Phosphorylation-Based Signaling in Human Immune Cells – A Systems View

by

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“Science is the poetry of reality”

(Richard Dawkins)

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Maria Elisabeth Kalland

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Abbreviations

$A_{2A}R$	adenosine receptor 2A
AA	arachidonic acid
Ab	antibody
AC	adenylate cyclase
actTregs	activated Tregs
ADAP	adhesion and degranulation promoting adapter protein
Ag	antigen
AHR	aryl hydrocarbon receptor
AITCP	adhesion-induced T cell priming
AKAP	A-kinase anchoring protein
AMP	adenosine monophosphate
AP1	activator protein 1; composed of members of the Fos and Jun families of DNA binding proteins
APC	antigen-presenting cell
ATP	adenosine triphosphate
BASH	B cell adaptor containing SH2 domain
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
BL	Burkitt's lymphoma
BLNK	B cell linker
Btk	Bruton's tyrosine kinase
C	catalytic subunit of PKA
Ca^{2+}	calcium ions
cAMP	cyclic adenosine 3', 5' monophosphate
CCR7	CC chemokine receptor 7
CD	cluster of differentiation
CD40L	CD40 ligand
COX	cyclooxygenase
coxib	selective COX-2 inhibitior
Cpb	Csk-binding protein
CREB	cAMP response element binding protein
Csk	C-terminal Src kinase
CSR	class-switch recombination
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCR5	CXC chemokine receptor 5
DAG	diacylglycerol
DC	dendritic cell
EBV	Epstein-Barr virus
EP	E prostanoid
EPAC	exchange protein directly activated by cAMP
Erk	extracellular signal-regulated kinase
FCB	fluorescent cell barcoding
Fos	Fos family of DNA binding proteins; see AP1
FOXP3	forkhead box P3, the human protein
FOXP3	FOXP3, the human gene
Foxp3	FOXP3, the mouse gene; *scurfin*
Foxp3	FOXP3, the mouse protein; scurfin
FRET	fluorescence resonance energy transfer
Gads	Grb2-related adaptor protein
GATA3	GATA binding protein 3
GC	germinal center
GDP	guanosine diphosphate
GEM	glycosphingolipid-enriched microdomain
GITR	glucocorticoid-induced TNF-receptor-related
GPCR	G-protein-coupled receptor
Grb2	growth-factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HePTP	hematopoietic tyrosine phosphatase
I	ionomycin
ICAM1	intercellular adhesion molecule 1
ICOS	inducible costimulator
ICOSL	inducible costimulator ligand
IDO	indoleamine 2,3-dioxygenase
IFN	interferon (e.g., IFN-γ)
Ig	immunoglobulin (also IgA, IgD, IgE, IgG, IgM)
IKK	IκB kinase
IL	interleukin (e.g., IL-2)
$InsP_3$	inositol-(1,4,5)-triphosphate; IP_3
IP_3	inositol-(1,4,5)-triphosphate
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
Itk	IL-2-idicible T cell kinase
iTregs	induced regulatory T cells
IκB	inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
Jun	Jun family of DNA binding proteins; see AP1
KD	knock down
LAB	linker for activation of B cells
LAG3	lymphocyte-activation gene 3
LAT	linker for activation of T cells
LAX	linker for activation of X cells
Lck	lymphocyte-specific protein-tyrosine kinase
LFA1	lymphocyte function-associated antigen 1
LIME	Lck-interacting molecule
MAPK	mitogen-activated protein kinase
Mek	MAPK- or Erk kinase; MAPK kinase
MHC	major histocompatibility complex
MS	mass spectrometry
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK	natural killer
NSAID	non-steroidal anti-inflammatory drugs
NTAL	non-T cell activation linker
nTregs	natural occurring regulatory T cells
pAb	phospho-specific antibody
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PAMP	pathogen-associated molecular pattern
PCs	plasma cells
PD-1	programmed cell death-1
PDE	phosphodiesterase
PDK1	phosphoinositide-dependent protein kinase 1
PDL-1	PD-1 ligand
PFA	paraformaldehyde
PG	prostaglandin (also PGH_2, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2)
PGE_2	E_2 type prostaglandin
PGS	prostaglandin synthases
PH	peckstrin homology
phospho	phosphorylated; phosphorylation-based

PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-(3,4)-biphosphate
PIP_3	phosphatidylinositol-(3,4,5)-triphosphate
PKA	cAMP-dependen protein kinase; protein kinase A
PKA-I	PKA type I
PKB	protein kinase B
PKC	protein kinase C (e.g., PKCθ)
PLC	phospholipase C (e.g., PLCγ1)
PMA	phorbol 12-myristate 13-acetate
PMT	photomultiplier tube
PRD	proline-rich domain
PRR	pattern recognition receptor
PTB	phosphotyrosine-binding
$PtdIns(3,4,5)P_3$	phosphatidylinositol-(3,4,5)-triphosphate; PIP_3
$PtdIns(4,5)P_2$	phosphatidylinositol-(3,4)-biphosphate; PIP_2
PTK	protein tyrosine kinase
PTM	post-translational modification
PTPase	protein tyrosine phosphatase
pY	phosphorylated tyrosine
RAG1	recombination-activating gene 1
RAG2	recombination-activating gene 2
RasGRP	Ras guanyl nucleotide-releasing protein
RhoA	Ras homolog gene family, member A
RNA	ribonucleic acid
ROR	retinoic acid receptor-related orphan receptor
rTregs	resting regulatory T cells
SFK	Src family of PTK
SH2	Src homology 2
SH3	Src homology 3
SHM	somatic hypermutation
SHP	SH2-domain-containing tyrosine phosphatase
SHP1	SH2 domain-containing tyrosine phosphatase 1
siRNA	small interfering RNA
SIT	SHP-2-interacting transmembrane adaptor protein
SLAMF	signaling lymphocyte activation molecule family
Slp-65	SH2-domain-containing leukocyte-specific protein of 65 kDa
Slp-76	SH2-domain-containing leukocyte-specific protein of 76 kDa
SMAC	supramolecular activation cluster
SOS	Son of Sevenless
Stat	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
T-bet	T box transcription factor
Tc	cytotoxic T
TCR	T cell receptor
Tfh	follicular helper T
TGF-β	tumor growth factor β
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAP	transmembrane adaptor protein
Tregs	regulatory T cells
TRIM	TCR-interacting molecule
WB	western blot
ZAP70	ζ-chain-associated protein of 70 kDa

List of publications

I. **Nikolaus G. Oberprieler[#], Simone Lemeer[#], Maria E. Kalland, Knut M. Torgersen, Albert J. R. Heck, and Kjetil Taskén** (2010). High-resolution mapping of prostaglandin E2-dependent signaling networks identifies a constitutively active PKA signaling node in $CD8^{+}CD45RO^{+}$ T cells. *Blood 116(13):2253-65.*

II. **Maria E. Kalland, Nikolaus G. Oberprieler, Torkel Vang, Kjetil Taskén, and Knut M. Torgersen** (2011). T Cell-Signaling Network Analysis Reveals Distinct Differences between CD28 and CD2 Costimulation Responses in Various Subsets and in the MAPK Pathway between Resting and Activated Regulatory T Cells. *J Immunol. 187(10):5233-45.*

III. **Maria E Kalland[#], Silje A Solheim[#], Sigrid S Skånland, Kjetil Taskén, and Torunn Berge** (2012). Modulation of proximal signaling in normal and transformed B cells by transmembrane adapter Cbp/PAG. *Exp Cell Res. 318(14):1611-9.*

[#] These authors contributed equally

1 Introduction

1.1 Functional specialization of the immune system

The human body has developed a wide range of defense mechanisms in order to survive in a hostile environment. These mechanisms protect us against attacks from infectious agents, such as bacteria, viruses, fungi and parasites, and collectively constitute what is known as the immune system (Medzhitov, 2007;Litman et al., 2010). The immune system is crucial to human health in preventing pathogenic organisms from entering the body and causing harm to cells and tissues. In addition, it plays an important role in identifying and eliminating nascent tumors (Zitvogel et al., 2006). If the immune system were to fail, even minor pathogenic threats would pose a fatal risk (Medzhitov, 2007;Litman et al., 2010).

1.1.1 General overview of the innate and adaptive immune systems

The vertebrate immune system includes two functional divisions; the innate – or natural – immune system and the adaptive immune system, which is sometimes referred to as acquired or specific. While the innate immune system may counter an infection in its nascence through preformed cells and factors, the adaptive immune system has a slow but powerful onset and is responsible for clearing established infections. Both levels of protection involve a variety of blood-borne immune cells known as leukocytes or white blood cells. Whereas the key players of the innate immune system are phagocytes such as granulocytes, macrophages and dendritic cells, the adaptive immune system relies on the actions of B- and T cells. The B- and T cells, also known as B- and T lymphocytes, originate from common lymphoid progenitor cells in the bone marrow and are responsible for all of the three hallmarks of adaptive immunity: specificity, immunological memory, and enhanced secondary immune responses.

The bone marrow and thymus are termed the primary lymphoid organs. This is where the lymphoid progenitor cells differentiate into mature B- and T cells (Ceredig et al., 2009;Miller, 2011) (Figure 1). The secondary lymphoid organs include the lymph nodes and lymphatic structures present in the mucosa and the gut, as well as the spleen. These are nodes in the lymphatic circulation, which serve to capture antigens brought to them through lymph or blood and present them to circulating lymphocytes. An adaptive immune response is initiated

when a lymphocyte encounters its cognate antigen in one of these secondary lymphoid tissues (Figure 1), as detailed later. However, the time required for such a response to mount is significant, and while the lymphoid activation and maturation proceeds, the body relies on innate immune mechanisms for its protection (Medzhitov, 2007). As opposed to adaptive immunity, the innate immune response is initiated at the site of infection with preformed cells and immune factors, thus responding without delay. Importantly, cues from the innate immune response serve to direct and influence the nature of the resulting adaptive immune response elicited in the secondary lymphoid organs against the same infection (Iwasaki and Medzhitov, 2010;Medzhitov, 2007).

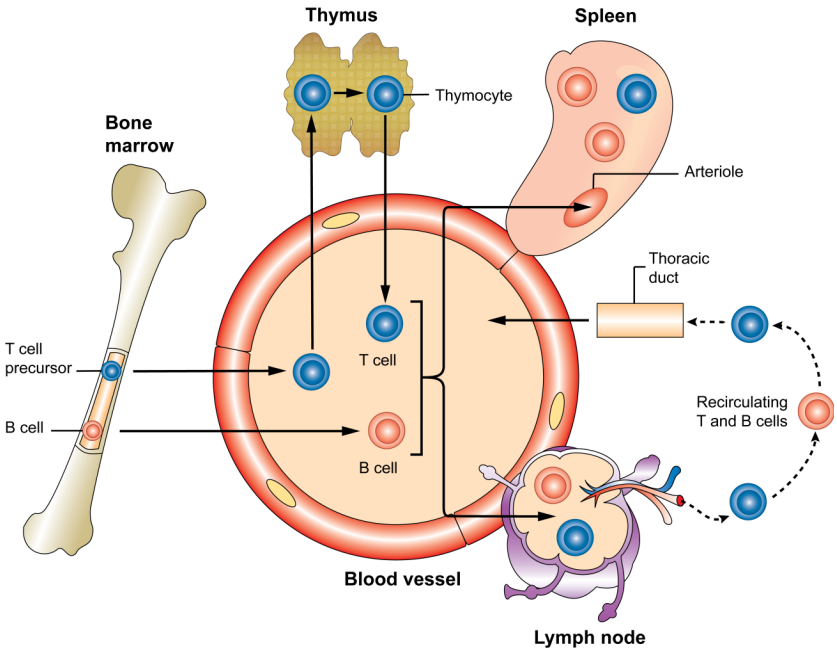

Figure 1. Overview of B- and T cell production and maturation in primary lymphoid organs and their migration and recirculation to secondary lymphoid organs. The B- and T cells originate from pluripotent stem cells in the bone marrow. In contrast to B cells (red), which complete their maturation processes in the bone marrow, T cell precursors leave the bone marrow at an early stage and migrate to the thymus where they differentiate into mature T cells (blue). After maturation, B- and T cells are transported via the blood to secondary lymphoid organ such as the spleen and lymph nodes, in search for their cognate antigen. From the secondary lymphoid organs, mainly T cells, but also a fraction of B cells, recirculate back to the blood via the lymph and the thoracic duct, as depicted in the figure. Adapted from (Miller, 2011).

The innate defense mechanisms include complement proteins and inflammatory cytokine responses, as well as innate immune cells such as macrophages, neutrophils and natural killer

(NK) cells. The macrophages and neutrophils are phagocytes, which destruct invading pathogens by engulfing and digesting them along with tissue debris and dead cells. The NK cells are small granular cytotoxic lymphocytes that migrate into infected tissues in response to inflammatory cytokines and kill infected cells without prior sensitization (Sun and Lanier, 2011). As opposed to adaptive immunity, innate immunity does not produce a specific immunological memory of the infection.

The specificity and memory of adaptive immunity arise from the recombination of genes coding for segments of the B- and T cell antigen receptors (Brack et al., 1978;Gellert, 2002;Tonegawa, 1983). The recombination relies on a recombinase complex of two lymphoid-specific proteins encoded by the recombination-activating gene 1 (RAG1) and RAG2 that serves to generate antigen receptors with a multitude of specificities (McBlane et al., 1995;Schatz et al., 1989;Oettinger et al., 1990). Once the antigen receptor recognizes its cognate antigen in the context of sufficient costimulation (see below), the intracellular signaling emanating from the activated receptor triggers proliferation and differentiation of the lymphocyte, resulting in a clone of lymphocytes specific for the same antigen. This process is known as clonal selection and results in the generation of short-lived effector cells and long-lived memory cells. As described later on, the memory cells are capable of mounting an enhanced secondary immune response if the same antigen is encountered again (Ahmed and Gray, 1996;Zinkernagel et al., 1996).

1.1.2 Innate immune mechanisms regulate adaptive immunity

Sufficient costimulation is a prerequisite for adaptive immunity. This involves presenting the pathogen in the context of an antigen–major histocompatibility complex (MHC) on the surface of an antigen-presenting cell (APC) such as dendritic cells (DC), macrophages and B cells (Davis and Bjorkman, 1988;Neefjes et al., 2011). In addition to the costimulatory signals afforded by the MHC molecules, other cell-bound and soluble signals play an important role in directing adaptive immunity. Such signals include the "danger signals" elicited by the innate immune system in response to foreign materials (Matzinger, 2002). Innate immune cells respond to dangers through a set of pattern recognition receptors (PRRs) that recognize different types of pathogens through conserved pathogen-associated molecular patterns (PAMPs) – such as lipopolysaccharides and mannose – that are shared by bacteria, viruses and parasites (Iwasaki and Medzhitov, 2010;Medzhitov, 2007). The PRRs are subdivided into

secreted, transmembrane and cytosolic pattern recognition receptors, among which the best characterized receptors are the transmembrane PRRs of the Toll-like receptor (TLR) family.

Detection of a viral particle or a microbial cell component through PRRs expressed on the surface of an APC induces endocytosis or phagocytosis of the pathogen, resulting in presentation of pathogen-derived antigens to T cells in the context of MHC molecules. Simultaneously, the APC up-regulates its expression of costimulatory molecules and begins secreting various cytokines with the aim of priming nearby T cells for activation. The nature of the pathogen determines the PRRs it activates, which in turn dictates the immune response elicited against it (Iwasaki and Medzhitov, 2010;Medzhitov, 2007). Thus, antigens derived from invading pathogens are discriminated from self-antigens in part by the expression of stimulatory coreceptors that results from the activation of PRRs.

DCs are the archetypal professional APC and superior to other APCs in activating naive T cells (Steinman, 2008;Pulendran and Ahmed, 2006). They are strategically placed in both lymphoid and non-lymphoid sites as immune sentinels, and perform sampling of the microenvironment by phagocytosing extracellular material. The recognition of pathogens through PRRs leads to the activation of immature DCs. This results in more efficient endocytosis, followed by secretion of inflammatory cytokines and acquisition of migratory properties (Iwasaki and Medzhitov, 2010;Medzhitov, 2007). The activated DCs migrate from the infected tissues to draining lymph nodes where they mature into cells capable of presenting pathogen-derived antigens to circulating lymphocytes and initiating adaptive immune responses (Cavanagh and von Andrian, 2002;Randolph et al., 2008). When the presented antigen is recognized by a cognate T cell, a signal initiated by the interaction of the intercellular adhesion molecule 1 (ICAM1) on the APC with the lymphocyte function-associated antigen 1 (LFA1; also termed αLβ2 integrin) on the T cell induces a stable interaction between the cells that consequently leads to retention of the T cell (Dustin and Springer, 1989;Pribila et al., 2004;Dustin et al., 1997). This interaction, which is referred to as either the supramolecular activation cluster (SMAC) (Monks et al., 1998) or the immunological synapse (Grakoui et al., 1999), is essential for the activation of naive T cells (Dustin, 2009). Multiple signaling pathways are initiated at this contact site, all of which influence the fate of the T cell. The final outcome depends on the quality, strength, and duration of the T cell–APC interaction, resulting in proliferation, anergy, or apoptosis (Iezzi et al., 1998;Huppa et al., 2003;Gett et al., 2003).

1.1.3 Immunological memory

A fundamental feature of the adaptive immune system is the capacity to form immunological memory, resulting in long-lasting protection from recurrent infections by the same pathogen (Dutton et al., 1998;Gray, 1993;Dutton et al., 1999;Wherry et al., 2003). Immunological memory depends on the generation of memory B- and T cells as well as long-lived plasma cells that continuously secrete high-affinity neutralizing antibodies (Sallusto et al., 2010). Memory B- and T cells develop from naive progenitor cells throughout the course of a primary infection. These memory cells remain even after the infection has been cleared (Figure 2).

Both naive and memory T cells are long-lived cells that circulate the blood and lymph systems in a resting state. However, in response to a secondary challenge by the same pathogen, resting memory T cells differentiate more rapidly into effector T cells and perform their effector functions faster than their naive counterparts. Accordingly, memory T cells offer a more potent and efficient immune response in the early phases of the secondary infection, when the amounts of antigen are limited. This provides enhanced protection to the host and leads directly to reduced disease severity (Ahmed et al., 2009;Harty and Badovinac, 2008;Jameson and Masopust, 2009;Kaech and Wherry, 2007;Williams and Bevan, 2007).

As Figure 2 illustrates, the generation of memory T cells occurs in two stages (Salek-Ardakani and Croft, 2006;Williams and Bevan, 2007). The first phase, called the effector phase, is characterized by expansion and differentiation, and usually takes place the second week after infection until the pathogen is cleared (Figure 2). Throughout this phase pathogen-derived antigens are brought to the secondary lymphoid tissues by APCs. Here, naive circulating T cells scan the surface of lymph node-resident APCs with their TCRs, and are retained by the formation of immunological synapses upon antigen recognition. This initiates proliferation and differentiation of the naive T cell into an expanded population of effector T cells that migrate to the site of infection. These effector cells are the ones that perform specialized T cell functions, such as secretion of cytokines, in order to facilitate the activation of B cells ($CD4^+$ T cells) or acquire cytolytic capacity ($CD8^+$ T cells). Although the effector T cells are in a highly active state, they require continuous stimulation through the TCR and costimulatory receptors (e.g. CD28 and CD2) in order to carry out their immune functions (Iezzi et al., 1998). As the levels of antigen diminish, the majority of effector cells enter into

apoptosis and the immune response proceeds to a second phase of contraction and generation of memory cells (Dooms and Abbas, 2002;Seder and Ahmed, 2003) (Figure 2).

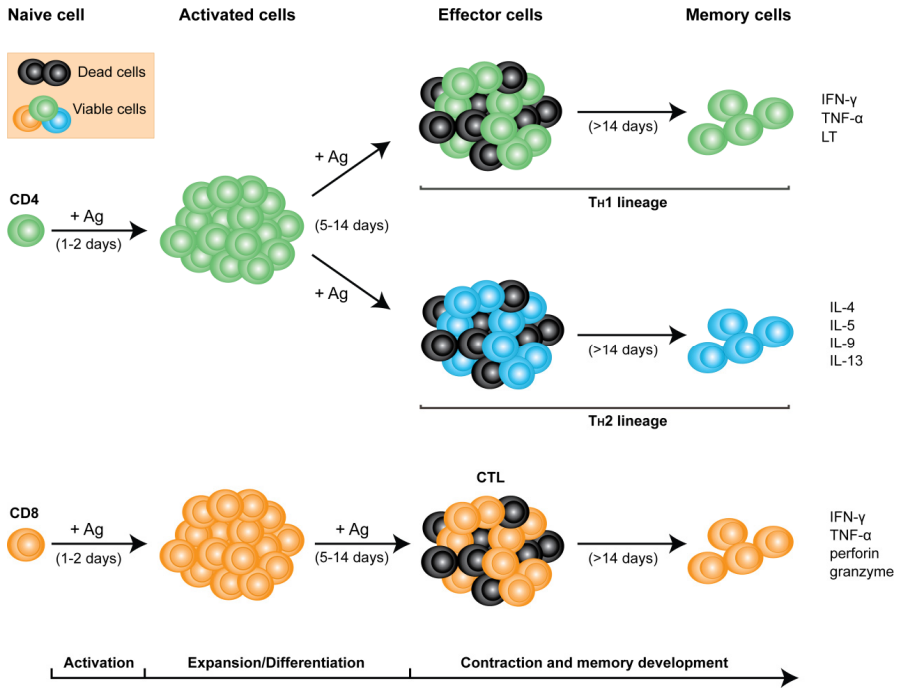

Figure 2. Differentiation of naive CD4$^+$ and CD8$^+$ T cells into effector and memory cells. Naive CD4$^+$ T cells first encounter their cognate antigen in the spleen or in a lymph node draining the site of infection. Here, they recognize the peptide antigen as bound to MHC class II on an APC, leading to clonal expansion of antigen-specific T helper (Th) cells. Naive CD8$^+$ T cells, on the other hand, recognize their cognate antigen in the context of MHC class I and generate clones of antigen-specific cytotoxic T (Tc) cells, also referred to as cytotoxic T lymphocytes (CTL). The T cell receptor (TCR) constitutes the antigen-binding receptor for both lineages. Depending on the nature of the invading agent and the cytokines secreted at the site of infection, the clonally expanded and fully differentiated effector T cells may acquire different effector functions. The cartoon illustrates the divergence of CD4$^+$ T cells into the classical Th1 and Th2 lineages and the differentiation of CD8$^+$ T cells into CTLs in the presence of antigens. After the pathogen becomes eliminated, most of the effector cells enter apoptosis, while a small fraction of approximately 10% survive and become long-lived memory cells. Adapted from (Salek-Ardakani and Croft, 2006;Williams and Bevan, 2007;Zhang and Bevan, 2011).

T cells at different stages of activation may be phenotypically distinguished by their expression of different CD45 isoforms. Naive T cells express the CD45RA isoform, whereas effector/memory T cells typically express CD45RO (Dutton et al., 1998). CD45 is a transmembrane protein tyrosine phosphatase (PTPase) abundantly expressed in all nucleated hematopoietic cells (Figure 3). Other than being a phenotypic marker CD45 is also known as an important inducer of signaling through the TCR (Desai et al., 1994;Volarevic et al.,

1993;Hovis et al., 1993;Koretzky et al., 1990). Due to alternative splicing of its extracellular domain, the enzyme exists in several isoforms where the largest isoform includes all three alternatively spliced exons (CD45RABC) and the smallest contains none (CD45RO) (Trowbridge and Thomas, 1994) (Figure 3). As antigen-inexperienced naive T cells become activated and differentiate into effector/memory T cells, their CD45 expression switches within 3-5 days from the larger CD45RA isoform to the smaller CD45RO isoform (Akbar et al., 1988;Birkeland et al., 1989). We took advantage of this alternate splicing in Paper I-II, where we used CD45RA and CD45RO as markers to distinguish between naive and effector/memory T cells ($CD45RA^+/CD45RO^-$ and $CD45RA^-/CD45RO^+$, respectively).

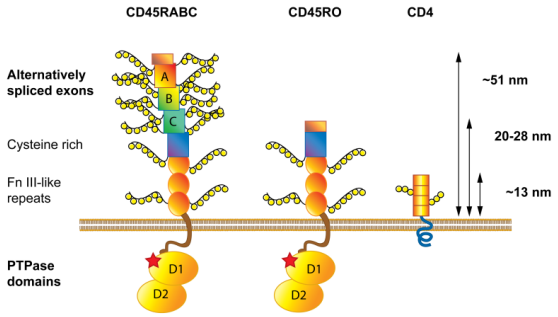

Figure 3. The molecular structure of CD45. As a result of alternative splicing of the extracellular CD45 exons, CD45 comes in multiple isoforms. Depicted in this figure is the size comparison of the largest CD45RABC isoform with the smallest CD45RO isoform, and that of the CD4 coreceptor expressed on the surface of $CD4^+$ T cells. Adapted from (Penninger et al., 2001).

1.1.4 Specialization of the $CD4^+$ effector T cells

Following antigenic activation, $CD4^+$ T cells may acquire a number of different phenotypes and functions. Several lineages of Th cells have been identified, each of which secrete a distinct set of cytokines (Zhou et al., 2009;Zhu et al., 2010;O'Shea and Paul, 2010). The different Th lineages are thought to fulfill different functions and may have become specialized towards particular types of infections. Thus, depending on the type of pathogen encountered, the resulting pool of pathogen-specific effector Th cells may be skewed towards one or more of the Th lineages. These include, among others, the classical Th1 (Szabo et al., 2003) and Th2 cells (Ansel et al., 2006), as well as the more recently identified Th17 (Chen et al., 2007) and follicular helper T (Tfh) cells (Crotty, 2011;King, 2009) (see Figure 4).

The cytokine environment in combination with the strength of the antigen–TCR interaction dictate the functional fates of activated T cells in a particular infection (Zhou et al., 2009;Zhu et al., 2010;O'Shea and Paul, 2010) (Figure 4). Among the many combinations of cytokines possible, the pro-inflammatory cytokine interferon γ (IFN-γ), produced by both NK cells and

T cells, in combination with interleukin (IL)-12 produced by innate immune cells will polarize the Th differentiation towards that of Th1 cells. This process involves activation of the transcription factors signal transducer and activator of transcription 4 (Stat4), Stat1 and the T box transcription factor T-bet. The cells that belongs to the Th1 lineage are known to be important for development of cellular immunity against intracellular microorganisms, and are characterized by predominant production of IFN-γ (Szabo et al., 2003).

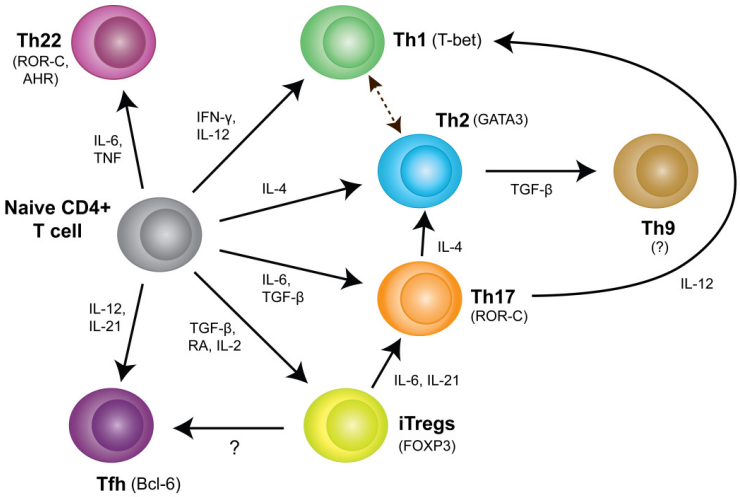

Figure 4. The cytokines induced in the microenvironment in response to features of the invading pathogen determine the functional fates of the responding T cells. Naive $CD4^+$ T cells responding to an infection proliferate and differentiate into effector T cells with helper functions specially suited to that infection. Depending on the cytokine milieu, the differentiating $CD4^+$ T cells acquire different functions. For instance, in the presence of IFN-γ and IL-12, the T cell may differentiate into and produce cytokines of the Th1 lineage. In the same way, IL-4 facilitates differentiation into Th2 cells, while IL-6 and TGF-β promote differentiation towards Th17 cells. The recently identified Th22 lineage seems to be favored by the combined secretion of IL-6 and TNF at the site of activation (Duhen et al., 2009). Tfh cells represent another recently discovered lineage that appears to result from the combined presence of IL-12 and IL-21. In addition, T cells with regulatory capacity (iTregs) may be induced from naive T cells in the presence of TGF-β, retinoic acid (RA) and IL-2. Finally, it has been hypothesized from recent findings that Th2 cells may differentiate into IL-9 producing Th9 cells, although this remains to be confirmed. The adaptations described here depend on, but are not restricted to, the expression of the following lineage-defining transcription factors: T-bet for Th1, GATA3 for Th2, ROR-C for Th17, ROR-C and aryl hydrocarbon receptor (AHR) for Th22 (Trifari et al., 2009), and FOXP3 for iTregs. Recent findings indicate a high degree of plasticity and inter-convertability between the functionally distinct Th lineages. Arrows in the figure indicate the observed lineage interchangeability. Adapted from (Zhou et al., 2009).

The Th2 cells, on the other hand, which produce high levels of IL-4, IL-5 and IL-13, are required for development of humoral immunity controlling helminths and other extracellular pathogens. Differentiation of Th2 cells is directed by the transcription factors GATA binding

protein 3 (GATA3) and Stat6, both downstream targets of IL-4 (Ansel et al., 2006). Differentiation towards the more recently identified Th17 cell lineage appears to be important for clearance of certain extracellular bacteria and fungi, particularly at the mucosal surfaces. The cells of this lineage are characterized by the simultaneous production of IL-17A, IL-17F and IL-22 cytokines. Their differentiation is linked to the expression of the transcription factor retinoic acid receptor-related orphan receptor (ROR)-C, induced by tumor growth factor β (TGF-β) in concert with the pro-inflammatory cytokines IL-6, IL-21 and IL-23, all of which are known to induce phosphorylation of Stat3 (Chen et al., 2007). Differentiation of Tfh cells, being essential regulators of B cell differentiation and memory B cell development, requires the cytokine IL-21 and induction of the transcription factor B cell lymphoma 6 (Bcl-6) protein (Crotty, 2011). Further details about the possible functional fates of naive $CD4^+$ T cells are indicated in Figure 4.

1.1.5 Humoral immunity

As described in section 1.1.3, the key feature of the adaptive immune system is the ability to remember experiences in the past and respond with heightened kinetics and efficacy to re-challenges by the same pathogen. In addition to inducing activation of $CD4^+$ T cells, as described earlier, infections caused by extracellular pathogens also initiate production of high-affinity antibodies by antigen-specific B cells (Rajewsky, 1996). This is essential for clearing extracellular infections and constitutes the basis for humoral immunity as well as design of efficacious vaccines. The high serum stability of antibodies and their continuous replemenishment by long-lived plasma cells (PCs) ensure lasting protection (McHeyzer-Williams and McHeyzer-Williams, 2005;Tarlinton, 2006).

The antigen receptor expressed on the surface of naive B cells is called the B cell receptor (BCR) and is composed of membrane bound immunoglobulins (Igs) of the IgM and IgD classes (Goding et al., 1977;Meffre et al., 2000). Despite some structural differences, these molecules are considered to be the counterpart of the TCR in T cells. Consequently, BCRs are the receptors responsible for recognition of soluble antigens in the initial phase of B cell-mediated immune responses. The BCR constitutes two identical heavy chains and two identical light chains linked together by disulfide bounds (Reth, 1992). The light chains, which are expressed as either kappa (κ) or lambda (λ) subunits on individual B cells, constitute the BCR unit responsible for antigen recognition. The heavy chains, on the other

hand, dictate the functional properties of the Igs. These exist in five different isotypes, designated by the Greek letters as α, γ, δ, ε and μ that define the corresponding Ig classes: IgA, IgG, IgD, IgE and IgM, respectively.

Characteristically, B cells release their antigen receptor into the surrounding environment in the form of pathogen-specific antibodies that may neutralize or, with the aid of complement factors or cellular mediators, kill the target pathogen. Thus, B cells are the cells responsible for humoral immunity. The B cells are activated as their BCR recognizes an extracellular pathogen. This leads to activation of intracellular signaling cascades, resulting in B cell proliferation and differentiation (Kurosaki et al., 2010). For details about the signaling events induced in response to antigen-recognition by the BCR, see section 1.2.5.

During a primary immune response, naive B cells may choose either one of three fates upon activation through the BCR (Nutt and Tarlinton, 2011) (Figure 5). Most of them will follow the extra-follicular pathway and rapidly differentiate into short-lived plasmablasts, with a half-life of approximately 3-5 days, which give rise to the first transient wave of antibody defense against the invading pathogen (McHeyzer-Williams and McHeyzer-Williams, 2005;Ho et al., 1986;Jacob et al., 1991). Notably, the antibodies produced in this stage are of low-affinity for the specific antigen. Although most of the activated B cells follow the extra-follicular pathway, a few of them will follow the follicular one, undergoing intense proliferation and migrate from the outer face of the B cell follicle to the center of it where they start forming a specialized structure known as germinal center (GC) (Allen et al., 2007;MacLennan, 1994) (Figure 5). Several essential molecular processes will take place within the GC in order to develop efficient long-lasting serological immunity. These processes include class-switch recombination (CSR) and somatic hypermutation (SHM) of the constant and variable regions of the Ig genes, respectively, in addition to clonal selection of high-affinity B cells (Berek et al., 1991;Jacob et al., 1991;Tarlinton and Smith, 2000;Liu et al., 1996). Thus, the antibodies produced by the terminally differentiated GC B cells have a drastically improved affinity for the activating antigen and with isotypes that are relevant for more efficient clearances (Niiro and Clark, 2002). Following these processes, GC B cells will emigrate from the follicle and differentiate into either high-affinity memory B cells or long-lived PCs (Figure 5), both contributing to enhance protection to the host for protracted time (Nutt and Tarlinton, 2011). The long-lived PCs primarily migrate to and reside in the bone marrow, where they continuously secrete high levels of antibodies for prolonged periods of

time well after the primary response have subsided. The memory B cells, on the other hand, will remain circulating in the periphery and maintain their ability to rapidly proliferate and differentiate into plasmablasts following re-exposure to the same antigen (Tarlinton, 2006;Good-Jacobson and Shlomchik, 2010). Hence, memory B cells and long-lived PCs will contribute to efficient elimination and reduced collateral tissue damage upon re-challenges by the same pathogen. This ability of naive B cells to develop into either memory B cells and long-lived PCs is the basis for the design of the majority of the vaccines that are currently in use (Plotkin, 2008).

Although the majority of the activated B cells undergo isotype switching in the GC, some will undergo isotype switching without prior SHM and give rise to early memory B cells (Figure 5). During this process, the variable portion of the Ig that binds to the antigen maintains its antigen specificity. Nevertheless, the humoral immune response diversifies as the distinct classes of Igs have different functional properties. In the first phase of the immune response the abundance of antigens allows for both low- and high-affinity interactions with antigen-specific B cells, as the affinities of the antibodies produced are heterogeneous. However, as the response progresses and a large number of antibodies are bound to antigens, the amount of available antibodies gets markedly reduced. At this point, a process called affinity maturation starts, wherein the B cells expressing the higher affinity antibodies are preferentially stimulated. This process, which is dependent on help from Tfh cells localized in the T cell zone of secondary lymphoid organs (Crotty, 2011), increases the affinity of the secreted antibodies for a certain antigen, and is the result of SHM in the variable portion of the Ig gene during clonal expansion. Positive selection ensures that only the B cells capable of producing antibodies with higher affinity for the specific antigen survive. Interaction between B cell-expressed CD40 and Tfh-expressed CD40L (Bishop and Hostager, 2001), as well as the presence of IL-4 and IFNγ secreted by activated Tfh cells, serve to facilitate CSR and formation of GC (King, 2009).

The differentiation of B cells is a highly controlled process (Niiro and Clark, 2002). Consequently, signaling through the BCR is tightly regulated in order to prevent erroneous B cell development and ensuing tissue damage. In Paper III, we used an established phospho-specific flow cytometry protocol to compare early BCR signaling in both normal and transformed B cells in an attempt to elucidate the role of the adaptor protein phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) in BCR signaling. Notably,

this adaptor protein has been shown to negatively regulate proximal TCR signaling in T cells. For further details about the function of this transmembrane adapter protein, see section 1.2.2.

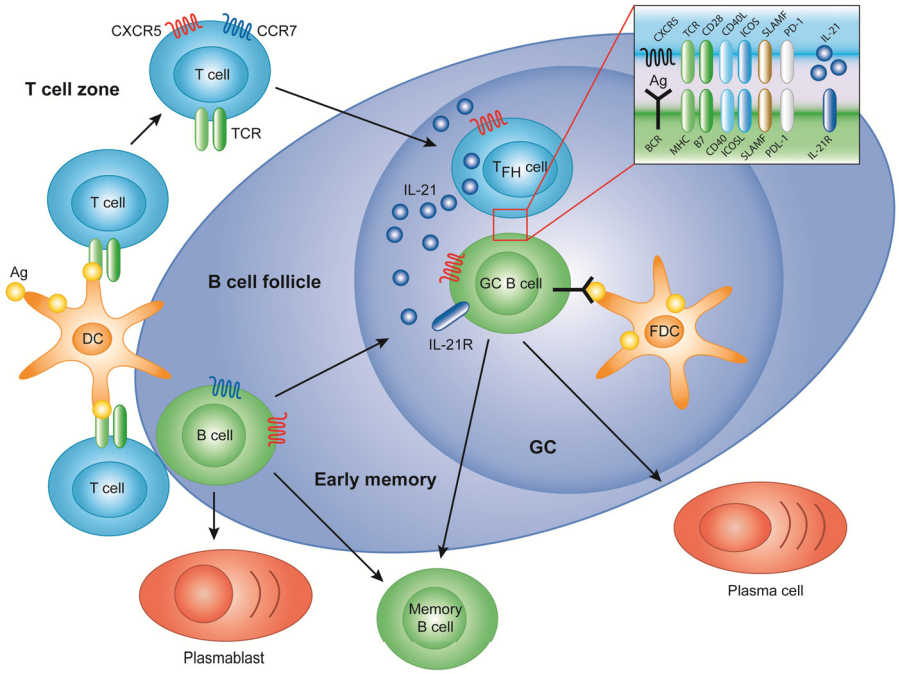

Figure 5. B cell follicles are necessary for development of humoral immunity. Shortly, the lymphocytes in the secondary lymphoid organs are segregated into distinct zones based on their sensitivity to various chemokines (Cyster, 2005). These zones are called B cell zones (follicles) and T cell zones that are specialized regions for B- and T cells, respectively. Initially priming of antigen-specific T cells by DC in the T cell zone induces up-regulation of ICOS, PD-1 and the chemokine-receptor CXCR5 on the T cells, which subsequently migrate toward the interface between the zones. In this interface, the T cells will interact with cognate B cells and mature into Tfh cells. The follicular B cells will migrate in response to antigen-induced up-regulation of the chemokine receptor CCR7 to the border of the T cell zone in search for T cell help. Upon interaction with cognate T cells, the activated B cells will differentiating into plasmablasts, giving rise to early memory B cells that have not undergone affinity maturation or they will return back to the center of the follicle to form specialized GC. The B cells of the GC interact with Tfh cells by means of multiple molecular pairings, including among others the interactions between the TCR and MHC, the CD28-B7 family members, ICOS and its ligand ICOSL, and CD40L and CD40 (see insert). These pairings induces secretion by Thf of IL-4 and IL-21, which dictate the B cell to differentiate into either affinity-matured memory B cells or long-lived PCs. Adapted from (Nutt and Tarlinton, 2011).

1.2 Immune cell signaling and activation

The antigen-specific cells of the immune system, which are divided into the B- and T cells, are maintained in the quiescent G_0 stage of the cell cycle and require potent antigen stimulation to progress from this phase. Of note, the antigen receptors of the B- and T cells recognize fundamentally distinct forms of the antigens: whereas the BCR recognizes the native or denatured form of proteins, or carbohydrates in soluble, particulate or cell-bound form, the TCR recognizes short, proteolytically processed peptide antigens (8 ~ 15 residues) bound to MHC molecules on the surface of an APC. Although the structures of these two receptors are quite different, the signal transduction events initiated by their engagement are remarkably similar.

1.2.1 Signal integration by post-translational phosphorylation

Among the various mechanisms for regulating cellular processes, post-translational modifications (PTMs) have a central role in creating highly dynamic and largely reversible relay systems that interpret and respond to alterations in the cellular microenvironments. The PTMs include modifications such as proteolytic cleavage or the addition of chemical groups or proteins to one or more amino acids of the target protein. To date, over 200 different PTMs have been reported (Deribe et al., 2010), contributing among others to the diversity in inter- and intracellular signaling and relay of fast messages throughout the cells. Whereas some PTMs are stable, like those involved in maturation and folding of newly synthesized proteins (e.g., glycosylation, lipidation and formation of disulfide bridges), others such as phosphorylations are more transient, but nonetheless crucial for efficient intracellular signaling (Deribe et al., 2010). In the present Thesis, we have studied phosphorylation-based signal integration in response to a variety of stimuli in functionally distinct immune cell populations. Thus, the following focuses on post-translational phosphorylation processes.

Intracellular phosphorylation of serine, threonine and tyrosine residues in eukaryotic cells (Deribe et al., 2010;Narayanan and Jacobson, 2009) is one of the main mechanisms by which a cell modulate the intrinsic biological activity of proteins, as well as their turnover, localization and interaction with other proteins (de et al., 2006;Sefton and Shenolikar, 2001). Accordingly, reversible phosphorylation controls a multitude of cellular processes. In the initiation of diverse phosphorylation responses, extracellular stimulation of cells trigger

signals which are transduced, amplified and regulated by kinase cascades that are turned on and off by means of reversible addition (by kinases) or removal (by phosphatases) of phosphate groups (White, 2008;Hunter, 2000;Cohen, 2000). As such, phosphorylation and dephosphorylation processes are known to regulate the majority of the aspects of cell function, including cell growth, metabolism, apoptosis, and intercellular communication and coordination (White, 2008;Schlessinger, 2000). Of the entire proteome, the enzymes that control phosphorylation events in eukaryotic cells amount to 1.5-2.5% (Manning et al., 2002), indicating that post-translational phosphorylation is an important and ubiquitous regulatory mechanism in eukaryotic cells. Whereas cellular processes affected by phosphorylation of serine and threonine residues include cell-cycle progression and control of cellular growth, phosphorylation of tyrosine residues are initiated predominately by surface-bound receptors involved in the regulation of cellular proliferation and differentiation (Ullrich and Schlessinger, 1990;Deribe et al., 2010).

Modulating a protein by the addition of a phosphate group gives it an extra charge of -2 at physiological pH, which provokes a perturbation of the tertiary structure through a conformational change that may either affect its enzymatic activity and/or interactions with other molecules (Narayanan and Jacobson, 2009;Johnson and Lewis, 2001). Thus, in addition to inducing changes in intrinsic activity, phosphorylation may also initiate creation of docking sites for interactions with other molecules. For example, phosphorylated serine residues appear to interact with forkhead-associated domains. Furthermore, phosphorylated tyrosine residues are known to interact with Src homology 2 (SH2) domains or other phosphotyrosine-binding (PTB) domains of the interacting proteins (Deribe et al., 2010). In the case of interaction between pTyrosine (pY) residues and SH2 domains, the pY residue binds to a conserved pocket in the cassette-like structure of the SH2 domain by ionic interactions with a strictly conserved arginine (Johnson and Lewis, 2001;Pawson, 2004). This interaction is further strengthened by the ability of the SH2 domain to interact with other N- and C-terminal residues in juxtaposition to the pY-binding domain.

Around 30% of all cellular proteins are estimated to be phosphorylated in vivo (Cohen, 2000). In this context, abnormal phosphorylation patterns have been demonstrated to be either the cause or result of numerous diseases, e.g., diabetes, rheumatoid arthritis and several types of cancer (Blume-Jensen and Hunter, 2001;Ostman et al., 2006). Drugs affecting kinases or phosphatases have been used to treat different diseases, and new modulators of these may

thus have therapeutic potential. In spite of the recent intensity with which phosphorylation events have been studied, extensive work remains in order to map signaling pathways associated with diverse biological processes and responses, and to identify the phospho-proteins involved (Kosako and Nagano, 2011;White, 2008).

1.2.2 PAG - a transmembrane adaptor protein

The process of immune-cell activation relies on organized and coordinated assemblies throughout the cell of both kinases and phosphatases, ultimately leading to the induction of cellular responses, which depend on the character of the stimulus and the receptors involved. Critical in this context are the transmembrane adaptor proteins (TRAPs), which act as molecular scaffolds that do not possess enzymatic activity, but provide docking sites for cytosolic effector molecules (Horejsi et al., 2004). The TRAPs ensure clustering of signaling complexes in the proximity of the plasma membrane, and contribute to co-localization and integration of the signals initiated by these. This condensation of signals is crucial for the efficient transduction of antigen receptor-induced signaling responses.

Currently, at least seven different TRAP members have been identified. These may be divided into two groups based on their ability to target specialized microdomains of the plasma membrane called lipid rafts. The lipid rafts serve as platforms for antigen receptor signaling in both B- and T cells, clustering the antigen receptors with effector molecules necessary for the initiation of productive signaling (Pierce, 2002;Simons and Toomre, 2000). One of these two TRAP groups encompasses the adaptor proteins that associate with the glycosphingolipid-enriched microdomains (GEMs). The members of this group include linker for activation of T cells (LAT) (Zhang et al., 1998), phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG; also referred to as the Csk-binding protein [Cpb]) (Brdicka et al., 2000;Kawabuchi et al., 2000), non-T cell activation linker (NTAL; also called the linker for activation of B cells [LAB]) (Brdicka et al., 2002;Janssen et al., 2003), and finally the Lck-interacting molecule (LIME) (Hur et al., 2003;Brdickova et al., 2003). The rest of the TRAP members belong to the group of TRAPs that are excluded from the lipid rafts, called the non-GEM associated TRAPs; the TCR-interacting molecule (TRIM) (Bruyns et al., 1998), SHP-2-interacting transmembrane adaptor protein (SIT) (Marie-Cardine et al., 1999) and linker for activation of X cells (LAX) (Zhu et al., 2002).

The GEM-associated TRAPs consist of a short extracellular domain, a single transmembrane region terminated by a CxxC palmitoylation motif, and a long cytoplasmic tail that contains a varying number of tyrosine based signaling motifs, which dictate their docking properties (Horejsi et al., 2004). The protein tyrosine kinases (PTKs) of the Src and spleen tyrosine kinase (SYK) families phosphorylate and regulate the binding properties of the GEM-associated TRAPs. Thus, these adaptor proteins serve an important regulatory function through their interactions with PTKs, playing a crucial role in connecting signals initiated by cell-surface receptors with downstream signaling pathways. Our investigations into the in vitro regulatory function of PAG in early BCR signaling are presented in Paper III. Thus, the remainder of this section will described this particular TRAP member in more detail.

The adaptor was identified simultaneously by two independent groups and was named PAG (Brdicka et al., 2000) or Cbp (Kawabuchi et al., 2000). The two discovering labs characterized PAG as a heavily phosphorylated protein with the ability to target lipid rafts and provide docking sites for the negative regulatory C-terminal Src kinase (Csk). As described in section 1.2.3, TCR-transduced signaling is initiated by the activation of the Src family of PTKs (SFKs) Lck and Fyn. The activities of these kinases are under negative control by Csk, which phosphorylates a conserved, inhibitory C-terminal tyrosine residue of Lck and Fyn, and thereby prevents their activation (Chow et al., 1993;Okada et al., 1991).

Compared with the other TRAPs, PAG appears to be unique in being ubiquitously expressed rather than restricted to specific hematopoietic cell lineages (Brdicka et al., 2000;Kawabuchi et al., 2000). In spite of being expressed by several different cell types, investigations regarding its function have mainly been performed using T cells and T cell derived cell lines. In resting T cells, the Src kinase FynT (the T cell specific form of Fyn) is responsible for keeping PAG in a heavily tyrosine-phosphorylated state (Yasuda et al., 2002;Shima et al., 2003). Human PAG carries ten tyrosine-based motifs where nine of these have been demonstrated to be susceptible for phosphorylation by PTKs (Ingley et al., 2006). In particular, phosphorylation of Y317 in human PAG (Y314 in murine PAG) provides a docking site for the SH2 domain of Csk (Brdicka et al., 2000;Kawabuchi et al., 2000). Association of Csk with PAG enhances the catalytic activity of this kinase, bringing it into the vicinity of its substrate and facilitating phosphorylation of the inhibitory tyrosine (pY505) of Lck (Takeuchi et al., 2000;Vang et al., 2001;Vang et al., 2003). In response to TCR stimulation, PAG is rapidly and transiently dephosphorylated, presumably by CD45, resulting

in dissociation of the PAG/Csk complex (Brdicka et al., 2000;Davidson et al., 2003;Torgersen et al., 2001). This is necessary in order for proper T cell activation to occur, indicating that Csk-mediated inhibition of Lck, being regulated by PAG, provides a negative regulatory feedback loop which sets the threshold for TCR activation. Accordingly, PAG, through its capacity to recruit Csk and inactivate the Src kinases, appear to negatively regulate T cell activation (Figure 6). Surprisingly, no effect was observed on embryogenesis, development of the thymus and the function of T cells in PAG knockout mice (Dobenecker et al., 2005;Xu et al., 2005), although a reduced level of raft-associated Csk was demonstrated (Xu et al., 2005). Taken together, these findings indicate that the development and activation of T cells is safeguarded by other compensatory mechanisms that have the ability to regulate the activity of Csk.

As mention in the beginning of this chapter, the signaling responses initiated by engagement of the TCR- and BCR receptors are remarkably similar. Combined with the fact that PAG is ubiquitously expressed, we attempted in Paper III to investigate whether this adaptor protein exhibit a similar regulatory role in antigen-induced signaling in B cells as has been found in T cells.

1.2.3 Overview of TCR signaling

During an immune response, T cells become activated by antigens through the engagement of the TCR and the associated CD3/ζ complex. This eight-chain complex constitutes at least two functional units. The extracellular domain of the clonotypic TCR αβ chains is involved in binding of the antigen, while the ζ chains and the CD3 subunits (γ, δ and ε) participate in signal transduction. All of the TCR/CD3 complex subunits are transmembrane proteins. The antigen-recognizing α and β chains each contains a single membrane-spanning region with very short cytoplasmic domains. The CD3/ζ subunits have larger cytoplasmic tails that participate in signal transduction. Experiments performed nearly three decades ago in an attempt to test the independent role of the different components of the TCR, unraveled that the co-expression of the CD3/ζ subunits with the αβ chains were necessary for an efficient expression of the receptor to occur (Weiss and Stobo, 1984). Although it was discovered quite early that the CD3/ζ subunits following TCR ligation transduce the signals, it remains controversial exactly how the first signal is transduced (Smith-Garvin et al., 2009).

Upon binding to the MHC complex, the TCR undergoes conformational changes leading to activation through phosphorylation of the activation site of either one or both of the SFKs Lck and Fyn (Straus and Weiss, 1992;Veillette et al., 1988b;Veillette et al., 1989), which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth, 1989) in the cytoplasmic portions of the TCR-associated CD3- and ζ chains (Samelson et al., 1986). Lck and Fyn have been reported to associate with both the TCR (Samelson et al., 1990) and the CD4- and CD8 coreceptors (Veillette et al., 1988a;Barber et al., 1989). Nevertheless, earlier studies have demonstrated a more specific role for Lck than Fyn with regard to regulation of the tyrosine phosphorylation of the ITAMs (van Oers et al., 1996). Phosphorylated ITAMs contain two tyrosine residues which serve as docking sites for the tandem SH2 domains of the PTK ζ-chain-associated protein of 70 kDa (ZAP70) (Chan et al., 1991;Chan et al., 1992;Weiss and Littman, 1994), a member of the SYK family of non-receptor tyrosine kinases. Consequently, ZAP70 associates with the TCR only when the cell becomes activated following ligation of the receptor (Chan et al., 1991). Importantly, this positions ZAP70 in the vicinity of Lck, which activates the former through phosphorylation of key tyrosine residues (Chan et al., 1992) (Figure 6). Activated ZAP70 phosphorylates the transmembrane adaptor molecule LAT of 36-38 kDa (Zhang et al., 1998) and subsequently the cytosolic adaptor molecule SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (Slp-76) (Bubeck et al., 1996), leading to formation of a LAT/Slp-76 nucleated signaling complex (Smith-Garvin et al., 2009). This complex orchestrates the activation of signaling proteins involved in a number of processes important for T cell activation including reorganization of the cytoskeleton through activation of phospholipase Cγ1 (PLCγ1), which controls the calcium- and diacylglycerol (DAG)-mediated activation of calcineurin, nuclear factor of activated T cells (NFAT), protein kinase C θ (PKCθ) and Ras guanyl nucleotide-releasing protein (RasGRP) (Figure 6). RasGRP is a positive regulator of Ras and hence the Ras-Raf-Mek-Erk signaling pathway, leading to activation of the transcription factor activator protein 1 (AP1) (Smith-Garvin et al., 2009). PKCθ, on the other hand, is implicated in signaling events eventually activating the transcription factor, nuclear factor κB (NF-κB), which is a key regulator of immunological reactions. However, in contrast to NFAT and AP1, NF-κB activation is very weak when only the TCR is triggered (Kane et al., 2001).

A central theory in T cell activation is that induction of the TCR in the absence of proper co-ligation of other cell surface receptors keeps the T cells in a non-responsive state called anergy, in which the cells become refractory to restimulation rather than get activated and

proliferate (Appleman and Boussiotis, 2003). For this reason, it is accepted in the field that additional signals to TCR-induced signaling is necessary in order to ensure productive T cell activation instead of inducing anergy (Smith-Garvin et al., 2009). Costimulation is discussed in the next section.

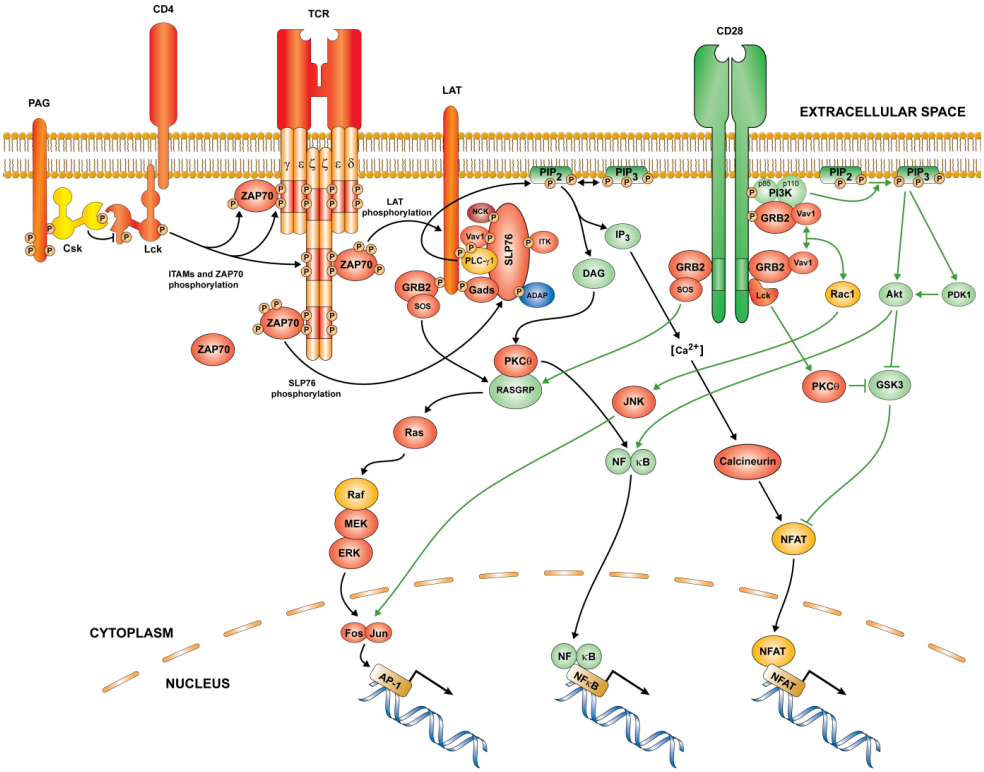

Figure 6. Overview of some of the signaling pathways involved in T cell activation. See the text for further details. This grafic illustration combines information from numerous publications from the T cell signaling field.

1.2.4 Coreceptors for signal transduction initiated by the TCR

Based on the fact that TCR signaling alone is not sufficient to induce activation of NF-κB, concurrent costimulatory signals have been suggested to play a key role in the ultimate activation of this transcription factor. The role of costimulatory molecules is at least two-fold, involving both reduction of the threshold for TCR signaling and contribution of additional signals necessary for optimal T cell activation, pushing the cells from a resting state to commit to proliferation and subsequent differentiation (Iezzi et al., 1998). The underlying

molecular mechanisms for the effects of costimulation remain in part elusive, but most probably involve augmentation of signaling pathways that also are activated by TCR stimulation alone (Acuto and Michel, 2003). For instance, costimulation with CD28 will both lower the threshold for TCR signaling and induce robust activation of NF-κB (Kane et al., 2001;Kane et al., 2002;Schulze-Luehrmann and Ghosh, 2006). Two signaling pathways have been implicated to explain the link between CD28 and NF-κB, and there may even be crosstalk between these. The first pathway involves growth-factor receptor-bound protein 2 (Grb2)/Vav-mediated activation of PLCγ1/PKCθ (Marinari et al., 2002;Zhang et al., 1995;Tarakhovsky et al., 1995), while the second encompasses activation of phosphatidylinositol 3-kinase (PI3K) (Figure 6). As CD28 lack enzymatic activity, it mediates signaling through the binding of SH2 and SH3 domains of intracellular signaling proteins to corresponding tyrosine and proline-based motifs of its cytoplasmic tail (Ward, 1996). Phosphorylation by either Lck or Fyn of one well-studied motif of CD28, the YMNM sequence (Prasad et al., 1994;Hutchcroft and Bierer, 1994), leads to binding of the SH2 domain of PI3K (August and Dupont, 1994;Pages et al., 1994;Prasad et al., 1994;Stein et al., 1994;Truitt et al., 1994) and the adaptor protein Grb2 (Raab et al., 1995;Schneider et al., 1995). Grb2 also bind via its two SH3 domains to the C-terminal diproline motif of CD28 (Kim et al., 1998;Okkenhaug and Rottapel, 1998). Activation of PI3K initates phosphorylation of phosphatidylinositol with the resulting production of phosphatidylinositol-(3,4)-biphosphate (PIP_2) and phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) (Okkenhaug and Vanhaesebroeck, 2003;Rudd et al., 2009). These lipids, known as D-3 lipids, will associate with the plasma membrane and subsequently recruit other signaling proteins such as phosphoinositide-dependent protein kinase 1 (PDK1) through the binding of their pleckstrin homology (PH) domains (Okkenhaug and Vanhaesebroeck, 2003). Activation of PDK1 facilitates activation of protein kinase B (PKB/Akt), which in turn induces auto-phosphorylation, activation and regulation of other downstream signaling events (Riha and Rudd, 2010;Rudd et al., 2009). Recruitment of Grb2 to the YMNM motif of CD28 initates signaling through activation by phosphorylation of Vav1 or the binding of the exchange factor Son of Sevenless (SOS) (Riha and Rudd, 2010). SOS is an activator of the Ras GTPase (Boriack-Sjodin et al., 1998) and consequently induces activation of the MAPK/Erk pathway (Figure 6). Cooperation of Grb2 with Vav1 leads to activation of Rac1, which in turn activates c-Jun N-terminal kinase (JNK) (Kim et al., 1998) that is necessary for translocation of PKCθ to the site of activation (Riha and Rudd, 2010). Studies have shown that Vav1 and

Rac1 coordinately facilitate micro-clustering of kinases and adaptor proteins into immunological synapses that are necessary in order to ensure optimal T cell activation (Monks et al., 1998;Grakoui et al., 1999).

The importance of the costimulatory contribution of CD28 has been tested in CD28-deficient mice, where a reduced proliferation of T cells in response to antigenic stimulation has been observed (Green et al., 1994;Shahinian et al., 1993). However, repeated antigen stimulation or long-term viral infections have shown to overcome CD28 deficiency. Interestingly, the relatively mild phenotype associated with CD28 deficiency suggests that other costimulatory molecules can compensate for the loss of CD28, and CD2 has been proposed to play such a role (Green et al., 2000;Sasada and Reinherz, 2001;Shahinian et al., 1993). CD2 has been demonstrated to act both as an adhesion molecule as well as a signaling molecule (Bachmann et al., 1999;Bierer et al., 1988;He et al., 1988;Kanner et al., 1992;Moingeon et al., 1989), although its role in T cell activation is not clearly defined. Results from previous studies indicate that CD2 cross-linking can induce proliferation of T cells and secretion of cytokines, both events mediated through the activation of ZAP70 (Martelli et al., 2000;Meinl et al., 2000). Independent of TCR stimulation, interactions between CD2 and its ligand CD58 appear to induce signaling through formation of CD2 clusters in distinct membrane microdomains (Kaizuka et al., 2009). Additionally, transgenic expression of CD2 in developing thymocytes results in increased apoptosis at the double positive stage, suggesting that CD2 generates signals that resemble the ones derived from the TCR (Melton et al., 1996). Along these lines, both Lck and Fyn have been shown to interact with CD2 and to become activated in response to CD2 cross-linking (Bell et al., 1996;Carmo et al., 1993;Danielian et al., 1992;Lin et al., 1998). Interestingly, the ITAM domains of the TCR-associated CD3- and ζ chains appear to be required for propagation of CD2-mediated signals (Bockenstedt et al., 1988;Moingeon et al., 1992;Alcover et al., 1988). Additionally to be important for the formation of IS (Kaizuka et al., 2009), some studies indicate that activation through CD2 induces exclusion of CD45 and enrichment of PKCθ in the interface between the T cells and their cognate APCs (Dustin et al., 1998;Bagnasco et al., 1989;Zaru et al., 2002). Notably, the identification of the function of this coreceptor has been complicated by the fact that CD2 knockout mice only show partial defects in immune responses (Killeen et al., 1992). Thus, similar to CD28-deficient T cells, CD2-deficient T cells are only mildly affected, indicating some redundancy. However, the combined lack of CD28 and CD2 led to profound defects in activation and proliferation of T cells (Green et al., 2000), suggesting the necessity of at least

one of these two costimulatory molecules for induction of proper T cell function. In Paper II, we investigated the relative contribution of CD2 and/or CD28 in induced activation or inactivation by means of phosphorylation of known signaling molecules downstream of the TCR.

1.2.5 BCR-induced signaling responses

Similar to the TCRs, the BCRs are composed of two functional units. As described in section 1.1.5, the membrane bound Ig constitutes the antigen-binding subunit of the BCR, ensuring antigen recognition. The other functional unit of the antigen receptor is composed of a non-covalently disulphide-linked Igα/Igβ heterodimer, which is responsible for initiating intracellular signaling upon antigen binding (Figure 7). Comparable to activation of the TCR, ligation of the BCR initially leads to phosphorylation of the Igα/Igβ ITAMs with resulting conformational opening of their cytoplasmic domains (Reth and Wienands, 1997;Tolar et al., 2005). In contrast to TCR-induced signaling, this initial phosphorylation process is mediated preferentially by the SFKs Lyn rather than Lck (Dal Porto et al., 2004;Sohn et al., 2006). B cells do also express other SFKs, specifically Fyn, Blk, Fgr and Hck, but in contrast to Lyn, mutations of these have demonstrated minor effect on B cell development and activation (Lowell and Soriano, 1996;Texido et al., 2000).

The phosphorylated ITAMs in the open conformation serve as docking sites for the SH2 domain-containing molecule SYK, which sequentially leads to enhanced catalytic activity of the kinase (Rowley et al., 1995). Particularly, activation of SYK has been demonstrated to be one of the key mediators in antigen-induced B cell activation, as disruption of *SYK* inhibits activation of more distal effector molecules (Jiang et al., 1998;Takata et al., 1994) as well as markedly blocks B cell development (Turner et al., 1995). Notably, in BCR signaling, SYK acts as the counterpart of ZAP70 in T cell activation. Whereas ZAP70 is exclusively expressed in T and NK cells (Chan et al., 1992), SYK appears to be the SYK family of PTKs that is preferentially expressed in B cells, myeloid cells and thymocytes (Taniguchi et al., 1991). The recruitment of SYK to the BCR complex leads to phosphorylation of the cytosolic adaptor protein B cell linker (BLNK), also known as SLP65 or BASH, which is the functional counterpart of the combined complex of LAT and SLP76 in T cell activation (Fu et al., 1998;Wienands et al., 1998;Goitsuka et al., 1998;Kurosaki and Tsukada, 2000). Employment of BLNK to the BCR complex leads to SYK-dependent activation of the TEC-family of PTK

Bruton's tyrosine kinase (Btk) (Kurosaki and Kurosaki, 1997;Baba et al., 2001). Clustering of Btk, SYK and BLNK results in a nucleated signaling complex that orchestrates binding and activation of key effector molecules such as Vav, Grb2 and PLCγ2 (Fu and Chan, 1997;Fu et al., 1998;Hashimoto et al., 1999;Ishiai et al., 1999;Wienands et al., 1998;Chiu et al., 2002). PLCγ2 in B cells corresponds to PLCγ1 in T cells (Kurosaki and Tsukada, 2000). Hence induced PLCγ2 increases the production of inositol-(1,4,5)-triphosphate (IP_3) and DAG. IP_3 activation causes a surge in intracellular Ca^{2+} levels (Scharenberg et al., 2007) that result in differential activation of at least two divergent signaling pathways: calmodulin-calcineurin-NFAT and PKCβ-NF-κB (Dolmetsch et al., 1997;Petro and Khan, 2001;Saijo et al., 2002;Su et al., 2002). Production of DAG leads to recruitment of PKCβ and RasGRP3, where the former phosphorylate the latter in order to ensure full activation of RasGRP3. Immediately after activation, GTP-bound Ras directly bind Raf1, initiating the Mek-Erk signaling pathway that activates AP1 (Dal Porto et al., 2004;Hashimoto et al., 1998) in a similar manner as induced upon TCR stimulation.

Although the BCR complexes are the primary signal transducers in B cell activation, receptor-associated adaptors and coreceptors may significantly influence BCR-induced signaling both qualitatively and quantitatively. One important coreceptor in this respect is the transmembrane protein CD19, a crucial contributor in B cell activation (Dal Porto et al., 2004;Carter and Fearon, 1992). Ligation of CD19 leads to enhanced activation of B cells much in the same way as CD28 influences T cell activation (Buhl et al., 1997;Carter and Fearon, 1992;Otero et al., 2001;Tuveson et al., 1993). Upon ligation of the BCR, Lyn rapidly phosphorylates multiple tyrosine residues of the cytoplasmic domain of CD19 (Fujimoto et al., 2000). This brings about the recruitment of SH2-domain-containing molecules such as the p85 subunit of PI3K, Lyn, Btk and Vav (Fujimoto et al., 2000;Tuveson et al., 1993;Weng et al., 1994;Buhl and Cambier, 1999;Wang et al., 2002). As in T cells, induction of PI3K leads to activation of PKB/Akt (Figure 7), which further inhibits the glycogen synthase kinase 3 (GSK3) by means of phosphorylation (Gold et al., 2000). Notably, the induction of the PI3K-PKB/Akt initiates numerous signaling cascades that ultimately activate the transcription factors NFAT and NF-κB, both essential for proper B cell activation (Beitz et al., 1999;Gold et al., 2000). In the same way as for T cell, NF-κB is important for both B cell development and proliferation (Schulze-Luehrmann and Ghosh, 2006). Although activation of PI3K is mainly mediated through CD19, modest PI3K activity is observed in CD19 deficient B cells following BCR ligation (Buhl and Cambier, 1999). This may partly be explained by the weak

ability of SYK to activate PI3K upon BCR-engagement. In addition to activation of the PI3K-Akt-GSK3 pathway, associated linking of CD19 to ligation of the BCR contributes to enhancement of the intracellular calcium flux and activation of the Erk, JNK and p38 MAPK pathways (O'Rourke et al., 1998;Li and Carter, 2000).

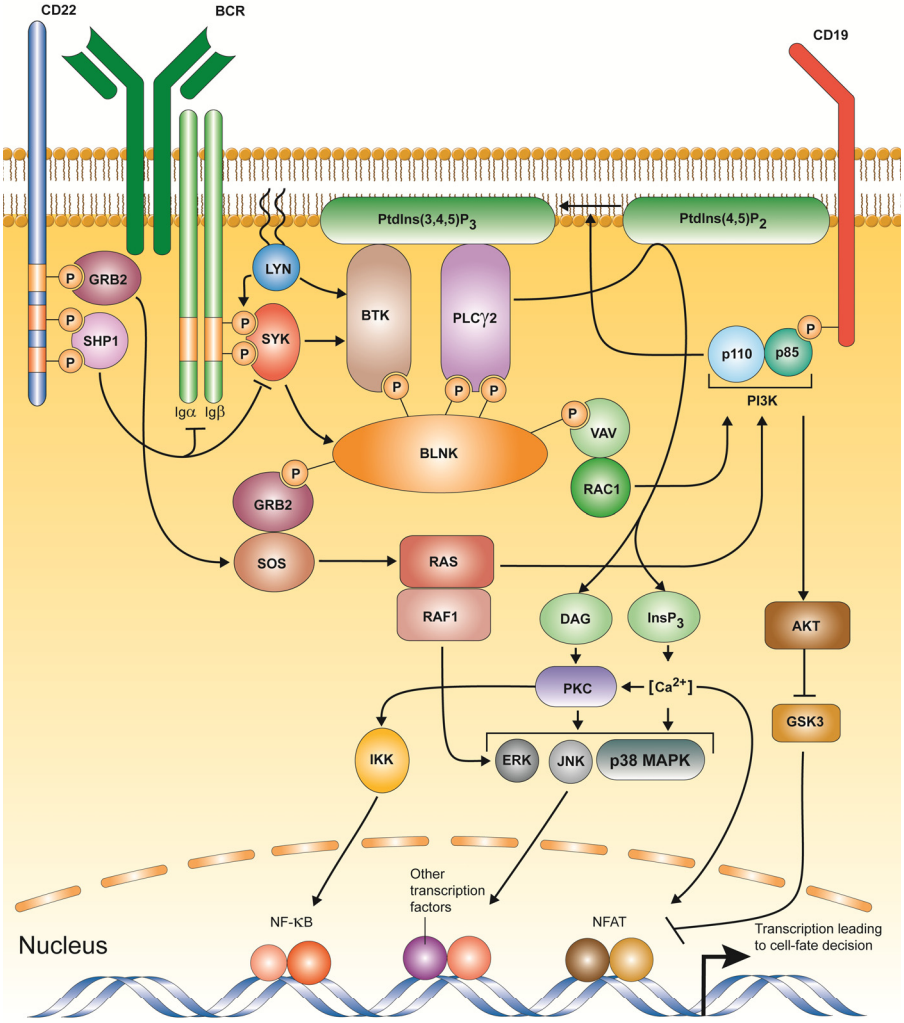

Figure 7. Overview of signaling pathways induced following ligation of the BCR. See the text for further details. Adapted from (Niiro and Clark, 2002;Scharenberg et al., 2007).

The transmembrane immunoglobulin-like lectin CD22 is another crucial coreceptor in B cell activation. In contrast to CD19, which facilitates B cell activation, CD22 has the ability to modulate B cell activation negatively as well as positively (Nitschke, 2005;Sato et al., 1996).

Activation of CD22 induces the phosphorylation of one or more of its cytoplasmic ITAMs, thereby promoting the binding of the adaptor protein Grb2. The SH2 domain-containing tyrosine phosphatase 1 (SHP1) is also recruited to the cytoplasmic domain of the rapidly phosphorylated CD22 (Blasioli et al., 1999;Doody et al., 1995). However, in contrast to Grb2, SHP1 is recruited to two of the inhibitory tyrosine residues of CD22 collectively known as immunoreceptor tyrosine-based inhibitory motifs (ITIMs), rather than to its ITAMs (Blasioli et al., 1999;Otipoby et al., 2001). Thus, docking of SHP1 to CD22 inhibits rather than promotes BCR signaling (Figure 7), thereby providing a negative feedback loop which contribute to tightly regulation of the BCR response (Nitschke, 2005).

As outlined in section 1.2.2, PAG may be yet another modulator of BCR-induced signaling. It should be noted that the final outcome of BCR activation depends on the concomitant activation and recruitment of several coreceptors and adaptor molecules (some of which may still be unknown), and that any observed regulatory effect therefore should be interpreted with caution. See the discussion of Paper III (chapter 4.3) for more details about the regulatory role of PAG in B cell activation.

1.3 Immune regulation by regulatory T cells

Regulatory networks are required throughout the body to maintain homeostasis in the tissues and ensure the integrity of the host. The immune system also contains a small fraction of cells which are capable of regulating other immune cells, thereby limiting tissue damage and autoimmunity. The most studied of these cells are the so called $CD4^+CD25^+$ regulatory T cells (Tregs) (Sakaguchi et al., 1995), which have the ability to suppress both activation, proliferation and function of other immune cells such as $CD4^+$ and $CD8^+$ T cells, NK T cells, NK cells, B cells, macrophages and DCs (Shevach, 2009). The function of Tregs has been demonstrated to be important for both maintenance of immune tolerance and prevention of autoimmune and inflammatory diseases (Sakaguchi et al., 2008;Horwitz, 2008;Viglietta et al., 2004;Ehrenstein et al., 2004;Lindley et al., 2005). Furthermore, Tregs appear to be key regulators of immune responses to infections of viral, bacterial or parasitic origin, in addition to possess the ability to restrain immunity directed towards both tumors and transplanted tissues (Belkaid, 2007;Nishikawa and Sakaguchi, 2010). The most characteristic feature of Tregs is their high expression of the transcription factor forkhead box P3 (FOXP3), known to confer to Tregs regulatory capacity by starting a Treg specific transcriptional program

(Fontenot et al., 2003;Hori et al., 2003;Khattri et al., 2003;Sakaguchi et al., 2010). The role of FOXP3 in Tregs is described in more detail in the following section.

1.3.1 FOXP3 – an essential regulator of regulatory T cells

The importance of Tregs has become clear through the appreciation that their dysfunction, due to e.g. mutations in the *FOXP3* gene, leads to fatal autoimmune diseases, immunopathology and allergy (Zhu et al., 2010), while adoptive transfer of Foxp3-expressing $CD4^+$ T cells inhibited development of autoimmunity (Tang et al., 2004;Mottet et al., 2003) and prevented graft rejection following organ transplantation (Waldmann et al., 2006).

In 2003, two years after several groups had characterized Tregs as $CD4^+CD25^+$ T cells (Dieckmann et al., 2001;Baecher-Allan et al., 2001;Levings et al., 2001;Ng et al., 2001;Jonuleit et al., 2001), *Foxp3* was described as the master control gene for both the development and function of Tregs in mice (Fontenot et al., 2003;Hori et al., 2003;Khattri et al., 2003). The key role of the *Foxp3* gene was first shown in the mouse strain Scurfy, which has a defective, X-linked mutant of the *Foxp3* gene (Brunkow et al., 2001). This mutant had earlier been found to be lethal in hemizygous males within 3-4 weeks of age (Godfrey et al., 1991), exhibiting elevated production of pro-inflammatory cytokines (Kanangat et al., 1996) and extensive activation of $CD4^+$ T cells (Blair et al., 1994;Clark et al., 1999). These consequences were characterized by scaliness of the skin and lymphatic infiltrates in multiple organs, hence the name Scurfy (Godfrey et al., 1991). A few years after the identification of Foxp3 (scurfin) as a determining factor in murine Tregs, the human homolog, FOXP3, was found (Roncador et al., 2005). As with Scurfy mice containing a dysfunctional *Foxp3* gene, mutations of the *FOXP3* gene in humans has been linked to a severe autoimmune disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett et al., 2001;Wildin et al., 2001). The regulatory role of FOXP3 in Treg function was further emphasized by the finding that ectopic expression of Foxp3 conferred suppressive properties to non-regulatory peripheral $CD4^+CD25^-$ T cells in mice (Hori et al., 2003;Khattri et al., 2003;Fontenot et al., 2003), with induced expression of various cell surface molecules such as CD25, cytotoxic T lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor (TNF)-receptor-related (GITR) protein – all of which are characteristically expressed at high levels in Tregs (Sakaguchi et al., 1995;Takahashi et al., 2000;Shimizu et al., 2002;McHugh et al., 2002).

In spite of the fundamental role of FOXP3 in immune homeostasis, some discrepancies have been identified between human and mouse Tregs regarding FOXP3 expression. In mice Foxp3 seems to be a robust marker for both induced and thymus-derived Tregs (Hori et al., 2003;Khattri et al., 2003;Fontenot et al., 2003). In humans, however, the expression of FOXP3 may become up-regulated not only in Tregs but also in non-regulatory effector T cells. Hence, when effector T cells are activated through the TCR, the expression of FOXP3 increases, though generally at lower and more transient levels than in Tregs (Gavin et al., 2006;Yagi et al., 2004). These data indicate that elevated levels of FOXP3 in humans could reflect an immune activation rather than the induction of regulatory mechanisms. Moreover, functional studies of FOXP3 have revealed that transient expression of the protein is insufficient to confer regulatory properties to human $CD4^+$ T cells (Allan et al., 2005;Allan et al., 2007;Gavin et al., 2006;Morgan et al., 2005;Tran et al., 2007;Wang et al., 2007a). In contrast, a high and constitutive expression of FOXP3, independent of TCR stimulation, has been demonstrated to give rise to $CD4^+$ T cells exhibiting potent suppressive activity (Allan et al., 2008). It has been reported that effector T cells in patients with IPEX show an impaired capacity of cytokine production, which is not the case in Scurfy mice (Bacchetta et al., 2006). Overall, these findings suggest a more intricate relationship between FOXP3 and the regulatory capacity of Tregs in humans than in mice, and also that additional factors may be required for Tregs to acquire full suppressive capacity. Furthermore, the influence of FOXP3 in human cells may exceed its role in the development and function of Tregs, for example by contributing to enhanced cytokine production in effector T cells, as supported by the observation that $CD4^+$ effector T cells with cytokine-producing capability express low levels of FOXP3 (Miyara et al., 2009). Finally, studies performed recently demonstrate that the main function of FOXP3 may be to stabilize the Treg function once the fate of the Tregs are determined, rather than controlling the development and function of these cells (Gavin et al., 2007).

1.3.2 Regulatory T cell subsets and modes of action

A growing body of evidence indicates that Tregs, rather than being one distinct T cell lineage, comprise a number of different cell populations that collaboratively enforce immune homeostasis. Briefly, the Tregs have been categorized as either naturally occurring Tregs (nTregs), which are derived from the thymus and express FOXP3 (Sakaguchi et al., 1995;Sakaguchi et al., 2001;Shevach et al., 2001;Shevach, 2002), or inducible Tregs (iTregs),

which are induced in secondary lymphoid organs or peripheral tissues in response to inflammatory stimuli (Apostolou and von, 2004;Curotto de Lafaille and Lafaille, 2009;Knoechel et al., 2005;Kretschmer et al., 2005). Thus, the main distinction between iTregs and nTregs is that nTregs originate in the thymus as a distinct T cell lineage with specialized suppressive properties (Itoh et al., 1999;Sakaguchi et al., 1982), whereas iTregs acquire their regulatory capacity in the periphery when stimulated by specific antigens under certain conditions defined by the presence of particular sets of cytokines (Curotto de Lafaille and Lafaille, 2009;Josefowicz and Rudensky, 2009;Sakaguchi et al., 2008). As a result, iTregs are generated by some of the same processes that lead to the development of effector T cells and may therefore represent a natural consequence of protective immunity. Accordingly, the TCR repertoire of iTregs are similar to, whereas those of nTregs significantly differ from, the TCR repertoire of conventional T cells (Hsieh et al., 2006;Pacholczyk et al., 2006;Scheinberg et al., 2007;Wong et al., 2007). Importantly, the broad TCR repertoire of nTregs, with specificities that matches both naive and autoreactive T cells, may enable them to control immunological as well as immunopathological reactions.

Although nTregs and iTregs are difficult to discriminate phenotypically, even with respect to their expression of FOXP3, they may differ in function and stability (Curotto de Lafaille and Lafaille, 2009). It has been shown that iTregs contribute minimally to the pool of Tregs in homeostatic conditions and that they are more likely to hold back an escalating immune response than to prevent it in the first place. Importantly, it has been hypothesized that the main function of nTregs is to protect against autoimmune responses in the periphery by neutralizing self-reactive T cells that have escaped negative selection in the thymus. However, recent studies indicate that nTregs are preferentially recruited and expanded in infectious settings instead of the conversion of conventional T cells to iTregs (Feuerer et al., 2009). Along this line, iTregs appear to act locally and preferentially at mucosal tissues, such as the intestines and the lungs, and in the skin (Campbell and Koch, 2011). In these tissues, they are destined to modulate an ongoing immune response in order to prevent the infected tissues from being needlessly damaged once an infection has been cleared.

The activity of Tregs depends on several factors: the cytokine environment, the strength and duration of TCR stimulation, and the nature and extent of costimulatory signals. A number of mechanisms for Treg-mediated suppression have been proposed (Figure 8), including the secretion of immunosuppressive cytokines, killing through cytolysis, disruption of the

metabolic function of target cells, and inhibition of DC maturation and/or functional modification of DCs (Campbell and Koch, 2011;Shevach, 2009;Vignali et al., 2008). Notably, a recent study in mice (Wing et al., 2008) has demonstrated that one of these mechanisms, mediated by CTLA-4 which is constitutively expressed by Tregs (Read et al., 2000;Takahashi et al., 2000), is crucial for their suppressive function both in vitro and in vivo. It is not yet clear whether one or several of the proposed mechanisms need to be activated at the same time in order to ensure maximal suppressive function (Vignali et al., 2008). However, both the site of action and the inflammatory environment are likely to influence the mechanism(s) of suppression exerted by the Tregs (Campbell and Koch, 2011).

As indicated in section 1.3.1, FOXP3 may be expressed by suppressive as well as non-suppressive T cells in humans, suggesting that $FOXP3^+$ T cells are functionally heterogeneous and consequently that FOXP3 does not equivocally identify human Tregs. A recent publication by Miyara et al. suggested that $CD4^+$ T cells in human blood may be divided into five different categories based on their combined expression of CD45RA/RO, CD25 and FOXP3 (Miyara et al., 2009) (Table 1).

Table 1. Phenotypes of defined subsets of $CD4^+$ T cells.

Cell subset	Phenotypic features
conventional naive T cells	$CD45RA^+/CD45RO^-CD25^-FOXP3^-$
conventional effector/memory T cells	$CD45RO^+/CD45RA^-CD25^-FOXP3^-$
cytokine-producing effector T cells	$CD45RO^+/CD45RA^-CD25^+FOXP3^+$
resting regulatory T cells (rTregs)	$CD45RA^+/CD45RO^-CD25^+FOXP3^+$
activated regulatory T cells (actTregs)	$CD45RO^+/CD45RA^-CD25^{++}FOXP3^{++}$

Two of these populations constitute the nTregs compartment, specifically the resting Tregs (rTregs) and activated Tregs (actTregs). While the rTregs were considered “naive” Tregs expressing CD45RA, the actTregs appeared to have an effector/memory phenotype as they expressed CD45RO (Miyara et al., 2009) (Table 1). This is in accordance with earlier observations published by other groups (Fritzsching et al., 2006;Seddiki et al., 2006;Valmori et al., 2005). Hence, the "naive" rTregs may be considered functional precursors of actTregs (Fritzsching et al., 2006;Ito et al., 2008;Miyara et al., 2009). The observation that rTregs in response to activation can proliferate and differentiate into $Ki\text{-}67^+FOXP3^{++}CD45RO^+$ cells with suppressive capacities comparable to actTregs supports this notion (Miyara et al., 2009).

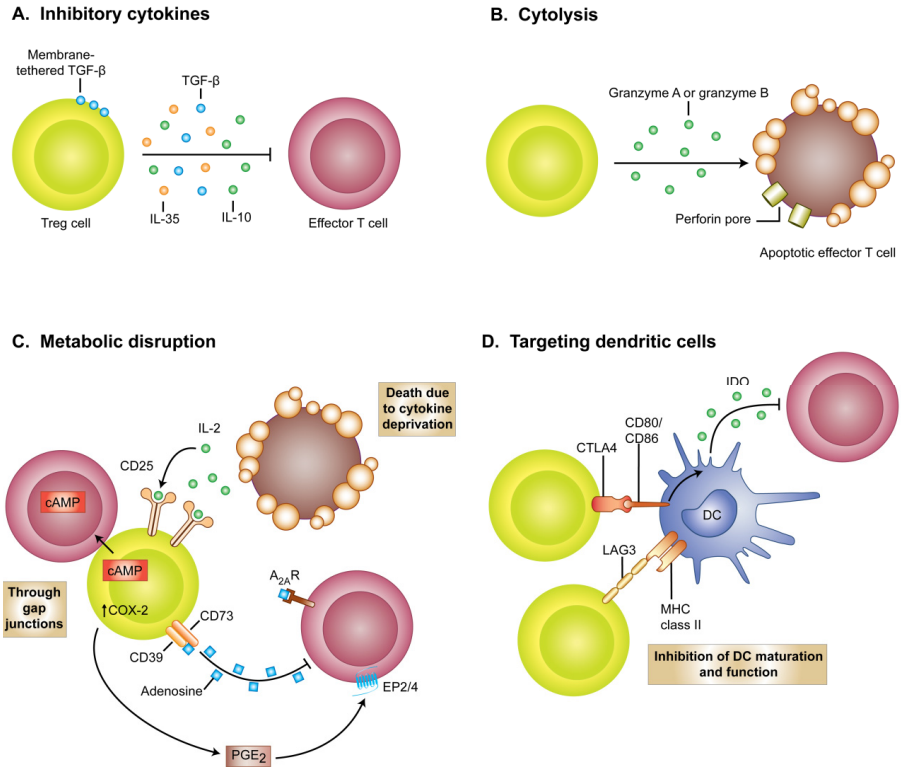

Figure 8. Overview of currently proposed suppressive mechanisms exerted by Tregs. The potential mechanisms for suppression exerted by Tregs can functionally be divided into four basic groups of action. (A) Secretion of inhibitory cytokines, such as the immunosuppressive cytokines IL-10, IL-35 and TGF-β. (B) Directly killing target cells by means of secreted serine proteases (granzyme A and B) together with perforin, leading to cytolysis. (C) Disruption of the metabolic activity of target cells, including induced apoptosis through CD25-dependent deprivation of IL-2 in the microenvironment; cyclic adenosine 3', 5' monophosphate (cAMP)-mediated inhibition by means of gap-junctions; adenosine receptor 2A ($A_{2A}R$)-mediated immune suppression generated by CD35- and/or CD73; and COX-2–PGE_2–$EP_{2/4}$-mediated T cell inhibition as observed in iTregs (see section 1.4.3.). (D) Functional modification or inhibition of maturation of the DCs required for activation of effector T cells. Suggested mechanisms include pairing of lymphocyte-activation gene 3 (LAG3) on Tregs with MHCII on the surface of the DCs; or interaction between CTLA-4 and CD80/86 on Tregs and DCs, respectively, leading to induced secretion by DCs of indoleamine 2,3-dioxygenase (IDO), a potent regulatory molecule that is known to exert immunosuppressive mechanisms. Adapted from (Vignali et al., 2008;Yaqub and Tasken, 2008).

However, rTregs in the periphery are not expected to be true naive T cells (i.e. cells that have not yet experienced maturation by TCR-stimulation) as Tregs require continuous stimulation through the TCR in order to preserve (Fisson et al., 2003;Darrasse-Jeze et al., 2009). Interestingly, it has been known for decades that a small proportion of activated $CD4^+$ T cells indeed express CD45RA and not CD45RO (Brod et al., 1989). Recently, a proportion of the

$FOXP3^+$ population of $CD4^+$ T cells was shown to constitute a non-suppressive subpopulation, $CD4^+CD45RA^-FOXP3^+$ T cells, which had the ability to secrete high levels of pro-inflammatory cytokines such as IL-2, IFN-γ and IL-17. This subset comprises cells with a Th17 lineage potential (Miyara et al., 2009).

Based on the fact that all the $CD4^+FOXP3^+$ T cells combined constitute less than 10% of all $CD4^+$ T cells in the peripheral blood (Miyara and Sakaguchi, 2011), traditional biochemical analysis of the signal transduction in these cells has been challenging. However, the recent design of a technique combining FCB (Krutzik and Nolan, 2006) with phospho-flow cytometry enabled us to increase the resolution to a level where signaling may be studied in small subsets of cells and linked to their functional properties (Kalland et al., 2011;Krutzik and Nolan, 2003;Krutzik et al., 2004). Understanding the mechanism of Treg-mediated suppression is a key issue of current immunological research. The recent findings that $FOXP3^+CD4^+$ T cells constitute a heterogeneous population of both regulatory and non-regulatory subsets of T cells highlight the necessity of being able to distinguish between the different subsets while analyzing their behavior, instead of examining the whole population as a uniform entity. In Paper II, we attempted to characterize the various T cell subsets on a "per cell" basis, studying the signaling pathways that were activated following stimulation through the TCR under a set of different conditions.

1.3.3 Antigen-induced TCR signaling in Tregs

Previous studies have demonstrated that human Tregs need to receive activation signals through the TCR in order to become functionally suppressive (Dieckmann et al., 2001;Levings et al., 2001). The antigen concentration necessary to activate Tregs has been estimated at 10- to 100-fold below the levels required to activate naive T cells with the same antigen specificity (Takahashi et al., 1998). However, once activated by one particular antigen, the Tregs are capable of suppressing other cells irrespective of antigen specificity. Importantly, the suppressive potency of Tregs depends on the strength and the quality of the stimulus provided (Shevach, 2009). In the presence of strong costimulatory signals and/or a powerful TCR signal, effector T cells may acquire resistance to the suppression mediated by Tregs, or this may even compromise the suppressive competence of the Tregs (Baecher-Allan et al., 2001;Sakaguchi et al., 2010). Thus, human Tregs are not able to suppress proliferation

of effector T cells under conditions where the immune cells are strongly activated, which is the case at the beginning of an infection.

Tregs have been demonstrated to display TCR responses that are different from those regularly observed in conventional naive and effector/memory T cells (see section 1.2.3). Although nTregs recognize antigens by means of the αβ TCR and show a similar repertoire in size compared to conventional T cells, the composition and characteristic of their TCR repertoire largely differ from that seen for conventional T cells (Hsieh et al., 2006;Pacholczyk et al., 2006;Wong et al., 2007). Whereas the engagement of the TCR induces activation and proliferation of conventional T cells, several human and murine studies have demonstrated that isolated Tregs fail to proliferate in response to stimulation through the TCR (Baecher-Allan et al., 2001;Itoh et al., 1999;Li et al., 2005); neither do they produce pro-inflammatory cytokines such as IL-2, IL-4 and IFN-γ (Allan et al., 2005;Jonuleit et al., 2001). Regardless of these observations, they can not be totally unresponsive to the TCR stimulation as this is required for the Tregs to acquire suppressive competence (Dieckmann et al., 2001;Levings et al., 2001). This implies that TCR stimulation in Tregs induces intracellular signaling pathways other than those known to be activated in conventional T cells, resulting in the induction of suppressor function rather than proliferation and differentiation. Indeed, murine Tregs demonstrate both reduced PLCγ1 activation and defective calcium mobilization in response to TCR stimulation (Carson and Ziegler, 2007;Gavin et al., 2002). Furthermore, a comprehensive study in mice demonstrated that the lack of IL-2 production by Tregs was due to failure in the activation of the PKC and Ras/Erk pathways in response to TCR stimulation (Hickman et al., 2006). Supporting these data, a previous report using freshly isolated, primary human T cells show reduced phosphorylation of the CD3/ζ chains, associated with poor recruitment of ZAP70 and lower phosphorylation of SLP76, indicating reduction of proximal signaling events in these cells following TCR stimulation (Tsang et al., 2006). As in the previously mentioned murine studies, the reduced proximal signaling after TCR stimulation led to defective activation of downstream signaling events, such as the elevation of intracellular calcium and the activation of Erk. In contrast to anergic cells, Tregs were unable to regain effector function upon adoptive transfer (Knoechel et al., 2006), indicating that Tregs do not belong to the anergic cell population, but rather demonstrate an altered proximal TCR signaling that could be critical for the induction of their suppressive function.

Given the therapeutic potential of targeting Tregs to treat autoimmunity, allergy, chronic infections and some types of cancer, the molecular mechanisms governing the function of these cells are of great interest. In Paper II, we aimed to define the molecular signaling pathways initiated in different Tregs subsets upon activation and study the functional implications of discernible differences in signaling between these subsets.

1.4 PGE_2 in immune regulation and tumor biology

Prostaglandins (PGs) are short-lived lipid mediators that are synthesized by most of the cells in the body in order to mediate diverse paracrine and/or autocrine effects. The E_2 type prostaglandin (PGE_2) is the one most abundantly produced in humans. The synthesis of PGE_2 is performed by various cell types and regulated by a variety of physiological and pathological processes, leading to a wide spectrum of biological outcomes (Matsuoka and Narumiya, 2007;Kobayashi and Narumiya, 2002). The role of PGE_2 in immune regulation is discussed in more detail below.

1.4.1 Synthesis of prostaglandins

Prostaglandin synthesis is initiated by activation of the enzyme phospholipase A_2, which produces arachidonic acid (AA) and other polyunsaturated fatty acids by hydrolysis of phospholipids located in the plasma membrane (Funk, 2001). The resulting AA is sequentially converted by the enzyme cyclooxygenase (COX) to the unstable endoperoxide intermediate PGG_2 and, subsequently, PGH_2 through two distinct enzymatic processes. A variety of cell- and tissue-specific prostaglandin synthases (PGS) transform PGH_2 into PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 or thromboxane A_2, all of which have a broad range of biological functions (Dey et al., 2006) (Figure 9).

The rate-limiting step in the prostaglandin biosynthesis is the conversion of AA to PGH_2 performed by the COX enzymes (Smith and DeWitt, 1996). To date, three different isoforms of COX have been indentified: COX-1, -2 and -3 (Legler et al., 2010;Park et al., 2006;Simmons et al., 2004;Smith et al., 2000). COX-1 is constitutively expressed in most tissues of the body, being responsible for the basal production of PGs and accordingly crucial to the maintenance of homeostatic PG-dependent processes.

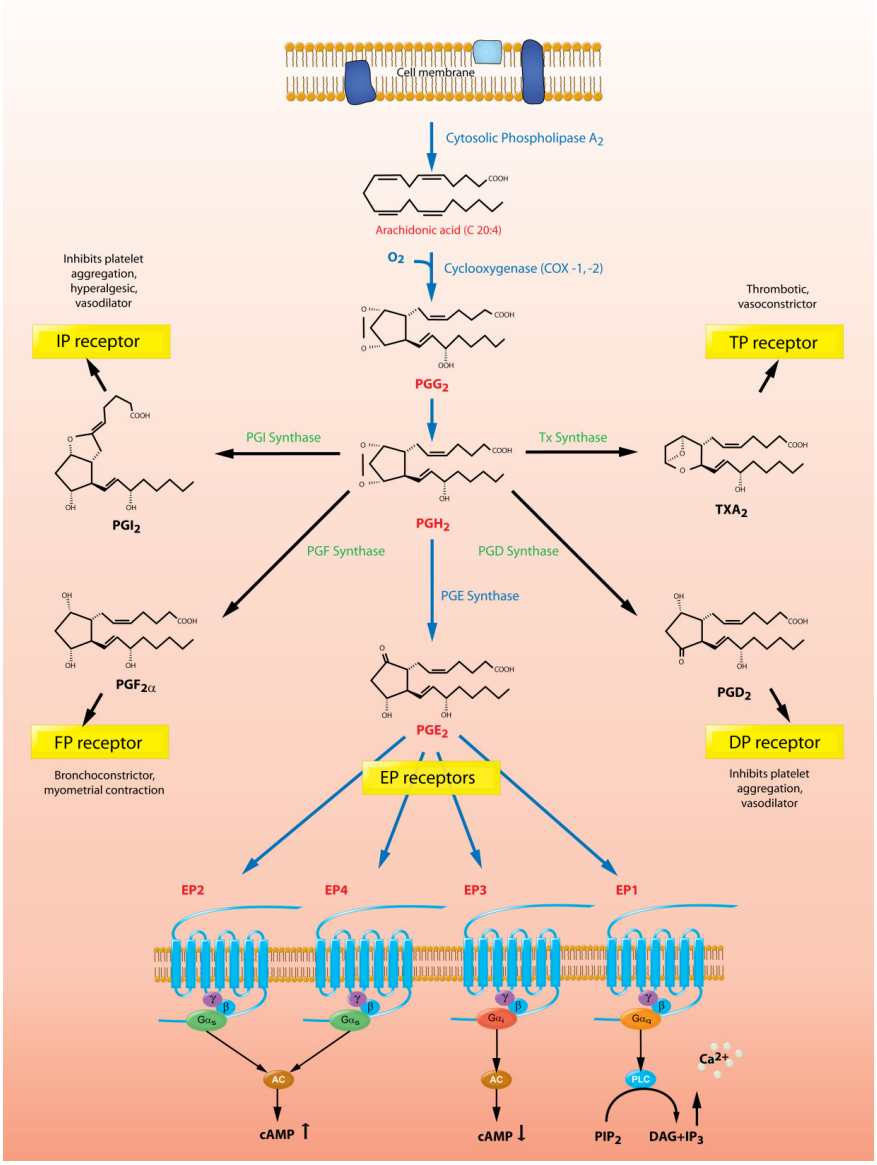

Figure 9. **The biosynthesis of PGs.** The common biosynthetic pathway of PGs is initiated by the activation of PLA_2 leading to liberation of AA from the plasma membrane into the cytoplasm. The next step in this biosynthesis, specifically the conversion of AA to PGH_2, constitutes the rate-limiting step in PG production. Depicted in this illustration are the various PGs produced, their respective receptors and related physiological phenomena. Emphasized in this figure is the initial events induced by PGE_2. PGE_2 exerts its large number of biological functions through binding to four different subtypes of EP receptors, $EP_{1\text{-}4.}$ These receptors are rhodopsine-type receptors with seven transmembrane domains. The different subtypes are known to utilize distinct sets of G proteins in order to initiate different signaling pathways, as illustrated in the figure. Adapted from (Dey et al., 2006;Legler et al., 2010).

COX-2, on the other hand, is induced by secreted cytokines and growth factors and is mainly involved in the regulation of inflammatory and proliferative responses. Some studies suggest that dysregulation of COX-2-expression may play a role in the development of cancer as this has been observed in e.g. colorectal tumors (Sinicrope and Gill, 2004;Tsujii et al., 1997) and breast cancer (Ristimaki et al., 2002;Wang et al., 2007b). COX-3, which is a splice variant of COX-1, is predominantly expressed in the brain and the heart (Chandrasekharan et al., 2002). Its functions, however, remain obscure. Following synthesis, the PGs transcend the plasma membrane either by diffusion or active transport (Park et al., 2006) and exert their biological actions through the selective binding of high-affinity prostanoid receptors located on the surface of the same or nearby cells (Sugimoto and Narumiya, 2007).

1.4.2 PGE_2-EP receptor signaling

The physiological effects of PGE_2 are conveyed through a group of seven transmembrane domain G-protein-coupled receptors (GPCRs) called E prostanoid (EP) receptors that are divided into 4 subtypes, $EP_{1\text{-}4}$, with unique patterns of expression (Sugimoto and Narumiya, 2007). The diversity in the cellular outcomes initiated by PGE_2 is attributed to its binding ratio to the four different receptor subtypes and the sites of action. Each of the EP receptors is linked to distinct G proteins and thereby initiates different signaling pathways (Breyer et al., 2001) (Figure 9). It has been demonstrated that signaling through the EP_1 receptor, which is coupled to a class of $G\alpha_q$ proteins, induces PLC-β/γ that mediates elevation of the free calcium concentration in the cytosol. The EP_2 and EP_4 receptors, which are both known to engage $G\alpha_s$ proteins, activate adenylate cyclase (AC) upon ligation and cause the intracellular levels of cAMP to rise (Figure 9). Such augmentation of cAMP has been shown to negatively influence the immune function of T cells by activating cAMP-dependent protein kinase (PKA) type I, leading to phosphorylation of numerous molecules downstream of the TCR (Figure 10). Of note, PKA type I has been demonstrated to constitute approximately 75% of the total PKA activity in human T cells (Skalhegg et al., 1992). The EP_3 receptor, which predominantly interacts with $G\alpha_i$ proteins, inhibits adenylate cyclase as it becomes activated, thereby causing a reduction in intracellular cAMP (Figure 9). The different EP receptors do not necessarily interact with only one G protein and may, through the binding of several G proteins, influence several different transduction pathways (Sugimoto and Narumiya, 2007;Breyer et al., 2001). Like other locally acting lipid mediators PGE_2 has a short half-life. It is rapidly inactivated – within seconds or a few minutes – by the cytosolic enzymes 15-

ketoprostaglandin 13-reductase and 15-hydroxyprostaglandin dehydrogenase (Funk, 2001;Legler et al., 2010).

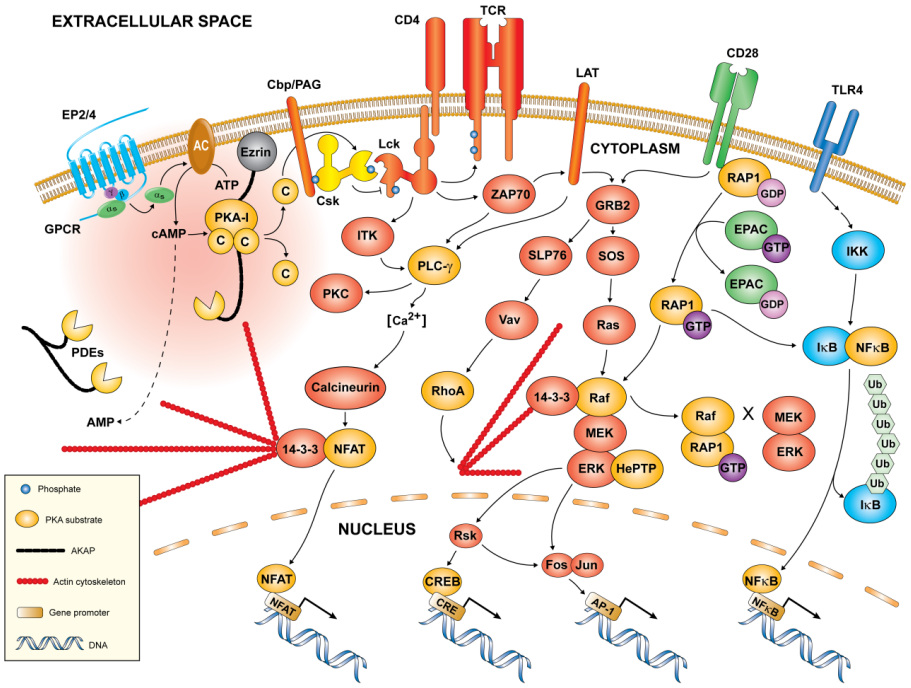

Figure 10. PGE_2 inhibits cell proliferation through a cAMP-PKA inhibitory pathway (Skalhegg et al., 1992;Vang et al., 2001;Vang et al., 2003). Engagement of the EP_2 and EP_4 receptors by PGE_2 on T cells initiate phosphorylation of proteins important in the regulation of a number of different signaling pathways, of which the most extensively studied is the one mediated by cAMP through PKA type I (Tasken and Aandahl, 2004). Elevation of cAMP leads to activation of PKA type I, which is known to phosphorylate a number of proteins involved in TCR signaling. One event extensively studied in the laboratory of Prof. Kjetil Taskén is the phosphorylation of the C-terminal Src kinase (Csk) at S364 (Torgersen et al., 2002;Vang et al., 2001). Phosphorylated Csk is recruited to lipid rafts by Cbp/PAG (Brdicka et al., 2000;Kawabuchi et al., 2000). Raft-associated Csk then maintain Lck in its inactive state through phosphorylation of its Y505 residue (Vang et al., 2003;Vang et al., 2004), thereby preventing it from phosphorylating and activating the ζ-chain of the TCR/CD3 complex (Vang et al., 2001). Thus, PGE_2 may potentially modulate the activation, differentiation and/or proliferation of T cells. Notably, PGE_2 may inhibit T cells also in cAMP-independent ways, e.g. by suppressing the release of intracellular Ca^{2+} or inhibiting the activity of Fyn. Adapted from (Torgersen et al., 2002).

1.4.3 The role of PGE_2 in immune regulation

Since PGE_2 can be produced by practically any cell in the body, it has the ability to regulate numerous physiological and pathophysiological processes. In Paper I, we aimed to investigate signaling networks induced by PGE_2 in various lymphocyte subsets, with a special attention

to T cell subsets. Accordingly, this section will focus on the role of PGE_2 in T cell-mediated immunity and tumor biology.

The production of PGE_2 is enhanced during inflammation in response to various immunological stimuli such as TNF-α and IL-1 (Dayer et al., 1985;Dayer et al., 1986). Although monocytes, macrophages, dendritic cells, fibroblasts and vascular endothelial cells are known as the main sources of PGE_2 in the immune system (Harris et al., 2002;Kalinski, 2012;Kurland and Bockman, 1978), lymphocytes may also up-regulate COX-2 and produce PGE_2 upon external stimuli (Iniguez et al., 1999;Mahic et al., 2006;Yaqub et al., 2008). The importance of PGE_2 as a pro-inflammatory mediator has been emphasized by the clinical efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) inhibiting COX either in an indiscriminate manner or by selective COX-2 inhibition (coxibs) (Simmons et al., 2004). Studies of knock-out mice deficient for the individual EP receptors have additionally revealed that PGE_2 not only acts as a pro-inflammatory mediator but also exerts anti-inflammatory functions (Sugimoto and Narumiya, 2007). In T cells, this effect relies predominantly on the EP_2 and EP_4 receptors (Nataraj et al., 2001). Although there appears to be some redundancy between these two receptors, they may contribute to varying extent (Sugimoto and Narumiya, 2007).

Dysregulation of COX enzymes has been linked to the pathogenesis of several diseases, including a variety of cancers (Greenhough et al., 2009;Wang et al., 2007b). In particular, the over-expression of the inducible isoform COX-2 – with increased production of PGE_2 as a consequence – has been associated with colorectal, pancreatic, lung and breast cancer (Wang et al., 2007b). In contrast to these observations, however, a recent study suggested a correlation between reduced expression of COX-2 and the development of primary breast cancer (Boneberg et al., 2008), implying a more complex role of PGE_2 in immune regulation. Overproduction of PGE_2 by tumor cells has been proposed as one of the strategies tumors use to evade anti-tumor immunity. In support of this, administration of antibodies against PGE_2 has been shown to delay tumor growth in mice (Greenhough et al., 2009). The underlying mechanisms by which PGE_2 prevents efficient anti-tumor immune responses remain poorly understood. However, some clues may be gathered from the finding that PGE_2 secreted from COX-2 over-expressing lung cancer cells significantly induced the expression of FOXP3 in human $CD4^+CD25^-$ T cells, resulting in a Treg phenotype (Baratelli et al., 2005;Sharma et al., 2005). Along this line, studies in the laboratory of Prof. Kjetil Taskén indicated that iTregs

that expressed high levels of COX-2 with subsequently high production of PGE_2, exhibited suppressive capacity (Mahic et al., 2006;Yaqub et al., 2008). These data suggest that PGE_2 may be responsible for both the acquisition of a Treg phenotype and the suppressive function of the resulting iTregs. Furthermore, PGE_2 has been implicated in the polarization of the immune response towards a Th2-type response that does not favor destruction of tumors. In this context, knock-out studies in mice have demonstrated that PGE_2, through activation of the EP_2 and EP_4 receptors, suppresses Th1 differentiation (Nataraj et al., 2001) and thereby facilitating Th2 responses. The ensuing activation of AC and resulting increase in intracellular cAMP leading to activation of PKA have been demonstrated to inhibit signaling mediated through the TCR and/or costimulatory molecules such as CD28 (Mustelin and Tasken, 2003;Chemnitz et al., 2006). Accordingly, the modulatory effects of PGE_2 on T cells are influenced by the simultaneous stimulation of the cell and the strength of the activation.

A study aimed at examining how the effect of PGE_2 was modulated by varying concentrations of stimulatory anti-CD28 antibodies (Yao et al., 2009), showed that in the presence of weak CD28 stimulation PGE_2 suppressed Th1 differentiation in a concentration-dependent manner, supporting earlier observations (Betz and Fox, 1991;Harris et al., 2002). Surprisingly, however, in the presence of stronger CD28 stimulation PGE_2 facilitated Th1 differentiation. This effect was mediated primarily through the EP_4 receptor, which instead of inducing intracellular cAMP levels led to activation of the PI3K pathway (Yao et al., 2009), as previously observed for EP_4 signaling in other biological systems (Fujino et al., 2003;Fujino and Regan, 2006). However, the relevance of this dual effect of PGE_2 on Th1 differentiation remains to be verified in vivo. Examination of the effect of PGE_2 on the differentiation of human $CD4^+$ T cells have also shown a dose-dependent facilitation towards Th17 cells in the presence of the cytokines IL-23 and IL-1β (Boniface et al., 2009). Supporting this observation, another study showed that PGE_2 together with IL-23 synergistically facilitated the expansion of Th17 cells from the memory ($CD4^+CD45RO^+$) T cell population. Unfortunately, the experimental setup in this study was not designed to detect the influence of PGE_2 on differentiation of naive T cells ($CD4^+CD45RO^-$) (Chizzolini et al., 2008). Importantly, the effects described above were mediated through both the EP_2 and EP_4 receptors resulting in the classical increase in intracellular cAMP levels. Overall, these observations indicate that PGE_2 in combination with other stimuli differently influence the differentiation of Th subsets, resulting from the fact that the EP_2 and EP_4 receptors may induce several distinct signaling pathways.

Investigating signaling differences between cell subsets, discrepancies in signaling pathways are desirable as these may represent potential targets for therapeutic intervention in disorders with deregulated signaling responses in one of the subsets. Although extensive research has been carried out in order to reveal the mechanisms underlying the inhibitory role of PGE_2 in T cells, our knowledge are still limited and further examinations of these mechanisms are warranted. Previous work has identified roles of PGE_2 in modulating proliferation, apoptosis and cytokine production in T cells (Harris et al., 2002). Furthermore, PGE_2 has been shown to suppress B cell function and act as an anti-inflammatory mediator on innate immune cells like granulocytes, monocytes and NK cells. Consequently, PGE_2 and the signaling pathways induced upon engagement of the individual EP receptors may represent promising targets for reversing immune suppression and preventing tumors from subverting the immune system.

2 Aims of the study

While both B- and T cell activating signal pathways and PGE_2 signaling has been extensively studied over many years, new technologies now allow the parallel analysis of many signaling readouts with the possibility of achieving more of a systems understanding of signal propagation in these cells. Furthermore, single-cell analysis allow for mapping signaling responses in specific subsets of immune cells. The aims for the work in the present Thesis, based on earlier work and focus in the group, were to:

1. Map phosphorylation-based signaling networks in response to PGE_2 in distinct T cell subsets.

2. Study the signaling patterns upon TCR triggering in the presence of CD28 and/or CD2 costimulation in different T cell subsets, i.e. naive and effector/memory $CD4^+$ and $CD8^+$ T cells as well as rTregs and actTregs.

3. As a consequence of our findings when addressing aim number 2, determine the influence of the Mek-Erk signaling pathway in up-regulating the Treg-specific transcription factor FOXP3 and controlling the suppressive capacity of rTregs.

4. Examine the interaction between the adaptor protein PAG and the Src kinase Lyn in B cells and its effect on B cell signaling.

5. Elucidate the regulatory potential of PAG in early BCR signaling in both normal and transformed B cells.

3 Summary of Results

Paper I: "*High-resolution mapping of prostaglandin E2-dependent signaling networks indentifies a constitutively active PKA signaling node in $CD8^+CD45RO^+$ T cells"*

In this study, we investigated PGE_2 signaling networks in lymphocytes by a novel combination of a quantitative MS-based phosphoproteomic approach, kinase prediction and high throughput phospho-flow cytometry. Using temporal quantitative phosphoproteomic MS, we monitored 247 unique phospho-sites on about 200 proteins regulated in response to PGE_2 treatment. Furthermore, by combining these phosphoproteomic MS data with stringent kinase predictions, we were able to predict signaling kinase networks for more than 20 kinases, with PKA as the strongest node and CAMKII and PKB/Akt as supplementary nodes. Based on these results we were able to select a series of phospho-epitope-specific antibodies, which were used in a high throughput phospho-flow cytometry-based method. A detailed analysis of naive and effector/memory $CD4^+$ and $CD8^+$ T cell populations revealed constitutively augmented PKA activity in effector/memory cells, especially in $CD8^+$ T cells. Moreover, the higher activation status of PKA in these cells raised the activation threshold, possibly providing a mechanism for avoiding non-specific T cell activation.

Paper II: "*T cell signaling network analysis reveals distinct differences between CD28 and CD2 costimulation responses in various subsets and in the MAPK pathway between resting and activated regulatory T cells"*

In order to unravel the signaling signatures of different human T cell subsets upon various T cell stimuli, the phosphorylation status of 18 signaling proteins was studied at six different time points following TCR triggering in the absence or presence of CD28 and/or CD2 costimulation. The combined use of phospho-flow cytometry and fluorescent cell barcoding provided a high-resolution signaling map, which served as the basis for our studies. Compared to effector/memory T cells, naive T cells displayed stronger activation of proximal signaling molecules after TCR triggering alone, while for distal phosphorylation events like pErk and pS6-Rp the situation was reversed. CD28 costimulation initiated the signaling events required for proper NF-κB activation, while activation of CD2 promoted signaling through S6-Rp. Analysis of signaling patterns in resting regulatory T cells (rTregs, $CD4^+CD45RA^+Foxp3^+$) and activated regulatory T cells (actTregs, $CD4^+CD45RA^-Foxp3^{++}$)

revealed that while rTregs had low basal, yet inducible Erk and Akt activity, actTregs displayed high basal Erk phosphorylation and little or no Akt activation. Interestingly, the use of Mek inhibitors to block Erk activation inhibited the activation-dependent up-regulation of FOXP3 in rTregs as well as their transition to actTregs with increased suppressive capacity.

Paper III: *"Modulation of proximal signaling in normal and transformed B cells by transmembrane adaptor Cbp/PAG."*

The SH3 binding sites for Lyn in PAG were identified using peptide binding arrays and Lyn SH3-GST protein. We observed that Lyn preferentially bound to the PRD1 domain in PAG and that this interaction was crucial for efficient phosphorylation of PAG by Lyn in vitro. We studied the BCR triggered responses in normal and transformed cells using both western blotting and phospho-flow cytometry, and observed that siRNA mediated knock down (KD) of PAG gave enhanced and prolonged activation of proximal BCR signaling in normal human B cells, but not in the Raji line of human Burkitt's lymphoma (BL) B cells with over-expressed PAG. Moreover, we examined the impact on BCR signaling of a PAG variant that contained two enhanced PRD motifs for Lyn binding (PAG**). In contrast to low expression of PAG, high expression resulted in decreased proximal BCR signaling, which was reinforced by increased association of Lyn with PAG. This effect appears to be linked to Csk activity as transient transfection of a PAG construct lacking binding sites for Csk resulted in stronger early BCR responses. In summary, our data indicate that PAG through interaction with the SH3 domain of Lyn and increased recruitment of Csk negatively regulates proximal BCR signaling in normal human B cells, at least in vitro.

4 Discussion

In the Introduction, I have provided an overview of the cellular organization of the immune system and the signaling responses elicited in both B- and T cells upon activation. I have outlined some of the functional importance of the systems studied in order to give background information relevant to the articles included in this Thesis and emphasize the unexplored potential of this research field. In the Discussion, I will go more deeply into the findings in each of the three individual projects and discuss them in separate chapters. Finally I will round off by discussing phospho-flow cytometry, which has been used as an investigation tool in the studies presented here, and compare this method with three other commonly used methods for signaling studies.

4.1 System views of PGE_2 signaling networks

The immunological research strategies have changed over the last decade from being performed based on hypotheses that rely on earlier results or published work by other investigators to utilization of high-throughput methods for investigating features of cell systems in an unbiased manner in order to produce new hypotheses (Benoist et al., 2006). In this context, proteomic studies are essential for the development of quantitative models by providing information about the cellular constituents at a molecular level as well as the biochemical states of proteins. A cell will respond to external stimuli through a complex interplay between several hundreds to thousands of proteins, ultimately instructing the secretion of cytokines specific for that particular cell, initiating cell proliferation and differentiation, or even committing the cell to apoptosis. Proteomic analysis provides a snapshot of the overall protein expression in a cell population under certain experimental conditions. In Paper I, we took advantage of a multipronged strategy with phosphoproteomic mass spectrometry (MS), kinase prediction algorithms and phospho-specific flow cytometry in order to study signaling networks induced by PGE_2 in primary human T cells. Indeed, temporal maps of PGE_2 signaling networks in healthy individuals may contribute to revealing aberrant responses to PGE_2 with relevance to pathological processes.

4.1.1 Phosphorylation-based signaling networks regulated by PGE_2

Phosphorylation-based signaling networks are defined by an inter-relationship between substrates of various effector kinases and phosphatases. The phosphorylation of kinase substrates is a highly transient process due to the rapid interplay between kinases and phosphatases. Hence, strategies employed to study phosphorylation-based signaling networks need to provide sensitive and reliable readouts to capture dynamic changes (Tan and Linding, 2009). In Paper I, we initially investigated the temporal regulation of PGE_2-induced signaling processes by utilizing a sensitive, high-throughput approach based on quantitative MS. As MS is not intrinsically quantitative (Choudhary and Mann, 2010), the introduction of chemical tags by triplex stable isotope dimethyl labeling of peptides allowed us to study 3 different time-points simultaneously, enabling investigation of temporal regulation (Boersema et al., 2008). Since phosphorylated proteins are present in small amounts and constitute only a minority of the proteome, the peptides resulting from multiple digestions were enriched after isotope labeling using a procedure involving sub-fractionation via strong cation exchange (SCX) and, subsequently, an optimized chromatographic technique based on complex formation between the phosphate groups and the metal oxide TiO_2 (Pinkse et al., 2008). This approach led to the identification of 796 triple-labeled phosphopeptides where phosphorylation of 247 of these, originating from 207 different phosphoproteins, appeared to be regulated by PGE_2 in primary T cells within one hour of stimulation. Although different patterns of temporal regulation were observed, the majority of the regulated phosphopeptides displayed considerably increased phosphorylation levels after 10 minutes of stimulation, which declined to near basal levels within 60 minutes, corresponding to the kinetics observed for PKA substrates containing the RRXpS/pT phosphorylation site.

Many of the discovered phosphorylation sites reported in Paper I were previously unknown mediators of PGE_2 signaling. In order to associate the 247 identified phosphorylation sites to their respective kinases and thereby reconstruct signaling networks regulated by PGE_2, we used 3 different algorithms for kinase prediction based on our MS data (Obenauer et al., 2003;Linding et al., 2008;Gnad et al., 2011). The combined results of the MS-based phosphoproteomics and kinase prediction algorithms highlighted PKA as one of the main kinases for the identified phosphorylation epitopes, along with CAMKII, PKB/Akt and GSK3. Based on the fact that PGE_2 mainly exerts its effects in T cells through stimulation of the EP_2 and EP_4 receptors, it was not a surprise that PKA was the kinase associated with a

majority of the phosphorylation sites. However, prediction of CAMKII, a known downstream mediator of EP_1, and PKB/Akt, which is downstream of EP_3, suggest that these receptors may also be operative in T cells. Although the algorithms employed in our study were based on sets of kinase phosphorylation sites identified by high-accuracy MS data, they cover only a minor part of the human proteome (Obenauer et al., 2003;Linding et al., 2008;Gnad et al., 2011). Moreover, the consensus sequences recognized by different kinases as predicted in the data sets underlying the algorithms used in such kinase predictions have considerable overlap, meaning that several of the predicted kinases phosphorylate either identical or similar phosphorylation sites. For these reasons, prediction of kinases based on bioinformatics analysis of proteomics data should ideally be verified experimentally. To remove some noise, we initially chose to include only kinases predicted by at least two of the algorithms included, and next set out to verify that the remaining kinases were in fact activated by PGE_2, either by looking at the same substrates or other readouts in the same pathways. Phospho-specific flow cytometry was used for this purpose, allowing the study of dynamic changes in a set of selected phosphorylation sites where phospho-specific antibodies were available.

Using the phospho-flow approach, we confirmed a concentration-dependent temporal activation of PKA in response to PGE_2 treatment. We also verified PGE_2-induced phosphorylation of GSK3α. Interestingly, in this respect, GSK3α phosphorylation at S21 exhibited a kinetic profile similar to PKA substrates with the RRXpS/pT phosphorylation site. In human embryonic kidney (HEK) 293 cells, PKA has previously been demonstrated to complex with GSK3α and inactivate its kinase activity by phosphorylating the S21 residue (Fang et al., 2000). The predicted GSK3α substrates recorded in our MS dataset show decreased phosphorylation in response to PGE_2, which is consistent with an inhibitory role of PKA on GSK3α activity, by phosphorylation of S21, also in T cells. This indicates a direct crosstalk between PGE_2, PKA and GSK3α signaling in primary human T cells. GSK3 is a multi-functional serine threonine kinase that is involved in numerous cellular processes including glycogen metabolism and glucose homeostasis, proliferation and regulation of gene expression, as well as development of tumors and neuronal function (Rayasam et al., 2009). As for PGE_2 signaling, the activity of GSK3 has been linked to a variety of diseases including inflammation and cancer. Hence, further investigations are required to resolve the functional implications of the observed PGE_2-PKA-GSK3α signaling pathway in primary T cells from healthy individuals.

Although strongly suggested by the kinase prediction algorithms, little or no changes in phosphorylation were detected for CAMKII or PKB/Akt upon a 60-minute time course of PGE_2 stimulation. Absence of CAMKII phosphorylation in our phospho-flow setup was further supported by the absence of intracellular calcium flux in response to PGE_2 treatment. Together, these results indicate that the two kinases were over-represented in the datasets generated by the algorithms. In fact, some of the phosphorylation sites identified for CAMKII and PKB/Akt overlap with sites identified for PKA and could therefore in reality be PKA targets, meaning that they were falsely attributed to CAMKII and PKB/Akt by the algorithms. On the other hand, nine unique phosphorylation sites were identified for CAMKII and six for PKB/Akt, suggesting that their kinase activities in response to PGE_2 treatment were below the detection limit of the phospho-flow technique applied in our study.

Collectively, our data indicate that PKA is the central node of PGE_2-regulated signaling networks in primary T cells, an effect that is known to be mediated through stimulation of the EP_2 and EP_4 receptors. These results are in accordance with a previous report published by Boniface et al., which demonstrated that both of these receptors are highly expressed in naive human $CD4^+$ T cells (Boniface et al., 2009). In comparison, the expression levels of the EP_3 receptor were found to be quite low whereas the EP_1 receptor appeared to be absent in this particular cell subset. The main advantage of the approach introduced in Paper I is its ability to provide a global view of regulated signaling networks in primary cells as opposed to most quantitative MS-based signaling studies of today, which have been performed using immortalized cell lines (Choudhary and Mann, 2010). Given the fact that phosphorylation-based signaling networks observed in cell lines may not be representative of those in normal cells, proteomic studies of primary cells are highly warranted. The dataset presented in Paper I reveals the complexity of PGE_2-induced signaling responses in human peripheral T cells and may serve as a valuable starting point for the development of new hypotheses regarding the role of PGE_2 in various disease contexts such as inflammation and tumor-immunity.

4.1.2 Detection of subset-specific PKA-regulated signaling effects

In our phosphoproteomic dataset from the initial MS study reported in Paper I, we identified a series of previously uncharacterized PGE_2-regulated phospho-sites, some of which have been reported to act downstream of the TCR (Torgersen et al., 2008). Specifically, new phosphorylation sites in CARMA1, PLCγ, Wiskott-Aldrich syndrome protein-interacting

protein, VASP, GADS, nuclear factor of activated T cells cytoplasmic 2, Fyn binding protein/adhesion and degranulation promoting adapter protein, and Nck were identified in our study. Considering that PKA was identified as one of the main activated signaling nodes in the PGE_2-regulated signaling network, we proposed that PKA might bridge PGE_2 stimulation to these downstream TCR signaling events in primary T cells. In Paper I, this hypothesis was explored in distinct subsets of lymphocytes by the use of phospho-flow.

Our phospho-flow analyses revealed a reduced basal phosphorylation of the ζ-chain in effector/memory subsets of both $CD4^+$ and $CD8^+$ T cells as compared to their naive counterparts. This finding appeared to depend on elevated PKA type I activity as the reduced ζ-chain phosphorylation was reversed by PKA type I inhibitors. Notably, this effect was most prominent in $CD8^+$ effector/memory T cells. Despite differential activities of PKA in naive and effector/memory T cells, pretreatment with PGE_2 prior to TCR stimulation significantly inhibited ζ-chain and Slp-76 phosphorylation in either subset. These results are in agreement with previous observations in that PGE_2 appears to be implicated in PKA type I-mediated inhibition of TCR signal transduction (Vang et al., 2003). Thus, they support the predominant paradigm in ascribing an inhibitory role to PKA type I in the context of T cell activation.

As described in the Introduction, naive and effector/memory T cells differ from each other in how they respond to antigens. Although it is well established that effector/memory $CD4^+$ and $CD8^+$ T cells respond more rapidly and efficiently to recall stimulation by the same antigen (Croft et al., 1994;Horgan et al., 1990;Iezzi et al., 1998;Luqman and Bottomly, 1992;Kaech et al., 2002), the underlying molecular mechanisms for these differences remain unclear. In this respect, the differences in basal PKA-substrate phosphorylation levels between naive and effector/memory T cells reported in Paper I might represent one biochemical mechanism that contributes to the observed differences in activation threshold. Still, as our study did not relate the observed phosphorylation events to actual immune responses in vivo, we can only speculate on their biological relevance. Interestingly, however, Conche et al. recently demonstrated a transient increase in PKA activity following adhesion-induced T cell priming (AITCP), which reduced the activation threshold and thereby facilitated antigen recognition (Conche et al., 2009). This is in contrast to the established idea that PKA serves to inhibit T cell activation through Csk (Vang et al., 2001), but may be explained either by differences in time kinetics (the effect reported by Conche et al. is fast and short-lived) or by the concomitant activation of Erk. Thus, the elevated levels of PKA observed in effector/memory

T cells may not only reduce proximal signaling events as described above, but could also increase the activity of Erk as a way of rendering the cells more sensitive to true antigenic stimulation. In line with these findings, an elevated basal phosphorylation level of Erk in effector/memory T cells was observed in Paper II. Accordingly, elevated PKA activity may be seen as a way of inhibiting inappropriate activation of effector/memory cells, while elevated Erk activity facilitates activation when proper antigen-binding take place. Thus, high basal phosphorylation of PKA might facilitate signal transduction upon antigen binding in antigen-cognate cells through activation of Erk, while at the same time inhibiting TCR signaling resulting from stimulation by for instance PGE_2. To our knowledge, Paper I is the first report to demonstrate differential basal PKA activity between naive and effector/memory T cells.

4.2 Signaling networks in T cell activation

T cell signaling has been extensively studied for many years and the various signaling pathways involved are well characterized. However, despite the fact that T cell signaling is a much researched subject, new discoveries are still being made and added to the web of knowledge. Furthermore, systems understanding of the process is still developing and there is a need for an overview of how distinct signaling pathways integrate and crosstalk in order to generate signaling networks in T cells. In addition, the increasing number of different T cell subsets being defined (as described in the Introduction) and the possibility of signaling differences between these subsets, warrant subset-specific signaling analyses to link signaling activity to functional properties of individual subsets of T cells. Paper II contributes to the understanding of both subset-specific signaling and how costimulation through CD2 and/or CD28 contribute to signal intensity and affect signal integration in functionally defined T cell subsets, as will be discussed in the following sections.

4.2.1 Signaling capabilities of the CD28 and CD2 coreceptors

In agreement with previous observations, the experiments presented in Paper II revealed an augmentation-effect of CD28 costimulation in all the defined subsets tested, both naive and effector/memory $CD4^+$ and $CD8^+$ T cells. In addition, we found that the signaling responses in these subsets were even stronger with concomitant stimulation through the CD2 coreceptor. Although TCR signaling in the presence or absence of CD28 costimulation in conventional

naive/memory $CD4^+$ T cells is already well characterized (Acuto and Michel, 2003;Rudd et al., 2009) and CD2 though to be redundant versus CD28 (Green et al., 2000), we found distinct differences in signaling following CD28 and CD2 costimulation and between several distinct subsets of $CD4^+$ and $CD8^+$ T cells that have not been observed earlier. Notably, these signaling effects are presented with a higher resolution than previously published due to the power of the phospho-flow method, which gives information of signaling responses at the level of single cells.

Our study of the costimulatory effect of CD28 presented in Paper II showed that stimulation, through this coreceptor compared to TCR stimulation alone, induces higher levels of phosphorylation of many of the signaling molecules tested, including ζ-chain phosphorylation. These observations are consistent with data previously published by Andrew Shaw and colleagues, who demonstrated that Lck via the SH3 domain binds CD28 and gets activated by stimulation through this coreceptor (Holdorf et al., 1999). In contrast, our results are not compatible with the model proposed by Acuto and Michel suggesting that CD28 signaling first converges with the classical TCR signaling pathway at the level of Slp-76 and PLCγ1 and does not involve Lck (Michel et al., 2001). These differences may be explained by the fact that our observations were made in primary T cells, whereas Michel and Acuto's model was based on experiments from Jurkat cells, a transformed human leukemia T cell line that we now know carries a mutation in the PTEN gene making the PI3K pathway constitutively active (Shan et al., 2000) and which does not require costimulation in order to become activated. Hence, these observations may not reflect the true function of CD28 in primary human T cells. Furthermore, while earlier research mainly reflect signaling responses of the total $CD4^+$ T cell population, the technology now allows for analysis of subset-specific signaling effects. As the data presented in Paper I and II indicate, signaling responses in heterogeneous populations may cancel each other out due to variations in signaling effect and amplitude between the various subsets included in the analysis, an observation also reported by Adachi and Davis (Adachi and Davis, 2011). This could cause differences when findings in the older literature examining $CD3^+$ or $CD4^+$ T cell populations are compared to the observations made with the present technology.

As discussed in the Introduction, knock-out studies in mice of either the CD28 or CD2 coreceptors or both have indicated that at least some degree of costimulation is necessary in order to avoid anergy and ensure proper T cell activation for further differentiation (Green et

al., 2000;Sasada and Reinherz, 2001;Shahinian et al., 1993). These studies also suggest the existence of functional redundancy as loss of CD28 can be compensated for by CD2 and vice versa. The results presented in Paper II demonstrate that engagement of the CD28 coreceptor induces unique signals essential for NF-κB activation, whereas CD2 costimulation mainly enhances TCR-mediated signaling responses. A central issue raised by these observations is how CD2 compensates for its lack of ability to properly activate NF-κB, if the idea of redundancy is correct. The data gained from our study do not provide an answer to this question and further investigation is required to give a definitive answer. Nevertheless, we observed an augmented phosphorylation of proximal TCR signaling events, such as phosphorylation of the ζ-chain and activation of ZAP70, both Lck-mediated processes, in response to CD2, CD28 or combined CD2/CD28 costimulation. Accordingly, our data indicate that both coreceptors amplify early TCR signaling. However, the signaling responses to CD2 and CD28 appear to diverge downstream where different signaling responses are observed. Our data therefore suggest that CD28 and CD2 may play distinct roles in T cell activation even though they are largely able to compensate for the lack of the other. One explanation to this may be that both coreceptors function as amplifiers of TCR signaling and that they separately and in the absence of the other still are able to reduce the activation threshold to a sufficient level for a productive T cell activation response to occur.

The present knowledge of the signaling events induced in response to CD2 engagement is limited and further examination is required in order to evaluate the exact functional role of CD2 in T cell activation. Although signaling by CD28 is more extensively studied, the exact biochemical events it mediates remains to be fully understood and it is still debated whether CD28 support TCR signaling only qualitatively or if it also induces separate signaling pathways (Acuto and Michel, 2003;Rudd et al., 2009). Our findings strongly suggest that CD28 in addition to amplifying TCR signaling also supports T cell activation qualitatively by specifically inducing the activation of NF-κB. This observation is supported by a recent publication demonstrating that CD28, in an Lck-dependent manner, triggers a unique signaling pathway that involves recruitment and binding of PKCθ to the cytoplasmic tail of CD28 at the IS, which ultimately leads to efficient NF-κB activation (Kong et al., 2011). Previous studies by several investigators have provided convincing evidence that CD28 ligation indeed specifically regulates the NF-κB pathway and associated genes (Kane et al., 2002;Takeda et al., 2008;Tuosto, 2011), although the necessity of concomitant stimulation through the TCR remains controversial. Some of these studies have demonstrated that a PI3K-

Akt pathway may contribute to the connection between CD28 and NF-κB (Tuosto, 2011). Our study, however, demonstrates that activation of Akt did not show the same dependency on CD28 stimulation as the activation of NF-κB did. We therefore hypothesize that signaling molecules other than Akt are essential for bridging CD28 stimulation to NF-κB activation, although our data do not exclude the possibility that the increased CD28-mediated activation of Akt observed in the naive subsets may enhance the activity of NF-κB. In support of these notions, a number of earlier studies have shown that Akt is not a critical mediator for efficient TCR/CD28-mediated NF-κB activation, but instead appears to be an important regulator for the strength and/or duration of its activation (Tuosto, 2011;Takeda et al., 2008;Narayan et al., 2006).

4.2.2 Signaling profiles of naive and effector/memory T cells

Previous studies of signal transduction in human T cells have mainly been carried out in conventional or naive $CD4^+$ T cells due to insufficient amounts available for biochemical analysis of memory cells and especially of $CD8^+$ T cells. However, recent progresses enabling flow cytometry analysis of intracellular signaling responses have contributed to emerging knowledge of the biochemical changes induced also in less prevalent subsets. In Paper II, we revealed signaling differences between naive and effector/memory subsets of $CD4^+$ and $CD8^+$ T cells. Generally, we observed a stronger phosphorylation level in naive cells compared with effector/memory cells for proximal TCR-signaling molecules (e.g., the ζ-chain, ZAP70, and Slp76) upon activation. These findings are supported by earlier observations in primary human $CD4^+$ T cells (Hall et al., 1999). By contrast, the opposite phosphorylation pattern was found for more downstream signaling mediators, e.g. Erk and S6-Rp. These differences were identified in both $CD4^+$ and $CD8^+$ T cells, although the signals induced in $CD8^+$ T cells overall were reduced compared to those of $CD4^+$ T cells.

Adachi and Davis demonstrated in a recent publication that human peripheral naive and effector/memory $CD4^+$ T cells diverge in their initial response to activation at the level of Erk and p38 (Adachi and Davis, 2011). Interestingly, their data showed that TCR-mediated Erk phosphorylation is preferentially induced in naive cells, whereas p38 activation is predominantly observed in effector/memory cells after short-term stimulation (two minutes). Consistent with these findings, we observed a slightly lower responsiveness of p38 in naive cells compared with effector/memory cells. However, more importantly, we observed a

reduced ability of effector/memory cells to become Erk phosphorylated upon activation. Examination of the resting signaling states between these two subsets revealed, as mentioned previously, an augmented constitutive phosphorylation level of Erk in the effector/memory subsets. This high basal level of phospho-Erk might contribute to the stringent regulation of signal transduction observed in these cells. Adachi and Davis reported a negative link between activation of Erk and facilitated propagation of more downstream signaling events, which is compatible with this notion (Adachi and Davis, 2011). Further, we observed optimal Erk activation in effector/memory cells only when both coreceptors were stimulated simultaneously. Accordingly, under proper stimulatory conditions it might be that this high basal phosphorylation level of Erk in resting effector/memory T cells makes these cells better positioned to efficiently process downstream signals.

Despite differential regulation of Erk, all subsets in our study exhibited equal basal phosphorylation levels and proper induction of the ζ-chain, ZAP70, and Slp-76, although the levels were slightly reduced in the effector/memory cells. Adachi and Davis, on the other hand, demonstrated that differential Erk phosphorylation was mediated through the activation of Slp-76 as TCR-triggered naive $CD4^+$ T cells got strongly phosphorylated whereas the effector/memory cells did not. This distinct coupling of signaling appeared to be independent of ZAP70, which was equally activated in both subsets (Adachi and Davis, 2011). In contrast, Farber et al. demonstrated earlier a considerably higher induction of ZAP70 in naive murine $CD4^+$ T cells compared to the corresponding effector/memory population (Farber et al., 1997). However, Farber and colleagues only investigated CD3-induced signaling responses that are known to induce anergy. Comparison of the differential effects elicited by the different stimulatory conditions in our study revealed that costimulation was essential for optimal phosphorylation of ZAP70 in all subsets except the naive $CD4^+$ T cells. Hence, the differences in observations may be due to different stimulatory conditions being used. Alternatively, these differences reflect disparity between human and murine systems or simply occur as a result of differences in sensitivity of the techniques used for detection.

Taken together, our data reveal several interesting and novel features of the intracellular signaling capacities of naive and effector/memory $CD4^+$ and $CD8^+$ T cells. Our findings suggest that the differential activation responses displayed by these two subsets occur distally, as the most prominent differences in signaling were detected at the level of Erk. However, our study focused on immediate and early effects of T cell activation (from minutes to one hour

post-activation). It is therefore reasonable to expect that these signaling responses may be modulated during long-term stimulation by integration of other signals initiated for instance by auto- or paracrine effects of cytokines secreted during the course of activation. Furthermore, we cannot rule out that other regulatory phosphorylation sites not examined may play important roles in controlling the distinct functions and activation requirements of naive and effector/memory T cells. Nonetheless, our data indicate that Erk may be a central regulator of the immediate early signaling responses that functionally distinguish these cells.

4.2.3 Signaling in different Treg subsets, rTregs versus actTregs

As described in the Introduction, human FOXP3$^+$ T cells constitute a heterogeneous population of both non-suppressive and suppressive T cells with distinct functional properties (Fritzsching et al., 2006;Miyara et al., 2009;Seddiki et al., 2006;Valmori et al., 2005). For an intervention to be effective in the treatment of immunological disorders where Tregs have clinical impact, manipulation of distinct subpopulations may be necessary. As the FOXP3$^+$ subsets are quite recently defined, little is known about the underlying molecular characteristics that functionally differentiate them. In Paper II, we attempted to characterize and compare intracellular signaling cascades induced in these subsets upon activation with a main focus on the distinction between rTregs and actTregs.

Comparison of the signaling responses induced in rTregs versus actTregs in Paper II revealed a clear difference in the activation response of Akt. Whereas rTregs show a robust induction of phospho-Akt upon T cell activation, actTregs displayed little or no Akt phosphorylation. Interestingly, the phosphorylation state of Erk was constitutively elevated in actTregs to a level higher than that observed for effector/memory T cells. As a result of this high basal level, diminished activation response of Erk was detected in the actTregs in comparison to that found in the rTreg population. A previous study, designed to compare the signaling responses between conventional CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs, revealed a significantly lower induction of Akt by the co-engagement of TCR and CD28 in the Treg population (Crellin et al., 2007). As the Tregs here were defined as the CD4$^+$CD25^{high} T cells, these cells most probably correlate to the actTregs in our study and hence are in line with our observations. However, in contrast to our data, Crellin et al. did not detect any differential phosphorylation response for Erk. As the distinction between CD25^{low} and CD25^{high} is somewhat arbitrary, these differences may be due to the composition of the T cell population

analyzed by Crellin et al., which could contain both rTregs and cytokine-producing $FOXP3^+$ T cells. The functional importance of the observed differential signaling responses between rTregs and actTregs was examined in Paper II as described in the following section.

4.2.4 Regulation of Foxp3 expression and suppressive function

One of the main differences in phenotype characteristic between rTregs and actTregs is the expression level of FOXP3. We therefore hypothesized that the FOXP3 level influences the differential activation responses observed in these subsets. Although it is well documented that FOXP3 is one of the key regulators that preserve immune tolerance, the detailed molecular mechanisms governing its induction have just begun to be discovered (Josefowicz et al., 2012;Merkenschlager and von, 2010). Given the dominant role of Tregs in the maintenance of immune homeostasis, it is beneficial for development of new therapeutic strategies to gain more knowledge about the signaling pathways that either positively or negatively controls their function.

As rTregs are considered to be functional precursors of actTregs (Fritzsching et al., 2006;Ito et al., 2008;Miyara et al., 2009), we wanted in Paper II to identify signaling factors that might control up-regulation of FOXP3 in stimulated rTregs. In light of previous reports that have demonstrated that Akt activation dampened Foxp3 induction (Delgoffe et al., 2009;Harada et al., 2010;Haxhinasto et al., 2008;Sauer et al., 2008), it was somewhat surprising that our results indicated an independent role of Akt signaling in this respect. However, all of these studies have characterized the signaling effect of Akt in the differentiation process of murine Tregs and therefore do not necessarily mirror the functional role of Akt in human T cells. In addition, these studies have explored the mechanisms that control Foxp3 expression in peripheral $CD4^+CD25^-$ T cells (Delgoffe et al., 2009;Harada et al., 2010;Haxhinasto et al., 2008;Sauer et al., 2008), which may respond differently than natural committed rTregs. In line with this notion, one of these studies demonstrated a differential sensitivity between conventional and regulatory T cells in the activation of Akt, as nTregs appeared to be resistant to Akt-dependent inhibition of Foxp3 expression (Haxhinasto et al., 2008). This is supported by findings reported by Rubtsov and colleagues, which demonstrated a stable Foxp3 expression in mature murine Tregs even in the presence of immunological challenges (Rubtsov et al., 2010). Despite apparent lack of regulation of FOXP3 expression in our study, pretreatment with Akt-inhibitor upon subsequent stimulation of rTregs markedly impaired

their suppressive capacity, suggesting that defect Akt signaling inhibits the suppressive ability of these cells in a FOXP3-independent manner. This independent role of FOXP3 regulation in suppressive function has earlier been reported in human $CD4^{+}CD25^{high}$ Tregs (Crellin et al., 2007). However, the opposite observation with abrogation of suppression by activation of Akt was demonstrated in this report. Combined with our data, these findings suggest that Akt signaling may play a different role in rTregs versus actTregs, which may be reflected by the distinct activation responses observed in Paper II.

Based on the differential phosphorylation response of Erk between rTregs and actTregs, it was interesting to note that FOXP3 inducibility in rTregs appeared to be profoundly abrogated by perturbation of the Mek-Erk signaling pathway. Moreover, inhibition of this pathway reversed the ability of pre-activated rTregs to exert their suppressive function, suggesting that a Mek-Erk-dependent induction of FOXP3 is crucial for full development of these cells into actTregs. A recent publication by Beyer et al. showed that the Foxp3-controlled repression of the transcription factor SATB1, which acts as a chromatin organizer known to be involved in development and activation of T cells (Alvarez et al., 2000), is required for the suppressive function of murine Tregs (Beyer et al., 2011). This FOXP3-mediated inhibition of SATB1 activity appeared to be due to suppression of transcriptional programs that direct differentiation of human effector T cells (Beyer et al., 2011), indicating that the integrity of Tregs is dependent on continuous inhibition of effector T cell function. Interestingly, manipulation of human Tregs to ectopically express high levels of SATB1 led to reversal of FOXP3-mediated repression and induction of inflammatory effector cytokines (Beyer et al., 2011). This finding may in part explain why conventional T cells that temporarily express lower levels of FOXP3 upon early activation do not gain suppressive function. An alternative model for the terminal differentiation of peripheral T cells has earlier been proposed by Pan et al. (Pan et al., 2009). In this report, they demonstrated that the transcription factor Eos in primary murine Tregs directly interact with Foxp3 in order to repress genes associated with differentiation of effector T cells (Pan et al., 2009). Thus, together with our observation that elevated basal Erk activation is maintained only in the actTregs, it is tempting to speculate that differentiation of rTregs into actTregs involves a shift in Mek-Erk signaling that is necessary in order to keep the level of FOXP3 in actTregs high and confer these cells with unique suppressive capabilities in a similar manner as observed for the transcription factors Eos and SATB1. Mechanistically, the selective TCR and coreceptor

coupled activation of Mek-Erk and Akt signaling in rTregs might prevent untimely initiation of Treg-mediated suppression in the early phase of an immune response.

4.2.5 Controlling immune responses by targeting Tregs

Based on the key role of Tregs in the maintenance of peripheral tolerance (Bennett et al., 2001;Brunkow et al., 2001;Wildin et al., 2001) and their involvement in impairment of effective immunity against certain pathogens (Boettler et al., 2005;Weiss et al., 2004) and some types of tumors (Curiel et al., 2004;Nishikawa and Sakaguchi, 2010), therapeutic strategies aimed to modulate their development and function may be beneficial for the treatment of a variety of immune-related diseases. Thus, therapeutic interventions that impair the function and/or reduce the number of distinct Tregs may favor initiation of immune responses towards cancer and chronic infections. Conversely, immunotherapies leading to increased number and/or elevated suppressive function of Tregs may be useful for the management of abnormal or excessive immune responses, such as various autoimmune diseases and chronic inflammatory diseases including asthma and inflammatory bowel diseases (Miyara et al., 2009). Moreover, the latter therapeutic approach may also potentially prevent graft rejection (Trzonkowski et al., 2009;Waldmann et al., 2006) and increase maternal tolerance against the fetus (Guerin et al., 2009;Saito et al., 2007). Critical in the design of an efficient strategy is to tip the balance towards either heightened Treg or effector T cell activity, depending on the character of the immune dysfunction (Miyara and Sakaguchi, 2011). Accordingly, dominance towards Tregs is required in order to suppress ongoing pathological immune responses and induce tolerance to self-reactive effector T cells, whereas relative dominance of effector T cells is warranted to enhance or strengthen immune responses against tumors and some types of pathogens that have the potential to cause chronic infections. Thus, from a clinical point of view it might be desirable to control these T cell subsets disparately.

Although Treg-based cellular therapy involving ex-vivo expansion of antigen-specific Tregs is an area of extensive research, there are still many issues that have to be addressed before it is safe to use cell-based therapies with expanded Tregs in the clinic (Miyara and Sakaguchi, 2011;Sakaguchi et al., 2010). The challenges include successful expansion of a pure Treg population (as human $FOXP3^+$ T cells comprise a heterogeneous population), maintenance of functional properties during in vitro expansion and not least stability in vivo after infusion. It

is still an open question whether this approach will be effective and introduce long-lasting effect. For this reason therapeutic interventions with small molecules or biological that selectively modulate or reprogram the function of Tregs in vivo may be an easier way forward and have clinical applicability. A global inhibition of Tregs is not desirable as this may increase the risk for initiation of autoimmune diseases. Consequently, antigen-specific Treg-based therapies are warranted. The results reported in Paper II indicate a selective Erk signaling response in rTregs versus actTregs, an effect also observed between naive and effector/memory T cells. Inhibition of Erk may therefore not only influence conversion of rTregs to actTregs, but also inhibit activation of naive T cells and initiation of primary immune responses. Consequently, Mek and Erk merits further investigation for their potential as therapeutic targets for controlling various immune-related diseases, particularly those involving excessive activity of Tregs.

4.3 PAG regulation of early BCR signaling

Accumulating data have demonstrated that PAG functions as a negative regulator of proximal TCR-signaling and thereby participates in fine-tuning the threshold for T cell activation. This effect has been attributed to the ability of PAG to bring Csk into the proximity of activated SFKs, allowing Csk to reduce the activity of the kinases that are concomitantly bound to the adaptor protein. In Paper III, we wanted to explore whether a similar functional role of PAG could be identified in B cells. Amongst the different B cell-expressed SFKs, Lyn appears to be the only one indispensable to B cell development and activation. We therefore decided to examine the interaction of this particular SFK with PAG in order to explore the regulatory role of PAG in early BCR signaling (Paper III).

4.3.1 Formation of a Lyn/PAG signaling complex

Mapping of the interactions between the SH3 domain of Lyn and PAG in Paper III revealed an ability of Lyn to bind to two proline-rich domains (PRDs) of the cytoplasmic portion of PAG, termed PRD1 and PRD2. The affinity for PRD1 appeared to be stronger than that observed for PRD2. Interestingly, Lyn-mediated phosphorylation of PAG seemed critically dependent on the interaction with the first PRD, as disruption of this binding site totally abrogated initiation of PAG phosphorylation. The observed ability of Lyn to associate with both PRDs in PAG contrasts with earlier observations by Ingley et al., which suggested

involvement of only one motif, specifically PRD2, for the preliminary formation of a Lyn/PAG signaling complex. It should be noted, though, that these data were obtained in a preliminary yeast two-hybrid screen (Ingley et al., 2006). However, an ability of SKFs to interact with PAG via several distinct binding domains of the same character is supported by Oneyama et al., who demonstrate that PAG-mediated c-Src transformation is dependent on two independent SH2 binding sites (Oneyama et al., 2008).

Although the binding of Lyn to PRD2 in our study did not appear to be crucial for the initial phosphorylation of the adaptor protein itself, our data do not exclude a functional role of this binding in vivo. It is possible that this interaction enhances the phosphorylation of PAG that is initiated upon binding to PRD1 or it could cooperate with other signaling molecules that direct other functional outcomes. Another interesting possibility would be if the second PRD of PAG confers binding to another SH3 domain-containing molecule such as PI3K, which has been shown to differ slightly from Lyn with respect to PRD binding preferences (Rickles et al., 1994). Indeed, recent data published by Tauzin et al. indicate that PI3K may bind directly to phosphorylated PAG in some B lymphoma cell lines (Tauzin et al., 2011). Based on the fact that B cells express several SFKs, it is reasonable to assume that PAG through Csk could contribute to regulating B cell activation by modulating their activities as well. Our finding that Fyn co-immunoprecipitated with PAG in lysates from the B lymphoma cell line Raji is in line with this notion and supports earlier reports that have suggested a general SFK-associating capacity of PAG (Ingley, 2008).

4.3.2 The role of PAG in early BCR signaling

Data from earlier reports indicate a differential role of PAG in the activation of B- and T cells as an elevated phosphorylation level of PAG is observed upon triggering of the BCR, whereas the adaptor becomes transiently dephosphorylated by TCR stimulation (Awasthi-Kalia et al., 2001;Brdicka et al., 2000;Kawabuchi et al., 2000;Mutch et al., 2007). Despite these differences, our results in Paper III indicate that PAG negatively regulates antigen-induced signaling responses in B cells. This effect was observed in primary human B cells and the normal EBV-transformed B cell line Con. In contrast, no regulatory role of PAG in BCR signaling was detectable in the Raji line of human Burkitt's lymphoma (BL) B cells, which appeared to express excessive amounts of PAG in our study. Taken together, these observations suggest that PAG potentially may exert different functional roles in normal B

cells versus B lymphoma cell lines, an issue that is discussed in detail in the following section.

In our study, transient elevation of PAG resulted in decreased responses of early BCR signaling in Con cells. Moreover, increasing the interaction of Lyn with the adaptor using a PAG construct with improved affinity for Lyn further enhanced the inhibitory effect of PAG. In contrast, abrogation of Csk binding to PAG, by transient overexpression of a PAG construct lacking Csk binding sites, led to augmented BCR signaling. Accordingly, our data demonstrate a PAG-mediated BCR inhibitory role of Lyn and Csk in the normal B cell line Con. However, to which extent the inhibitory effects of the Lyn/PAG signaling complex may be transferred to primary human B cells remains unknown. Based on the fact that PAG is an adaptor protein with dual function, having the ability to control signaling processes by either regulating the activity of Csk or sequestering signaling mediators in lipid rafts, it is possible that it mediates its function in primary human B cells by localizing Lyn in the proximity of stimulated BCRs, thereby regulating the function of other signaling molecules. Consistent with this idea, activated Lyn has been demonstrated to rapidly phosphorylate tyrosine residues of the inhibitory BCR coreceptor CD22 following stimulation of membrane-associated IgMs (Chan et al., 1997;Leprince et al., 1993;Nitschke, 2005). As noted in the Introduction, CD22 has the ability to negatively modulate BCR signaling upon induced phosphorylation of its ITIM sequences. These phosphorylated ITIMs will recruit intracellular signaling proteins such as the tyrosine phosphatase SHP1, resulting in down-regulation of BCR signals (Nitschke, 2005). Since PAG may influence both the CD22 and BCR receptors through either ITAM or ITIM sequences, the final outcome of increased PAG phosphorylation upon BCR stimulation depends on the local distribution of these around the phosphorylated adaptor protein. Phospho-flow analysis of the signaling motifs involved could potentially provide further insight into the function of PAG in B cell activation. For the time being, however, phospho-flow antibodies that detect these motifs remain commercially unavailable, and such analyses were therefore precluded from the study presented in Paper III.

The observations that PAG knockout mice, in contrast to Csk knockout mice (Imamoto and Soriano, 1993), are viable (Dobenecker et al., 2005;Xu et al., 2005) and that Csk is recruited to the plasma membrane even in the absence of PAG (Dobenecker et al., 2005;Xu et al., 2005) imply that other membrane-bound Csk adaptor proteins may exist. In support of this, a recent study demonstrated that the membrane adaptor protein Caveolin-1 cooperates with

PAG in coordinating Csk-mediated inhibition of c-Src in fibroblasts and lung cells from mice (Place et al., 2011). It was found that depletion or functional inactivation of one of these two adaptors led to up-regulation or elevated phosphorylation of the other so as to ensure sufficient recruitment of Csk and thereby maintain normal c-Src activity. Furthermore, elevation of c-Src activity upon simultaneous removal of both of these Csk adaptor proteins, by a combination of knockout and knockdown, indicated that these two proteins were the only Csk adaptors available in the studied cell system (Place et al., 2011). Together these findings may explain why knockout of Csk in mice was fatal (Imamoto and Soriano, 1993) whereas removal of either Caveolin-1 (Murata et al., 2007) or PAG (Dobenecker et al., 2005;Xu et al., 2005) did not affect viability. Analysis of the overall tyrosine phosphorylation responses in murine $PAG^{-/-}$ T cells revealed that these cells responded normally upon activation (Dobenecker et al., 2005;Xu et al., 2005), again indicating that compensatory mechanisms are brought into play in these cells upon removal of PAG.

Redundancy between other B cell-expressed GEM-associated TRAP members such as NTAL/LAB and LIME may have contributed to the apparent minor changes observed in BCR signaling upon PAG-KD in Paper III. Whether these two TRAPs play an inhibitory role in B cell activation is a matter of controversy (Simeoni et al., 2008) and further investigations are required for addressing the relative contribution of PAG and other adaptor proteins in B cell activation.

4.3.3 Function of PAG in normal B cells versus B lymphoma lines

The observations that PAG can be both down-regulated and up-regulated in transformed cells (Feng et al., 2009;Kanou et al., 2011;Oneyama et al., 2008;Sirvent et al., 2010;Svec et al., 2005;Tauzin et al., 2008) suggest that PAG might exert dual regulatory functions in cell activation. Particularly, PAG has been found to be highly expressed in Burkitt's lymphoma and other lymphoma variants (Svec et al., 2005;Tauzin et al., 2008). A similar expression level was observed in kidney cancer (Feng et al., 2009). Under these circumstances it might be that PAG enhances cell activation either directly by interacting with SFKs or indirectly by organizing several signaling mediators in a membrane-associated signaling complex. The latter is confirmed by a recent publication by Tauzin et al., which demonstrates that activated Lyn/PAG signalosomes in some B lymphoma cell lines including Raji cells organize a set of

signaling molecules that appear to be crucial for the generation of survival signals (Tauzin et al., 2011).

In Paper III, we compared early BCR signaling responses between primary human B cells and immortalized, EBV-transformed Con and Raji cells. The expression levels of PAG in the EBV-positive, BL-derived Raji cells were significantly higher than those observed for primary B cells and the normal transformed Con line. Despite lower expression of PAG, however, primary B cells appeared to contain more Lyn than the transformed cells. A similar inverse relationship between the expression levels of Lyn and PAG has earlier been reported (Ingley et al., 2006). Nevertheless, in line with previous work published by Ke et al. (Ke et al., 2009), the kinase activity was found to be higher in the transformed cell lines. In summary, we observed that the kinase/adaptor ratio varied the most between primary B cells and transformed cells, indicating that PAG could potentially exert different functions in normal versus immortalized B cell lines. We studied these differences in terms of early BCR signaling.

Our data demonstrated that phosphorylation of the proximal signaling molecules SYK, BLNK, Btk and PLCγ2 were increased in primary B cells compared to transformed cell lines, possibly due to the fact that primary B cells were in a resting state whereas the cell lines were highly activated even at the outset of the experiment (Gururajan et al., 2006). SYK has previously been found to be constitutively active in B lymphoma cells (Chen et al., 2008;Gururajan et al., 2007) and specifically to be important for the survival of Raji cells (Tauzin et al., 2011). We observed some differences in signaling between Con and Raji cells with respect to these proximal mediators, but our data do not allow us to draw any conclusion regarding the involvement of PAG in these differences.

The phosphorylation of Akt in response to IgM cross-linking in the presence of H_2O_2 appeared to be higher and more sustained in the B cell lines compared to the primary B cells. This observation is in agreement with earlier findings that activated Akt promotes tumor survival through both anti-apoptotic and pro-proliferative effects that are utilized by a range of human cancer cells (Osaki et al., 2004;Nicholson and Anderson, 2002;Marone et al., 2008). Although the expression level of Lyn was markedly lower in transformed cells the kinase activity in these cells appeared to be higher. Lyn has previously been linked to the activation of the PI3K/Akt pathway in lipid rafts, resulting in the induction of small cell lung carcinoma

(Arcaro et al., 2007), colorectal carcinoma (Sirvent et al., 2010), chronic lymphoid leukemia (Contri et al., 2005) and acute myeloid leukemia (Dos et al., 2008).

To explore whether the differences in signaling responses observed between Con and Raji cells were due to PAG, we did siRNA KD of PAG in the Raji cells. However, we were unable to detect any changes in signaling events by knocking down PAG as measured by phosphorylation of proximal signaling mediators, phospho-Erk and intracellular calcium flux. It might be that the stimulatory conditions used in our setup were inappropriate for elucidating the functional role of PAG in the transformed cells. Despite repeated attempts with various RNAi strategies, we were only able to knock down about 50% of the amount of PAG in the Raji cell line, which could explain why were unable to detect any changes in PAG-dependent signaling response in PAG-KD Raji cells.

4.4 Experimental tools for signaling studies

The study of signal transduction in smaller subsets of cells using traditional biochemical methods has always been challenging. However, recent technical developments including fluorescent cell barcoding (FCB) (Krutzik and Nolan, 2006) and a growing number of phospho-epitope-specific antibodies have made it possible to use phospho-specific flow cytometry to study signaling processes at single-cell resolution in several phenotypically defined immune cell populations simultaneously (Kalland et al., 2011;Krutzik and Nolan, 2003;Krutzik et al., 2004;Oberprieler et al., 2010;Perez and Nolan, 2002). Moreover, these developments have enabled increasing the resolution to a level where signaling differences can be linked to functional properties in small subsets of cells as shown in Paper II (Kalland et al., 2011). For the three studies included in this Thesis, a phospho-flow technique was used as a tool for investigation. I will therefore compare this technique with three other methods, where one of these, immunoblotting, currently is regarded as the primary tool for examination of signal transduction in various immune cell populations.

4.4.1 Phospho-epitope-specific flow cytometry

The signaling processes originating at surface-bound receptors are highly regulated by post-translational phosphorylation events, conferring changes in terms of biological activity, positioning or ability to interact with other proteins. Phospho-flow is a phospho-epitope-

specific intracellular staining technique for flow cytometry that allows for examination of phosphorylation cascades in heterogeneous cell types at the level of single cells (Irish et al., 2006b;Krutzik and Nolan, 2003;Krutzik et al., 2004;Perez and Nolan, 2002). Thus, by measuring protein phosphorylation with phospho-specific antibodies in different immune cells, defined by distinct phenotype markers, we get an insight into the temporary dynamics of multiple phospho-sites under various experimental conditions. As we experienced while establishing the phospho-flow protocol applied to the studies included in this Thesis, and which is also reported by the group of Garry Nolan (Schulz et al., 2007), careful titration of antibodies directed against both the phospho-epitopes and the phenotype markers is essential for a successful analysis of the signaling nodes in the intended subsets of cells. Therefore, each antibody implemented in our studies was first tested for suitability with respect to our protocol, then titrated to identify the concentration that would simultaneously maximize the fluorescence intensity shift, achieve minimal background stain, minimize antibody-consumption and ensure staining reliability.

The specificity and sensitivity of phospho-specific antibodies against TCR-activated proteins such as the MAPKs (Erk, p38 and SAPK/JNK) and Akt, implemented in phospho-flow cytometric methods, had been validated by use of specific inhibitors and parallel Western blot analysis in previous studies (Perez and Nolan, 2002). Furthermore, antibodies specific for pStat3, pSYK/ZAP70, pNF-κB, pBtk and pPLCγ2 had also been widely used previously in studies for intracellular staining (Bendall et al., 2011;Gibbs, Jr. et al., 2011;Irish et al., 2004;Irish et al., 2006a;Irish et al., 2010;Krutzik and Nolan, 2003;Krutzik et al., 2004;Krutzik et al., 2008). Following our standard calibration setup for signaling studies in T cell, each antibody was tested for their concentration-response in freshly isolated T cells treated with pS/T kinase inhibitors (Calyculin A), pY kinase inhibitors (Na_3VO_4), physiological αCD3 and αCD28 antibodies, anisomycin, and the combined use of the PKC activator phorbol 12-myristate 13-acetate (PMA) and the intracellular calcium inducer ionomycin (I), as partly demonstrated in Figure 11 and Figure S5 in the supplementary of Paper I. The phospho-flow results from these calibration experiments where either compared with western blot (WB) analyses of the same sample and/or benchmarked against signaling patterns reported in earlier studies.

In order to increase the capacity of phospho-flow based methods, the group of Garry Nolan has developed another advanced cytometric technique called fluorescent cell barcoding (FCB)

(Krutzik and Nolan, 2006) that we made use of. Particularly, FCB encompasses the use of varying concentrations of bioreactive fluorescent dyes (FCB markers), staining cells that are treated differently with different intensities (Figure 11).

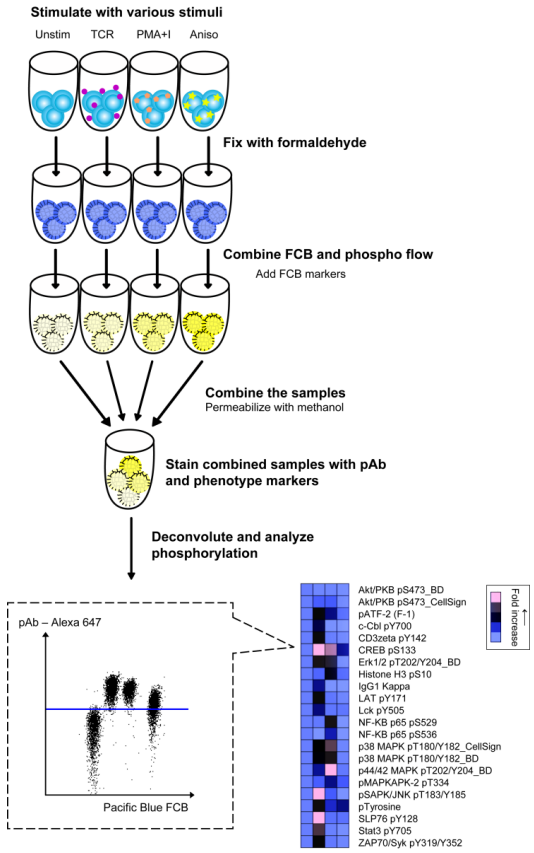

Figure 11. Flow diagram of the experimental setup using the phospho-flow technique in combination with FCB. The four samples were treated as follows (from the left): unstimulated (negative control); stimulated by the cross-linking of αCD3 and αCD28 Abs; treated with PMA and ionomycin (I); treated with anisomycin. After stimulation for a specific period of time, the cells were fixated in PFA in order to maintain their phosphorylation state. The fixated samples were then incubated with amine-reactive fluorescent dyes at varying concentrations for identification purposes. The samples were further permeabilized with ice-cold methanol, then washed, combined into one tube and stained with phospho-specific antibodies (pAb). During software analysis of the acquired data each of the original samples were identified according to their FCB signature and assessed separately with respect to each of the phospho-specific antibodies. In the representative FACS plot, each of the four different populations represent cells stimulated with one specific kind of stimulating agent, as noted above. The y-axis corresponds to the changes in phosphorylation of a specific epitope. Each column in the heatmap represents a different stimulus and each row represents a specific phospho-epitope. The color of each block is indicative of the fold change in mean fluorescence intensity in that channel.

This provides each sample with a unique fluorescence signature, enabling the combination and analysis of several distinct cell-samples at the same time, thereby eliminating assay-to-assay variation. The power of the FCB technique can be further enhanced by the use of two or more FCB reagents at the same time, generating a complex color signature that may be used to trace even more cell samples back to the sample from which they originated, on a per-cell basis. This technique has been taken advantage of throughout the present work, in particular in the studies reported in Paper I-II, and to some extent for the study reported in Paper III. Besides improving assay resolution, this technique also contributes to reducing antibody consumption and acquisition time. In multi-color phospho-flow analyses, multiple signaling nodes can be monitored simultaneously in a single cell using phospho-specific antibodies

labeled with distinct fluorophores. Hence, it is possible to obtain information on how the different signaling nodes interact in response to a given stimulus (Irish et al., 2004;Irish et al., 2006b;Perez and Nolan, 2002). In any case, the number of variables that can be studied at the same time is primarily subject to limitations in the instrumentation, such as the number of detectors (PMTs) in the FACS analyzer, as well as in the availability of reagents with appropriate fluorophores. Importantly, the difficulty of the experiment increases with each additional parameter as the spectral overlaps, which require compensation, gets more complicated.

In summary, phospho-flow is a semi-quantitative technique that provides relative quantification of the acquired data (Perez and Nolan, 2002). Its high throughput potential makes it a suitable tool for the investigation of complex signaling responses.

4.4.2 Comparison of immunoblotting and phospho-flow cytometry

Immune cell signaling, especially TCR signaling, has traditionally been studied using either immune-precipitation (pull-down) or immune-blotting techniques (e.g. WB). Our current knowledge on the signaling cascades initiated in response to TCR engagement is based heavily on results gained by the use of WB. While WB provides information on the phosphorylation of proteins in denatured cell lysates, phospho-flow allows for the detection of phosphorylated epitopes in cells that are fixed and permeabilized.

As detergent-insoluble fractions are removed by centrifugation or separation from the liquid phase of the lysate, at least part of the phosphorylated proteins that associate with lipid raft or the cytoskeleton may be removed and therefore not detected by standard WB analysis. The phospho-flow technique, on the other hand, detects the phosphorylation status in samples of stimulated cells that are fixed with paraformaldehyde (PFA), which cause protein-protein cross-linking and therefore are assumed to preserve both the localization and the interaction with other binding-partners of the targeted phosphoproteins. For this reason, in contrast to WB, phospho-flow gives information also of phosphorylated proteins that resides in detergent insoluble fractions, e.g. lipid rafts or those that associate with the detergent insoluble cytoskeleton, which is the case for certain signaling molecules after activation of antigen receptors (Goldman et al., 1997;Edmonds and Ostergaard, 2002). It should be noted, however, that the cell preparation involved in the phospho-flow protocol may mask or transform the epitopes to which the detecting antibodies bind, or impair the access of the phospho-epitope-

specific antibodies to their intracellular phosphorylated targets. Another caveat of this technique relies on the fact that access to the intracellular phospho-epitopes is gained by a methanol-based permeabilization that may cause some degree of protein denaturation. Generally, phospho-specific antibodies are verified by the manufacturers by their capacity to bind linear stretches of phosphorylated peptides. Thus, you can assume that the antibodies specifically made for phospho-flow cytometry will detect the fixed phospho-epitope while kept in its original state, as well as its denatured counterpart. The group of Garry Nolan has verified the specificity of several of the phospho-specific antibodies and designed an optimized phospho-flow protocol for these, comparing the results obtained by phospho-flow with WB results using the same cell samples (Krutzik and Nolan, 2003;Perez and Nolan, 2002). Both of these methods provided similar qualitative signaling patterns for pErk, pp38, pJNK, pStat1, pStat5 and pStat6, indicating that the results obtained by either method are equal for the phospho-specific antibodies tested (Krutzik and Nolan, 2003). Accordingly, in Paper III, we verified the antibodies specific for pBtk, pSYK, pBLNK and pErk in the context of PAG KD in normal B cells. However, based on the differences in the two experimental procedures as mentioned above, these methods may well yield different signaling patterns in response to the same stimulatory conditions, as described by Haas and colleagues (Haas et al., 2008). Interestingly, in this study, they demonstrated a gain in sensitivity for their phospho-flow approach compared to their WB analysis, phospho-flow revealing both concentration- and time-dependent changes dependent on the stimulatory conditions that was not even detectable with standard WB analysis.

One of the advantages of the phospho-flow technique over WB is its ability to analyze unsorted, complex mixtures of cells and its high-throughput potential, especially with the combined use of FCB as discussed in section 4.4.1. Of note, as reported by Perez et al. (Perez and Nolan, 2002), positive isolation of different cell populations may perturb the interpretation of the kinase activities of cells as antibodies used for the purpose of isolation upon binding can induce some sort of signaling itself. Hence, the permission of the phospho-flow technique to analyze heterogeneous mixtures of cells without prior cell sorting excludes this type of signaling disturbance. Furthermore, as FCB allows for the inclusion of both positive and negative controls in the same sample, pipetting and staining errors may be eliminated. For these reasons, phospho-flow represents an improvement over WB for studies of signal transduction that may also be beneficial in high-throughput setups for the discovery of new drugs in predefined populations of immune cells.

Although phospho-flow offers several advantages over WB analysis, some situations warrant the continued use of WB. Notably, while WB identifies proteins based on their molecular weight, phospho-flow does not. Hence, WB yields additional information about the mass of the phosphoproteins detected and thereby an indication of the specificity of the phospho-specific antibodies applied in the setup. Moreover, as WB is performed on cell lysates, the cell samples may be subjected to subcellular fractionation first, enabling the analysis of the subcellular localization of the phospho-proteins as well. These are all important points to bear in mind while selecting the most appropriate technique for a particular experimental setup.

4.4.3 Mass spectrometry (MS)

In principle, any soluble protein can be analyzed by MS. The main advantage with this approach is the unbiased and accurate characterization of proteins that relies on determination of peptide masses and identification of their sequences, which generally is performed by tandem MS (MS^2) (Germain et al., 2011). Still, as a tool for analyses of signaling networks MS suffer some weaknesses. One of these is low throughput. Sample preparations for MS analyses typically require extensive preprocessing, and both gradient separations by chromatography and data analysis are time-consuming processes. For system views in immunology an important disadvantage is the necessity of abundant starting material; for T cells that contain a small cytoplasmic volume, a cell number of at least 10^7 is often required. Furthermore, only a fraction of the sample is analyzed in one MS run, especially in complex samples, and there is a risk of assessing only the most abundant peptides (Germain et al., 2011). Thus, many peptides will go waste during the analysis and never be identified. For this reason, examination of samples run in parallel will give slightly different results as the sampling in each run will be incomplete. In order to achieve isolation of the less abundant phosphopeptides in a phosphoproteomic setup, enrichment techniques are utilized. In Paper I, we applied TiO_2 affinity chromatography to this end. Importantly, recent progress in MS instrumentation and technology has led to the emergence of high-sensitivity quantitative MS techniques that can be used on fewer cells (Luber et al., 2010;Iwai et al., 2010). Thus, while MS analyses had previously been performed predominantly on materials from cultured cells, it was now feasible to analyze primary cells, as had been done in Paper I. The major strength of MS with respect to phosphoproteomic analyses is that it allows for identification and comparison of thousands of proteins and phospho-sites in one run independently of antibody specificities. However, as opposed to phospho-flow cytometry that allow analysis of single-

cells, measurements from MS analyses, as those from WB analyses, reflects only averaging effects of the whole cell population analyzed.

4.4.4 Microscope techniques

Although phospho-flow cytometric approaches are valuable for analyses of phosphorylation-based signaling responses in primary immune cells, they are not able to provide information about localization, translocation or localized up- or down-regulation of the labeled phospho-sites over time. Despite the capability to analyze suspensions of heterogeneous cell populations, examination of cells located in a tissue environment is not possible. Furthermore, signaling responses detected by phospho-flow cytometry cannot directly be associated with cell behavior (e.g., changes in size, morphology, cell dynamic and motility). In cases where the above mentioned aspects are desirable to study, microscopic imaging is the preferred tool for investigation (Germain et al., 2011). As microscopic methods have not been utilized in the studies presented in this Thesis, further details about these methods will not be presented here. However, Sarris and Betz have reviewed the topic and provide an overview of the imaging techniques that have been applied in immunological research (Sarris and Betz, 2009). Of note, analyses of kinase-dependent intracellular signaling responses may be analyzed by a real-time FRET (fluorescence resonance energy transfer)-based imaging approach that relies on genetically encoded fluorescent indicators called Phocuses (fluorescence indicators for protein phosphorylation processes that can be custom-made), tracing phosphorylation-based alterations (Sato et al., 2002). In contrast to phospho-flow cytometry, this approach enables visualization and subcellular localization of intracellular phosphorylation processes using a confocal laser-scanning microscope. As a result, this method provides information not only of temporal but also of spatial dynamic changes of phosphorylation-dependent signaling at the single-cell level. Thus far, however, it is not possible to measure several kinases at the same time.

4.5 Closing comments and future perspectives

In the present Thesis, we have investigated phosphorylation-based signal integration in human immune cells. Signaling studies in T cells have previously demonstrated that approaching the cell from a systems perspective may be a prerequisite for understanding the functional implications of individual signaling processes. Notably, signaling responses may occur but

have no actual consequence. Therefore, although we examined the functional outcomes associated with the signaling responses observed in rTregs upon activation, further analyses of cellular behaviors linked to the signaling responses reported in Paper I-III would be beneficial. For instance, does the high basal level of PKA signaling in effector/memory T cells impair proliferation or cytokine production upon stimulation, or does their high constitutive PKA signaling and phopho-Erk levels enhance their responsiveness to true antigenic stimulation? Is the production of Igs enhanced upon PAG-KD or do the B cells become unresponsive? Given our in-depth data, the challenge now would be to assign a function to each of the signaling nodes observed. At present, many studies deduce novel biological mechanisms but do not validate them. Nevertheless, elucidation of these mechanisms will be essential in order to design modulation strategies for the generation and function of adaptive immune cells in the development of vaccines or therapeutics for the treatment of immunological diseases such as chronic infections, autoimmunity and cancers.

The work presented in Paper II led to a pending patent application on methods to inhibit activation of rTreg in a Mek-Erk-dependent manner, filed by the University of Oslo Technology Transfer Office, Inven2 AS. Future research should therefore reveal additional details surrounding the Mek-Erk-mediated inhibition of rTregs and will probably identify its potential in various clinical applications. This work is currently in progress in the laboratory of Prof. Kjetil Taskén, where several Mek-inhibitors have already been investigated (Lieske & Tasken, unpublished data). In addition, further investigation regarding the different signaling processes induced upon triggering of the CD28- and CD2 coreceptors are warranted, as these molecules influence the final outcome of T cell-mediated immunity. In this respect, a central issue introduced in the discussion is how CD2 compensates for its incapacity to properly activate NF-κB if the hypothesis of redundancy is correct. Further, a more detailed understanding of the mechanisms by which CD2 functions as a coreceptor in T cell activation will promote a clearer characterization of the interplay between antigen-recognition and CD28- and CD2 triggering in the initiation of T cell-mediated immune responses. Presently, the relative contribution of CD28- and CD2 costimulation in activated naive and effector/memory T cells is investigated in more depth in the laboratory of Prof. Kjetil Taskén (Skånland et al., manuscript in preparation). The PGE_2 signaling map obtained from the work presented in Paper I may serve as background information for studies of aberrant signaling responses to PGE_2 in immune-related diseases. The laboratory of Prof. Kjetil Taskén is at present using this study as a basis for studies designed to reveal signaling

differences between healthy individuals and patients with colorectal cancers (Moltu et al., unpublished data). Finally, the relative contribution of PAG and its cooperation with other adaptor proteins in B cell activation would be interesting to address in a follow-up study in order to elucidate its exact function in the activation processes of both normal B cells and various B lymphoma lines. Based on the fact that PAG is over-expressed in various B lymphoma cells, it would be of great interest to determine the impact of PAG on the aberrant signaling responses in these cells, as this eventually may lead to the identification of therapeutic targets for the treatment of these cancer types. As functional outcomes are expected to be dependent on the characteristics of the cells, detailed characterization of the studied tumor cells will be of importance in order to avoid misinterpretation of data. For future studies of the functional roles of PAG in lymphoma cells, it would be warranted to properly characterize the cells in order to decide whether the testing strategies or endpoints are appropriate for the indented purpose of the study.

5 Conclusions

1. 247 phosphorylation sites on 207 different proteins were found to be regulated by PGE_2 in primary T cells by means of a quantitative MS-based approach. Many of these were previously unknown phosphorylation sites (Paper I).

2. Based on the MS data, kinase pathway prediction and phospho-flow network analyses, PKA was identified as the central node of PGE_2-regulated signaling networks in primary human T cells (Paper I).

3. A constitutively active PKA signaling node was identified in effector/memory T cells (Paper I).

4. Engagement of the CD28 coreceptor was found to produce unique signals essential for NF-κB activation, whereas CD2 costimulation enhanced TCR-mediated signaling responses (Paper II).

5. CD28 costimulation potentiated TCR signaling in both naive and effector/memory $CD4^+$ and $CD8^+$ T cells and the signaling responses were further enhanced by concomitant stimulation through the CD2 coreceptor (Paper II).

6. Augmented constitutive phospho-Erk levels were identified in effector/memory T cells (Paper II).

7. Differences in signaling between rTregs and actTregs were identified. Whereas activation-induced Akt signaling was present in rTregs and diminished in actTregs, actTregs displayed constitutively high Erk phosphorylation with resulting diminished Erk responses compared to rTregs (Paper II).

8. A Mek-Erk-dependent induction of FOXP3 was found to be crucial for the development of rTregs into fully competent actTregs (Paper II).

9. Lyn was demonstrated to associate through its SH3 domain with two PRDs of PAG. The affinity for PRD1 was stronger than that observed for PRD2 and binding to PRD1 appeared to be essential for initiating PAG phosphorylation (Paper III).

10. PAG, by means of Lyn and Csk, negatively regulates proximal BCR signaling in normal human B cells, but not in the B lymphoma line Raji with over-expressed PAG (Paper III).

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