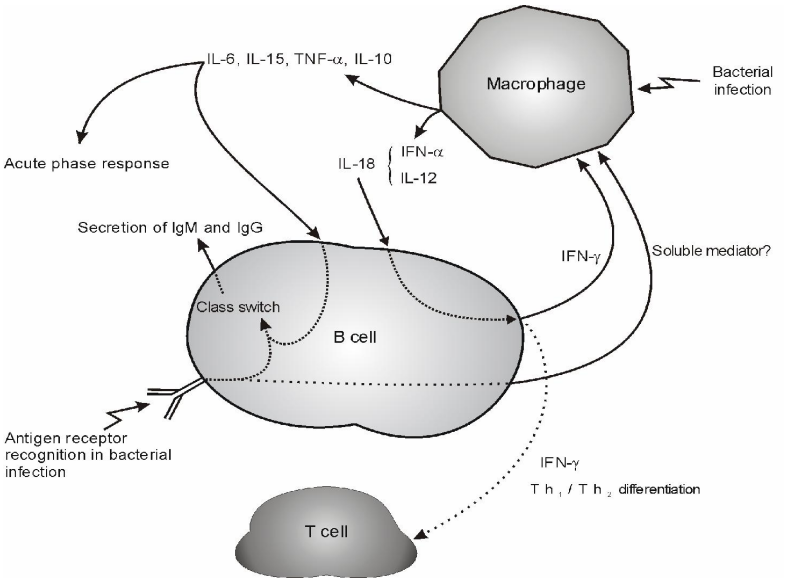

Figure 5. A model for the cooperation of B cells and macrophages during an inflammatory response. B cells are activated by polysaccharide-encapsulated bacteria through BCR and induced to produce soluble mediators. Simultaneously, Mϕ are activated by bacterial challenge to produce pro-inflammatory cytokines, which also stimulate B cells. Secretion of pro-inflammatory cytokines from Mϕ is enhanced by soluble mediators produced from activated B cells. BCR stimulation in the presence of Mϕ-derived cytokines leads to enhanced secretion of IgG, in addition to effective IgM production.

The other focus in the project was to study the regulation of BCR signaling by CD45 isoforms in human GC-derived follicular lymphoma B cell lines. Novel human B cell lines expressing distinct CD45 isoforms (RA and R0) were established. The distinct CD45 isoform expression was shown to play a role in fine-tuning of the basal, BCR- and cytokine-induced B cell proliferation, BCR-mediated cytokine production, and BCR-induced intracellular signaling. It seems that the CD45R0 has a role as a positive regulator in BCR-induced cellular events, whereas the CD45RA isoform has more an inhibitory role.

In the third study a novel combination of SYTO 17 staining with Annexin V-FITC and PI dyes was established and used in flow cytometric detection of BCR-induced apoptosis. The combination of SYTO 17 with the other widely used stains of apoptosis detection increased the sensitivity of the method. Furthermore, unfixed cells stained, detected and sorted with this method could be further analyzed by classical biochemical methods.

As a model for B cell targeted and specific adenoviral gene therapy, a novel fusion gene, hCAR-EGFP, was constructed and introduced into hCAR-negative human follicular B cell lymphoma cells with a lentiviral vector. In this experimental model it was indirectly shown that adenovirus retargeting changed adenovirus resistant cells to sensitive ones, suggesting that adenoviral gene therapy of B cell cancers is possible, but further development of appropriately targeted adenovirus vectors is still required to increase the cell-type specificity and efficacy of the method.

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APPENDIX: ORIGINAL PUBLICATIONS

Kuopio University Publications D. Medical Sciences

D 385. Perola, Outi. Hospital water supply as a source of nosocomial infections.
2006. 99 p. Acad. Diss.

D 386. Kuittinen, Taru. Autologous stem cell transplantation in patients with non-Hodgkin's lymphoma: progenitor cell mobilisation, toxicity of high-dose therapy, and progressive disease after transplantation.
2006. 115 p. Acad. Diss.

D 387. Siloaho, Maritta. Utilization of quality management systems in finnish medical laboratories.
2006. 105 p. Acad. Diss.

D 388. Haara, Mikko. Osteoarthritis and osteoporosis assessed from hand radiographs: prevalence, determinants, and associations with morbidity and mortality.
2006. 87 p. Acad. Diss.

D 389. Tenhola, Sirpa. Cardiovascular risk factors and adrenal function in 12-year-old children born small for gestational age or after a preeclamptic pregnancy.
2006. 85 p. Acad. Diss.

D 390. Zacharova, Jelena. Insulin-sensitizing genes and risk of type 2 diabetes: studies on the PGC-1α, PPARγ, adiponectin, leptin receptor and hepatic lipase genes.
2006. 103 p. Acad. Diss.

D 391. Rilla, Kirsi. Hyaluronan synthase: intracellular traffic, activity at plasma membrane, and impact on keratinocyte migration, proliferation and formation of microvilli.
2006. 119 p. Acad. Diss.

D 392. Pesonen, Tuula. Trends in Suicidality in Eastern Finland, 1988–1997.
2006. 119 p. Acad. Diss.

D 393. Tuhkanen, Hanna. DNA copy number changes in the stromal and epithelial cells of ovarian and breast tumours.
2006. 112 p. Acad. Diss.

D 394. Koskelo, Reijo. Säädettävien kalusteiden vaikutukset tuki- ja liikuntaelimistön terveyteen lukiolaisilla. 2006. 96 p. Acad. Diss.

D 395. Elo, Mika. Stress-Related Protein Synthesis in Mammalian Cells Exposed to Hydrostatic Pressure. 2006. 74 p. Acad. Diss.

D 396. Remes-Pakarinen, Terhi. Influences of genetic factors and regular exercise on bone in middle-aged men.
2006. 95 p. Acad. Diss.

D 397. Saarela, Tanja. Susceptibility genes of diabetes and endothelial dysfunction in preeclampsia.
2006. 103 p. Acad. Diss.

D 398. Piippo-Savolainen, Eija. Wheezy babies - wheezy adults? Adulthood asthma, bronchial reactivity and lung function after hospitalization for bronchiolitis in early life.
2006. 91 p. Acad. Diss.

D 399. Kauppinen, Anu. Lipocalin Allergen-Induced T Cell Response: Prospects for Peptide-Based Immunotherapy.
2006. 81 p. Acad. Diss.