

Immunity Leashed – Mechanisms of Regulation in the Human Immune System

by

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In memory of

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List of publications

1. **Torheim EA, Ndhlovu LC, Pettersen FO, Larsen TL, Jha AR, Torgersen KM, Kvale D, Nixon DF, Taskén K, and Aandahl EM (2009).** Interleukin-10-secreting T cells define a suppressive subset within the HIV-1-specific T-cell population. *Eur J Immunol*, 39(5): 1280-7.
2. **Torheim EA, Jarnæss E, Lygren B, and Taskén K (2009).** Design of proteolytically stable RI anchoring disruptor peptidomimetics for *in vivo* studies of anchored type I protein kinase A-mediated signaling. *Biochem J*, 424(1): 69-78.
3. **Pettersen FO, Torheim EA, Aaberge I, Holm M, Lind A, Taskén K, Kvale D (2009).** Cyclooxygenase type 2 inhibitor down-regulates chronic immune activation, CD38, and PD-1 in patients with untreated HIV-1 infection. Manuscript.

Abbreviations

AC	adenylyl cyclase	GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
Ad5	adenovirus type 5	GPCR	G protein-coupled receptor
ADP	adenosine diphosphate	GRAIL	gene related to anergy in lymphocytes
AIDS	acquired immunodeficiency syndrome	GRB2	growth-factor-receptor-bound protein 2
AKAP	A-kinase anchoring protein	GVHD	graft-versus-host disease
AKAP-IS	AKAP-in silico	H	histone
AMP	adenosine monophosphate	HEV	high endothelial venule
APC	antigen-presenting cell	HIV	human immunodeficiency virus
AR-HIES	autosomal-recessive hyperimmunoglobulin E syndrome	HLA	human leukocyte antigen
ATF-1	activator transcription factor-1	HPK-1	hematopoietic progenitor kinase-1
ATP	adenosine triphosphate	HSC	hematopoietic stem cell
Bcl-6	B-cell lymphoma-6	IBD	inflammatory bowel disease
C	catalytic subunit of PKA (isoform C α , C β , or C γ)	ICAM	intercellular adhesion molecule
cAMP	cyclic adenosine 3', 5' monophosphate	ICER	inducible cAMP early repressor
CARE	cAMP autoregulatory elements	IFN	interferon
CBL	Casitas B-lineage lymphoma	IKK	I κ B kinase
Cbp	Csk binding protein	IL	interleukin
CCL	CC-chemokine ligand	IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
CCR	CC-chemokine receptor	ITAM	immunoreceptor tyrosine-based activation motif
CD	cluster of differentiation	ITIM	immunoreceptor tyrosine-based inhibitory motif
CFSE	carboxyfluorescein succinimidyl ester	ITK	IL-2-inducible T-cell kinase
CNS	conserved non-coding sequences	iTreg	induced Treg
COX	cyclooxygenase	I κ B	inhibitor of kappa-light-chain-enhancer of activated B cells
CRE	cAMP response element	IRF-4	interferon regulatory factor-4
CREB	CRE-binding protein	JAK	Janus kinase
CREM	CRE modulator	LAG-3	lymphocyte activation gene-3
Csk	C-terminal Src kinase	LAT	linker for activation of T cells
CtBP-1	C-terminal binding protein-1	Lck	lymphocyte-specific protein tyrosine kinase
C-terminal	carboxy (COOH)-terminal	LFA	leukocyte function-associated antigen
CTL	cytotoxic T lymphocyte	LPS	lipopolysaccharide
CTLA-4	cytotoxic T-lymphocyte antigen 4	LYP	lymphoid tyrosine phosphatase
CXCL	CXC-chemokine ligand	MadCAM-1	mucosal vascular addressin cell adhesion molecule-1
CXCR	CXC-chemokine receptor	MAPK	mitogen-activated protein kinase
D/D	dimerization/docking	MHC	major histocompatibility complex
DC	dendritic cell	NFAT	nuclear factor of activated T cells
DC-SIGN	dendritic cell-specific ICAM-grabbing non-integrin	NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
DNA	deoxyribonucleic acid	NK	natural killer
DOK	downstream of kinase	NKT	natural killer T
DUB	deubiquitylating enzyme	NLR	nucleotide-binding domain and leucine-rich repeat-containing
DUSP	dual-specificity protein phosphatase	NOD	non-obese diabetic
EGFR	epidermal growth factor receptor	Nrp-1	neuropilin
ELISA	enzyme-linked immunosorbent assay	N-terminal	amino (NH ₂)-terminal
EP	E-prostanoid	nTreg	natural Treg
EPAC	exchange protein directly activated by cAMP	PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
ERK	extracellular signal-regulated kinase	PAMP	pathogen-associated molecular pattern
ERM	ezrin, radixin, and moesin	PBMC	peripheral-blood mononuclear cells
FOXP3	forkhead box protein-3	PD-1	programmed death-1
Gads	GRB2-related adaptor protein	PDE	phosphodiesterase
Gas	G protein α -subunit		
GATA-3	GATA-binding protein-3		
γ c	gamma-chain		
GEM	glycosphingolipid-enriched membrane microdomain		
GITR	glucocorticoid-induced TNF receptor		

PD-L	PD ligand	siRNA	small interfering RNA
PG	prostaglandin	SIV	simian immunodeficiency virus
PH	pleckstrin homology	SLE	systemic lupus erythematosus
PKA	cAMP-dependent protein kinase; protein kinase A	SLP76	SH2 domain-containing leukocyte protein of 76 kDa
PKC	protein kinase C	SNP	single-nucleotide polymorphism
PLC γ 1	phospholipase C γ 1	SOCS	suppressors of cytokine signaling
PMN	polymorphonuclear	SOS	sons of sevenless
PRR	pattern recognition receptor	STAT	signal transducer and activator of transcription
PTK	protein tyrosine kinase	STS	suppressor of TCR signaling
R	regulatory subunit of PKA	SUMO	small ubiquitin-like modifier
RA	retinoic acid	$t_{1/2}$	half-life
RA	rheumatoid arthritis	T1D	type-1 diabetes
RI	regulatory subunit of PKA type I (isoform RI α or RI β)	T-bet	T box expressed in T cells
RIAD	RI anchoring disruptor	TCM	central T memory
RIG-I	retinoic acid-inducible gene I	TCR	T-cell antigen receptor
RII	regulatory subunit of PKA type II (isoform RII α or RII β)	Teff	effector T cells
RLR	RIG-I-like receptor	TEM	effector T memory
RNA	ribonucleic acid	Tfh	follicular T helper
ROR γ t	retinoid orphan receptor-gamma-t	TGF-b	transforming growth factor-b
Rp-cAMPS	adenosine- 3', 5'-cyclic monophosphorothioate	Th	T helper
RT	reverse transcriptase	TKIP	tyrosin-kinase inhibitor peptide
SCF	S-phase kinase-associated protein-1- cullin-F box protein	TLR	Toll-like receptor
SCID	severe combined immunodeficiency	TNF	tumor necrosis factor
SH2	Src homology 2	Tr1	T regulatory 1
SHP	SH2-containing protein tyrosine phosphatase	Treg	regulatory T cells
		TSAD	T-cell specific adaptor protein
		Vav	Vav-1 oncogene
		ZAP70	ζ -chain-associated protein kinase of 70 kDa

1. Introduction

Three layers of protection serve to maintain the health of mammals living in a non-sterile environment (Fig. 1). First, physical barriers in the form of epithelial surfaces prevent microorganisms from entering the body. These barriers include the skin as well as the respiratory and intestinal mucosa. To further discourage colonization of mucosal surfaces by microorganisms, they are covered with anti-microbial agents. In addition, ciliary movement and muscular contractions in the form of bowel peristalsis and coughing clear the surfaces of mucus and trapped microorganisms. Whenever the physical barriers are breached, and microorganisms enter the underlying tissues, components of innate immunity are called into play. This second line of defense encompasses mechanisms of great diversity, ranging from molecular mechanisms such as the complement system, to cellular mechanisms in the form of phagocytic macrophages and natural killer cells. Typically, the effectors of innate immunity are pre-formed and expressed throughout the body, and may be recruited to the site of infection in a very short period of time.

The innate immune system constitutes a powerful and rapid defense that is able to overcome most invading microorganisms. However, in the event that an infectious agent manages to break through the first (innate) barriers, a secondary (adaptive) response is elicited. The inflammatory mediators provided by the innate effector systems serve to direct the adaptive immune response, which constitutes the third and final level of protection. For the adaptive immune system to be activated, the invading microorganism must be phagocytosed and processed by a dendritic cell (DC), which, in turn, translocates to draining lymph nodes while presenting fragments of the microorganism to circulating lymphocytes. Once a cognate lymphocyte is activated, it multiplies to create a clone of lymphocytes that all have the same antigen specificity. Eventually, the cells of the resulting lymphocyte clone differentiate into effector lymphocytes that attack the invading microorganism; either by the production of antibodies (B lymphocytes) or pro-inflammatory mediators (helper T lymphocytes), or by killing infected cells (cytotoxic T lymphocytes).

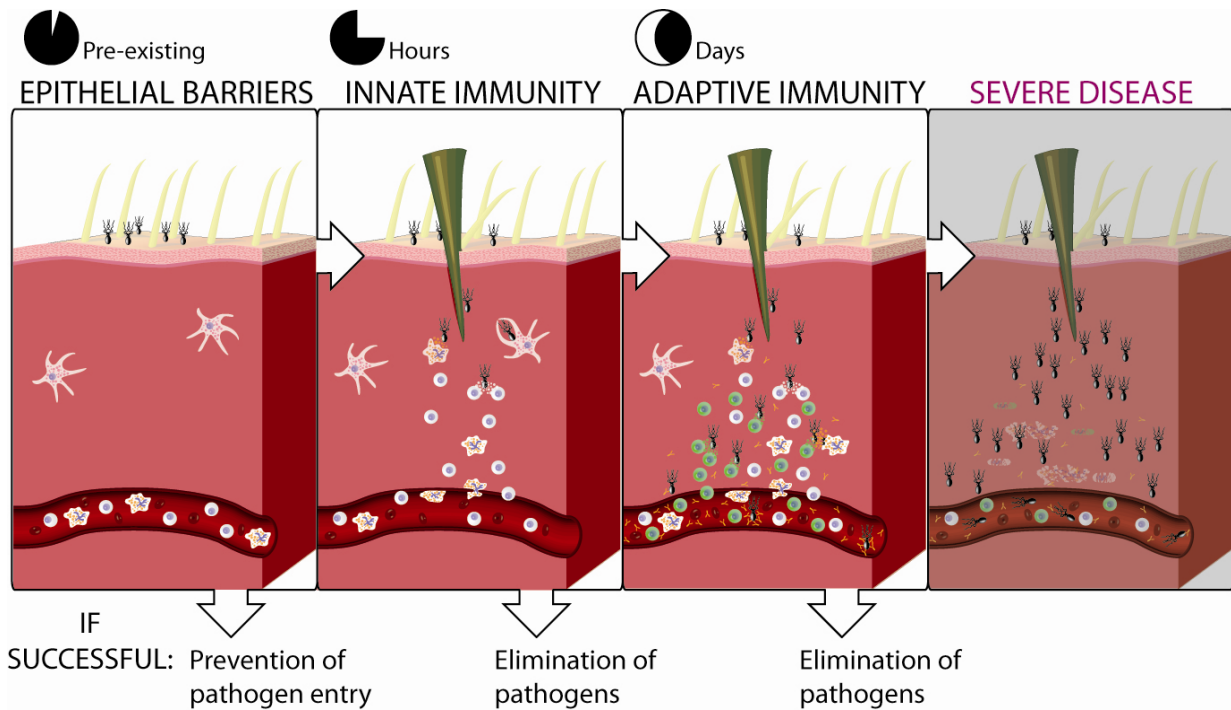


Figure 1: Major levels of protection in the human immune system. Three consecutive barriers prevent pathogens from entering the body. First, the epithelial barriers physically block the entry of microorganisms. Second, in the event that the epithelial barrier is breached, innate immune cells and other innate mechanisms constitute the immediate response against invading pathogens. Third, upon ingestion of pathogen by dendritic cells and presentation of peptide fragments to cognate T cells in draining lymph nodes, the adaptive immune response is initiated, involving clonal expansion of pathogen-specific lymphocytes and the generation of effector molecules such as cytokines, perforin/granzymes, and antibodies. The pathogen may be warded off at any of these levels. Should it succeed in evading these defensive mechanisms, however, severe disease may be the result.

1.1 Check-points of the normal immune response

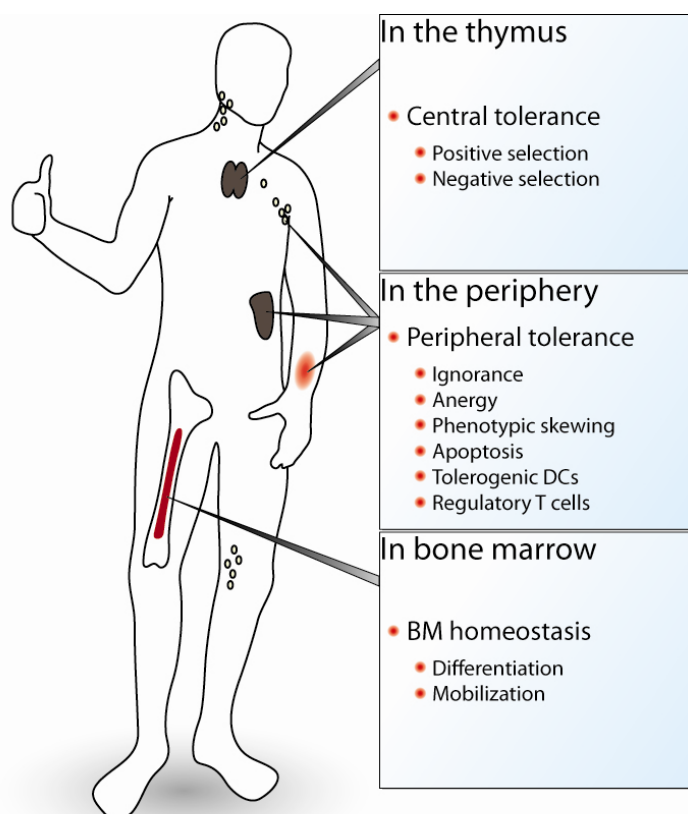
The series of events that was outlined above is what constitutes a normal immune response. It is well organized, and the different mechanisms of protection are called upon in an orderly manner. Most of the microorganisms that the host encounters are neutralized by these mechanisms. In the event that they are not, however, they may cause life-threatening and/or chronic infections. Thus, the importance of a functioning immune system can hardly be overstated. However, the defensive mechanisms entailed in the immune system have themselves, occasionally, been known to cause damage to the organism. Illnesses such as rheumatoid arthritis and diabetes type 1 are well-known examples of autoimmunity, resulting from targeted adaptive immune responses against components of articular or pancreatic tissues, respectively. These illnesses occur in spite of numerous safety mechanisms that have been put in place to avoid damage to self, and that will be discussed in the following sections.

1.1.1 The concepts of central and peripheral tolerance

Newly formed lymphocytes are tested early in life for their propensity to recognize determinants of self. B lymphocytes, being generated in the bone marrow, and T lymphocytes, which develop in the thymus, are both subjected to a selection process designed to rid the body of potentially self-reactive immune cells (276). This process is known as central tolerance and relies on deletion of cells carrying antigen receptors that recognize self antigens. The induction of Treg contributes to central tolerance as well, whereas processes such as anergy and receptor editing are believed to be of secondary importance (171). Since not all self antigens are expressed at the primary sites of lymphocyte development, mechanisms of peripheral tolerance have evolved to induce tolerance as the lymphocytes encounter self antigens in the periphery. Some of these mechanisms act directly on the responding T cell (T-cell intrinsic mechanisms like ignorance, anergy, phenotypic skewing, and apoptosis), whereas others evoke additional cell subsets, including tolerogenic DCs and Treg (T-cell extrinsic) (419). Figure 2 summarizes the major instances of central and peripheral tolerance.

Ignorance of self-antigens may represent the simplest setting of peripheral tolerance, yet has proven to be an important checkpoint in the development of, for example, murine diabetes (17; 170). In this case, tolerance may result from sequestration of the antigen in sites that are not easily accessible to the self-reactive T cell (12), or the antigen may not be present in sufficiently high concentrations to trigger a T-cell response (214). Anergy, constituting another form of peripheral tolerance, can manifest itself either intrinsically or in a T-cell-extrinsic manner, and results in a state of unresponsiveness to antigen. The induction of anergy was

Figure 2: Regulatory mechanisms that influence T-cell-mediated immunity. Mechanisms throughout the body contribute to maintaining immune homeostasis and keeping the immune system in check in the absence of pathogens. The generation of T cells and T-cell-mediated immune responses are controlled at multiple levels, from the mobilization of precursor cells in the bone marrow *via* differentiation in the thymus to effector functions in the periphery.



initially demonstrated *in vitro* following ligation of the T-cell receptor (TCR) in the absence of co-stimulation (183), but inhibitory co-receptors have since been discovered that are responsible for anergy *in vivo*, including cytotoxic T-lymphocyte antigen (CTLA)-4 (303; 422) and programmed death (PD)-1 (221). Notably, CTLA-4 may also be instrumental in the suppressive functions of Treg (433).

Treg play a prominent role in the maintenance of peripheral tolerance, as was demonstrated by the reconstitution of athymic nude mice – that lack T cells – with normal T cells (21). If the transplanted T cells were depleted of CD4+CD25+ Treg prior to reconstitution, the recipient athymic mice were shown to develop multiple autoimmune diseases. Importantly, any sign of autoimmunity was avoided by the concomitant administration of CD4+CD25+ Treg. These findings were substantiated by studies in mice that were thymectomized early in life. Thymic output of CD4+CD25+ Treg commences only at day 3 after birth – in contrast to CD4+CD25- T cells, which can be found circulating immediately after delivery (21). Thus, thymectomy at day 3 abrogates thymic generation of Treg and leaves an excess of self-reactive peripheral T cells largely unchecked, causing organ-specific autoimmune diseases. Depending on the mouse strain, this may affect the stomach, thyroid, ovary, testes, or prostate (204).

The role of innate immunity in directing peripheral tolerance remains somewhat unclear (419), although recent observations in for instance allergic diseases indicate that innate immune mechanisms involving cytokine production may be used to reinstate immune balance (230). Also, the successful use of adjuvants in vaccines to improve the generation of memory lymphocytes upon vaccination implicates modulation of peripheral tolerance by innate mechanisms (217). Notably, however, we remain largely ignorant of the operating mechanisms underlying the majority of adjuvants currently used in vaccines.

1.1.2 Molecular check-points in antigen presentation

Through the actions of tissue-resident antigen-presenting cells (APC), microorganisms and other potentially noxious agents are digested and presented in a highly specific manner to the cells that are best suited for neutralizing them. Thus, specialized APC like the DC infiltrate the peripheral tissues and serve as sentinels for the immune system. They continuously sample their surroundings and ingest any microorganisms that they come across. Receptors of the innate immune system that are expressed by the DC itself sense ‘danger signals’ in the surroundings and instruct the DC to bring its contents to the draining lymph node for presentation to circulating lymphocytes. The digested antigen (normally a peptide) is presented in the context of antigen-presenting molecules such as the major histocompatibility complex (MHC) class II, which is only expressed on the so-called ‘professional’ APC (DC,

macrophages, B cells, and basophils). Circulating lymphocytes access the presented antigen while passing through the lymph node and will, upon recognition, initiate an immune response then and there. In the case of viral infections, or infections with other intracellular pathogens, the cells that are infected (including non-hematopoietic tissue cells) will present fragments of digested antigen in the context of MHC class I. Peptide–MHC class I complexes are, in turn, recognized by antigen-specific CD8⁺ cytotoxic T cells, which initiate directional secretion of cytolytic products towards the infected cell in order to kill it by inducing apoptosis.

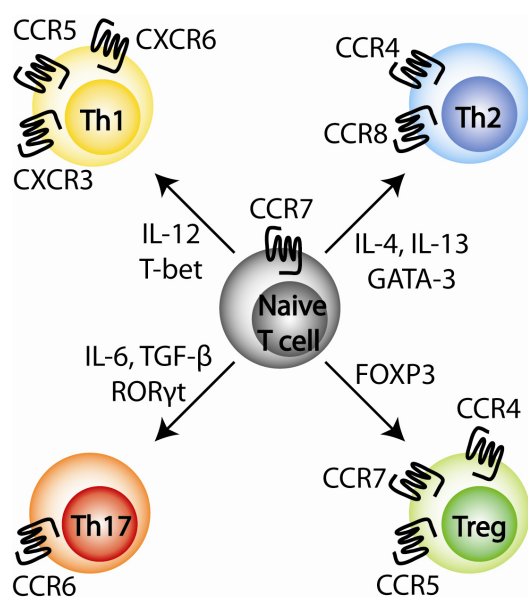
Dendritic cells play a major role in directing immune responses, and are known to be capable of inducing tolerance as well as immunity. The signals delivered by the DC to a responding T cell determine T-cell activation, clonal expansion, and differentiation (46). Depending on the activation status of the DC, three signals may be delivered. The first signal results from ligation of the TCR by a peptide–MHC complex. However, if delivered by itself, this signal will only result in tolerization of the T cell. On the other hand, if provided along with a second, co-stimulatory signal, the T cell may become activated. A third signal determines the differentiation fate of the T cell. This is transmitted in the form of soluble inflammatory mediators released into the surroundings by the DC and accessory cells of the lymph node. Both the second and third signals require prior activation of the DC or accessory cells through ligation of surface-expressed pattern recognition receptors (PRRs). The PRRs constitute a family of innate immune receptors that recognize structurally unrelated but evolutionarily conserved pathogen-associated molecular patterns (PAMPs) and include the membrane-bound Toll-like receptors (TLR), the cytosolic nucleotide-binding domain and leucine-rich repeat-containing (NLR) molecules and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (189; 295). Triggering of these receptors up-regulates the secretion of inflammatory mediators, as well as the expression on DCs of the co-stimulatory molecules that provide the second signal to cognate T lymphocytes. Thus, the PRRs are important regulators of antigen presentation, and constitute a significant means by which DCs are able to distinguish between self and non-self, so that only non-self antigens are presented to T cells in the context of potent co-stimuli. (46)

1.1.3 Leukocyte trafficking – homeostatic and inflammatory motility of immune cells

The continuous trafficking of immune cells between lymphoid and peripheral tissues plays a critical role in maintaining immunity. Even in the absence of inflammatory stimuli, some immune cells populate the peripheral tissues, either as resident sentinels or as patrolling surveyors. Both innate and adaptive immune cells contribute to this surveillance, which facilitates quick responses against potential threats. The polymorphonuclear (PMN) cells constitute the first line of cellular defense and are rapidly recruited to sites of infection by

locally produced inflammatory cues. The APCs, represented in the periphery by macrophages and dendritic cells, remain comparatively stationary as they sample their immediate vicinity for infectious material. As described above, the APCs will migrate to the draining lymph node in the event of pathogen recognition and fulfill its critical role in recruiting the adaptive immune system. Moreover, lymph-node resident B cells and basophils continuously engulf and present (on MHC II) soluble antigens that are brought to the lymph node through afferent lymphatics, thereby providing a second route to the activation of adaptive immunity (366).

The migration of circulating B- or T lymphocytes from the blood stream into a lymph node through high endothelial venules (HEVs) is subject to strict regulation [reviewed in (415)]. First, the lymphocyte adheres loosely to the endothelium through the interaction of L-selectin (CD62L) expressed on its surface with the HEV-expressed glycoprotein GlyCAM-1 (59; 219). This slows the cell down, and it starts rolling on the endothelial surface under the force of the blood stream. The intimate contact with the endothelium and the slow rotation of the cell facilitates interaction with chemotactic factors (e.g., chemokines) expressed on the endothelium. The expression of the chemokines CC-chemokine ligand (CCL) 21 and CCL19 on the HEV vessel wall serves to specifically recruit lymphocytes carrying the CC-chemokine receptor (CCR) 7 on their surface. Moreover, rolling B cells may also be recruited by CXC-chemokine ligand (CXCL) 12, which interacts with the B-cell expressed CXC-chemokine receptor (CXCR) 4. Recognition of these chemotactic factors results in upregulation of integrins on the surface of the lymphocyte, which mediates firm adhesion through the tethering of integrin receptors (intercellular adhesion molecule [ICAM]-1 or -2) presented on HEVs and, finally, extravasation of the cell. This three-step process of rolling, firm adhesion, and extravasation also applies to the migration of leukocytes from blood vessels into peripheral tissues, although the specific chemotactic factors will be different. Thus, the tissue-specific



expression of chemokines and adhesion molecules facilitates targeted trafficking of immune cells. Correspondingly, the migratory properties of a given leukocyte can be told by the range of homing receptors it expresses, which may, in turn, provide

Figure 3: Chemokine receptor expression profile of CD4+ T-cell subsets. The expression of chemokine receptors determines the migratory capacities of the CD4+ T-cell subset, and is influenced by the transcriptional regulation of the cell. Adapted from Ward *et al.*, 2009 (424).

important clues regarding its functional roles. The chemokine receptor expression profiles of central CD4⁺ T-cell subsets are presented in Figure 3.

1.1.4 Co-stimulation – the fruits of an inflammatory milieu

For a proper understanding of how the immune system is regulated, one fundamental idea that must be deliberated is the concept of co-stimulation. Co-stimulation lies at the very heart of immune regulation, being the decisive mechanism that balances tolerance and immunity in any given immune response. The importance of co-stimulation was first appreciated in 1970 by Bretscher and Cohn (60), who realized that the activation of an antigen-specific lymphocyte response required at least two distinct signals from the APC. It has since been determined that co-stimulation may be provided in several different ways – two of them being through ligands presented on the plasma membrane of APCs, or soluble factors released either by the APC itself or by accessory cells – and that the co-stimulatory factors may utilize a variety of intracellular signaling pathways to mediate survival or to offer proliferative cues (7; 209; 369).

The B7:CD28 family of co-stimulatory molecules plays a central role in regulating adaptive responses, and members of this family, in particular CD28 itself (see Fig. 4), directly control the activation status of T cells upon antigenic stimulation (7). Importantly, there is a quantitative relationship between the number of ligated TCR on a T cell and the likelihood of activating the cell – i.e., releasing it from the resting state (G₀ phase) to enter the cell cycle – resulting in proliferation and differentiation, and the initiation of an adaptive immune response. If the density of properly presented antigen is very high, it is actually conceivable that the T cell may proceed to proliferation and differentiation in the absence of co-stimulation. However, *in vivo*, the concurrent availability of co-stimulatory agents is an absolute prerequisite for maintaining a vigilant immune system and preventing disease. Mice lacking CD28 present with

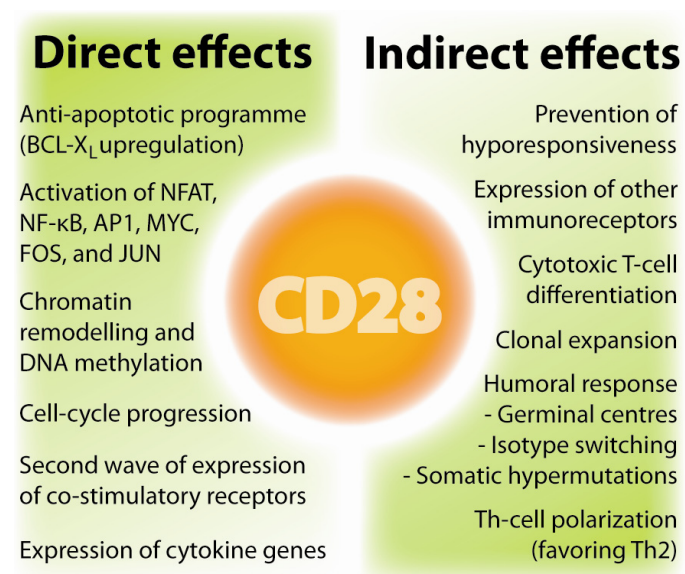


Figure 4: Effects of CD28 co-stimulation.

The prominent role of CD28 in promoting complete T-cell activation results from direct effects (left) on gene activation, cell cycle and survival, and from inducing the expression of a second wave of membrane receptors by activated T cells. The indirect effects (right) on clonal expansion and T-cell differentiation further underscores the importance of CD28 as a co-stimulatory factor. AP1, activator protein 1; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; Th, T helper cell. Adapted from Acuto *et al.*, 2003 (7).

reduced responses to a variety of immune challenges, as is also the case in mice that are devoid of the CD28 ligands CD80 and CD86 (7). Thus, co-stimulation through ligation of CD28 on the T cell by APC-expressed CD80 or CD86 is an important requirement for initiating a productive immune response. Other important co-stimulatory receptors include CD2 and the β_2 -integrin leukocyte function-associated antigen 1 (LFA-1), yet these cannot rival the stimulatory capacity of CD28 (444). Cytokines constitute another set of co-stimulatory molecules known to influence T-cell activation. In addition, the presence of inhibitory co-receptors may negatively regulate TCR stimulation, causing suppression of T-cell activation if qualitatively outnumbering the co-stimulatory receptors. Important co-inhibitory molecules include CTLA-4 and PD-1, also of the B7:CD28 family (148).

1.1.5 The importance of lymphoid tissues in directing immunity

The term “lymphoid tissue” denotes a collection of tissues dedicated to the generation of immune cells and induction of immune responses. The primary lymphoid tissues, including the bone marrow and the thymus, constitute veritable immune-cell cradles since this is where all blood cells originate. The secondary lymphoid tissues, encompassing the spleen and the peripheral lymph nodes, are hubs distributed throughout the body in which immune cells meet and adaptive immune responses are generated. In the primary lymphoid tissues, pluripotent hematopoietic stem cells (HSCs) differentiate and proliferate to replenish the blood cells that are lost in immune responses or due to homeostatic turnover. Thus, one fundamental step of immune regulation concerns the tightly regulated process of hematopoiesis, which involves the generation of new immune cells from HSCs in primary lymphoid tissues. Each subset of immune cells requires distinct growth factors, which are provided to the HSCs by the specialized microenvironment in niches created by committed accessory cells. These express and secrete factors that regulate the HSC homeostasis and regeneration, as well as its differentiation into specific hematopoietic lineages (195).

The secondary lymphoid tissues constitute hubs for the initiation of adaptive immune responses, and serve to facilitate the specific interactions required for activation of T- and B lymphocytes. This function is reflected in the lymph node architecture (Fig. 5): Naive T cells enter the lymph node from venous blood through the specialized HEVs (415). Following extravasation, which requires the expression of CD62L, CCR7, and LFA-1, the T cell enters the T-cell rich paracortex of the lymph node. Here it encounters antigen in the context of MHC class II as presented by either mature DCs that have migrated from the periphery through afferent lymph, or resident B cells or basophils that have captured antigen directly from lymph. In the event that the CD4⁺ T cell encounters its cognate antigen, it becomes activated and

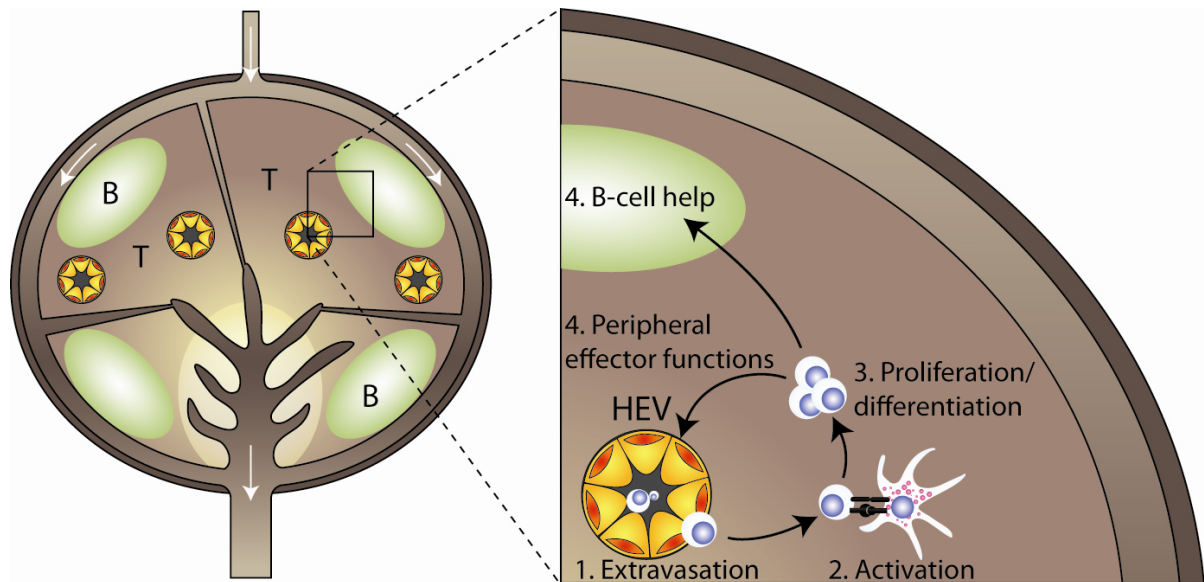


Figure 5: Lymph node architecture. The main routes of lymph flow are indicated by white arrows (left). Enlarged section: Circulating T cells enter the lymph nodes through high endothelial venules (HEVs) and encounter antigen-presenting cells in T-cell rich areas situated near B-cell zones (green spheres). T cells that meet cognate antigen and are activated undergo several steps of proliferation and differentiation, before progressing either to the adjacent B-cell zone, where they facilitate activation of antigen-specific B cells, or to the periphery, where they promote killing of pathogen and pathogen-infected cells. Adapted from von Andrian *et al.*, 2003 (415).

transiently expresses CXCR5 while down-regulating CCR7 to be able to leave the T-cell area and translocate to the B-cell rich primary follicles (339). Here, they interact with and stimulate cognate B cells to initiate a humoral immune response. The T cell may also exit the lymph node through an HEV and migrate to the area where the immune response originated, to direct immunity there.

The central role of lymph nodes in the generation of adaptive immune responses implies a role in controlling immunity. Importantly, the trafficking of immune cells through the lymph node is restricted to certain subsets of immune cells, and it may also be regulated upon inflammation to improve the chances of eliciting a productive immune response. Our knowledge concerning these processes remains limited, however, but the recent advent of multiphoton microscopy of intact tissues in live animals has greatly improved our understanding of the dynamics of antigen presentation (87).

1.2 *The players of adaptive immunity*

Keeping the body free of pathogens and avoiding the development of malignancies is a very demanding task, which has to be solved with a certain level of finesse in order to avoid exhausting the body's resources. While facing an enormous number of potential intruders, the challenge once lay in finding a way of neutralizing all conceivable threats at a minimum of energy expenditure. As previously outlined, the innate immune system serves an important role

in fending off most of the attacks. However, as soon as the pathogen has breached the innate defenses and established itself inside the body, another level of sophistication is required. While the organism continuously runs the risk of being overthrown by foreign and uninvited powers, simultaneously preparing for all of these risks by stock-piling immune cells in every flavor has, throughout evolution, proven too costly. Instead, the remedy to our needs evolved some 500 million years ago in the form of rearranging antigen receptors, resulting over the following years in an adaptive immune system capable of targeting and expelling any invader. The price to pay, however, is that it will only do so after a couple of rounds of cellular expansion and differentiation, resulting in a lag-time of about 4-7 days throughout which we depend on the innate immune mechanisms for our safety and wellbeing.

Another key feature of adaptive immunity is the ability to develop immune memory, enabling rapid and potent immune responses upon re-exposure to antigens that have been encountered previously (113). Following an immune response, most of the clonally expanded T- and B lymphocytes are killed in the interest of immune homeostasis. However, some cells are fed survival signals and redirected through a series of differentiation steps to become memory cells. These cells are maintained for future reference should the same antigen reoccur at a later time-point, and constitute what is known as immunological memory. In the event of a second response against the same pathogen, these memory cells will respond more quickly and with greater vigor than naive T cells.

1.2.1 The different faces of immunological memory

As mentioned in Chapter 1.1.3, tissue homing is an important mechanism for directing immune responses: Naive T cells are largely migratory cells that circulate the blood stream while searching for their cognate antigen, yet they require the ability to enter lymph nodes and encounter activated DCs in order to become properly activated. Once activated, they differentiate into effector T cells, which express homing receptors that allow them to home to lymph-node B-cell zones or the site of infection. Finally, as the infection resolves and most of the effector cells undergo apoptosis, two distinct sets of surviving memory T cells are distinguishable based on their expression of the lymph-node homing receptors CD62L and CCR7; central memory T (T_{CM}) cells (CD62L+CCR7+) and effector memory T (T_{EM}) cells (CD62L-CCR7-) (253; 333). Thus, whereas the T_{CM} cells have a tendency for accumulating in lymphoid tissues, the T_{EM} cells remain in the periphery as sentinel cells.

Interestingly, the T_{EM} cells may be targeted specifically to the organ in which they were originally primed (435). Thus, T_{EM} cells that were initially activated in gut-associated lymphoid tissues (GALT) will acquire a phenotype that allows them to home to the gut mucosa. This can

be achieved by the expression of $\alpha_4\beta_7$ -integrin and the CC-chemokine receptor CCR9, which will interact with the gut-associated mucosal vascular addressin cell adhesion molecule 1 (MadCAM1) and CCL25, respectively (70; 318). In contrast, skin-homing T cells express CCR4 and/or CCR10, which recognize the dermal-associated chemokines CCL17 and CCL27 (213; 449). It is the local microenvironment that instructs the expression of tissue-specific homing receptors on T_{EM} cells, a process that is influenced by the route of infection and the site of replication, yet independent of the type of pathogen (69; 156; 184; 265). Moreover, the homing phenotype is flexible and can be changed during a secondary pathogen challenge depending on the site of activation and replication of the T_{EM} cells (110; 266).

The memory T-cell pool is maintained over time by homeostatic proliferation driven by IL-7 and IL-15 (380). However, whereas the T_{CM} cells are maintained for a life-time, the peripheral T_{EM} cells gradually decrease in numbers (162; 169). It appears that the T_{EM} cells are eventually replaced by circulating T_{CM} cells, yet the T_{CM} cells are equally capable of mounting secondary immune responses once in the peripheral tissues (435). For several months after an infection, residual antigen expressed on activated DCs in the draining lymph node may contribute to the maintenance of the peripheral memory T-cell pool through the activation of T_{CM} cells (451). However, following the depletion of the residual antigen depots, T_{CM} cells continue to be exported to the periphery by an unknown, antigen-independent mechanism (203). Importantly, in the event of a secondary infection, the maintenance of a peripheral pool of memory T cells ensures not only rapid initiation of effector functions locally at the site of infection, but also the quick generation of an effective secondary immune response that does not require priming of naive T cells (435).

Memory T cells do not depend on co-stimulation for activation and may be activated in the periphery upon antigen recognition (93). Thus, although they still require antigen presentation in the context of MHC molecules, they are able to bypass some of the regulatory mechanisms that would normally safeguard the fidelity of immune activation. As a consequence, they have been suggested to mediate immune pathology upon viral infection (81; 240) and transplantation (190), as well as causing autoimmunity (403). The question is whether immunotherapeutic targeting of memory subsets may relieve clinical immune pathologies. This is further debated in the Discussion of this Thesis.

1.2.2 Polarization of the immune response

Upon activation by a DC, the CD4⁺ T cell may differentiate into one of several different effector T-cell lineages (Fig. 6). For the time being, the established lineages include the T helper type 1 (Th1), Th2, Th17, and follicular T helper (Tfh) lineages plus the induced Treg

(iTreg) lineage (459). As the CD4⁺ T cell is activated, its fate is decided mainly by the cytokines present in its environment and, secondly, the strength of the TCR signal it receives (57). The prevailing dogma, which was established in the late eighties, describes a dichotomy between the Th1 and Th2 lineages (268). It was believed that the CD4⁺ T cell chose one of these two fates upon activation, and that they would inhibit each other reciprocally through the secretion of lineage-specific cytokines. It was further believed that the choice of lineage represented a stable and irreversible differentiation state, and that the two lineages would maintain their cytokine profiles even under conditions that would normally promote the other lineage (272). However, the idea of stably differentiated lineages has been challenged with the recent discovery of another lineage of T helper cells, the Th17 cells, and the advent of inducible Treg cells. Moreover, the very recent identification of a ‘Th22’ lineage, and the suggestion that Th2 cells might differentiate into IL-9-secreting ‘Th9’ cells, further strains the current dogma (111; 400; 459).

The different T helper lineages have distinct cytokine profiles, and serve distinct tasks *in vivo*. Th1 cells secrete predominantly interferon (IFN)- γ and direct cellular immune responses against viruses and intracellular bacteria. Th2 cells, on the other hand, are mainly involved in humoral responses against helminths and extracellular pathogens, and produce a cocktail of interleukin (IL)-4, IL-5, and IL-13. The recently identified Th17 cells are believed to control responses against extracellular bacteria and fungi – especially at mucosal surfaces – and secrete IL-17A and IL-17F, as well as IL-22 (459). The production of cytokines and other effector molecules is regulated through the actions of lineage-specific transcription factors; T-box expressed in T cells (T-bet) in Th1 cells; GATA-binding protein-3 (GATA-3) in Th2 cells; and retinoid orphan receptor- γ -t (ROR γ t) in the Th17 lineage. The Tfh lineage secretes IL-21 and appears to require IL-12 and/or IL-21 plus the transcription factor B-cell lymphoma-6 (Bcl-6) for differentiation, and serves to regulate the maturation of B-cell responses in lymphoid tissues (117; 246; 285; 414). The iTreg may entail several different putative lineages, including T regulatory 1 (Tr1) and Th3. Whether or not these constitute distinct lineages remains a matter of debate – as goes for the fact that several of the characterized iTreg lack the expression of forkhead box protein-3 (FOXP3), even though this transcription factor has been hailed a specific factor defining the Treg population (349).

Extensive inter-lineage cross-regulation contributes to the consolidation and stability of the T helper lineages. For example, the Th2-expressed cytokine IL-4 represses commitment to the Th1 lineage by inhibiting the expression of IL-12R β , thereby antagonizing the important cues of IL-12 in Th1 lineage differentiation (383). Correspondingly, the induction of T-bet by IFN- γ inhibits Th2 differentiation by upregulation of the transcription factor Runx3, which

interacts with the *Ifng* promoter and the *Il4* silencer, respectively (107; 274). Furthermore, IFN- γ and IL-4 both inhibit the induction of IL-17 (161; 463). It is not clear, however, whether cells of the Th17 lineage cross-regulate the differentiation of Th1 or Th2 cells (459).

1.2.3 A new paradigm for polarized immunity

The T helper lineages, which were originally thought to represent stable commitments governed by epigenetic changes and controlled by specific “master regulator” transcription factors (18; 45; 272), have recently been challenged by the discovery of Th17 and iTreg lineages that demonstrate unprecedented instability (223; 229; 294; 438). For example, Th17

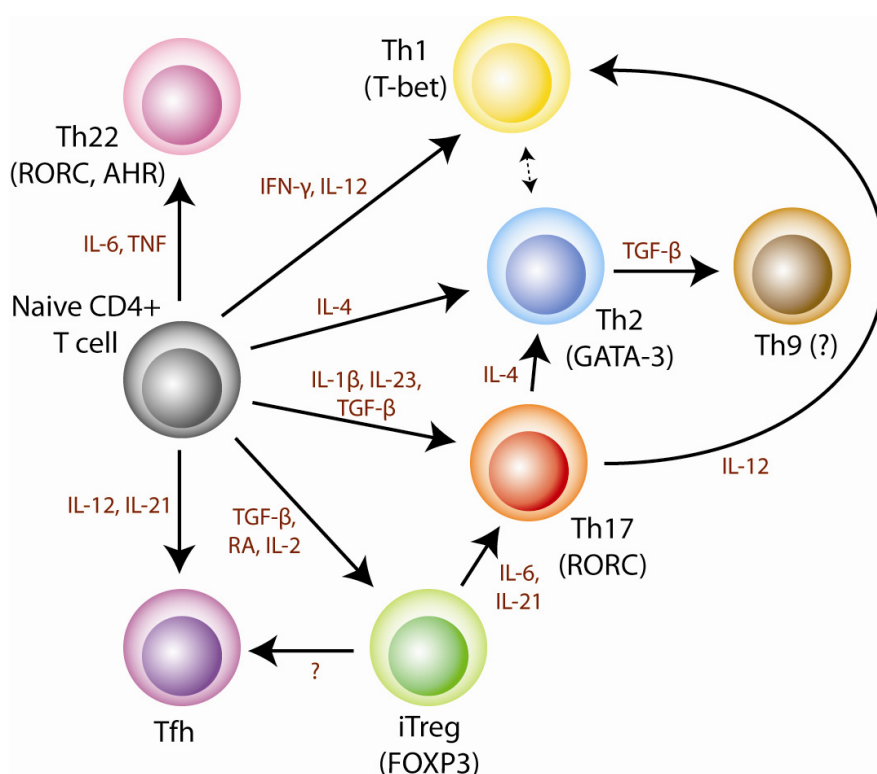


Figure 6: The cytokine milieu determines CD4+ T-cell differentiation and conversion. As the naive T cell encounters cognate antigen presented by antigen-presenting cells it undergoes activation, proliferation, and differentiation. Its fate, however, depends on the presence or absence of certain cytokines in the surroundings. In the presence of IFN- γ and IL-12, for instance, the T cell is likely to differentiate into and produce cells of the Th1 lineage. Correspondingly, IL-4 promotes differentiation of Th2 cells, whereas IL-6 and TGF- β favors the Th17 lineage. Furthermore, the recently identified Th22 lineage is advanced by a combination of IL-6 and TNF, while the Tfh cells result from the combined effects of IL-12 and IL-21. Moreover, the induction of iTreg cells benefits from a combination of TGF- β , RA, and IL-2. Finally, it has been suggested that cells of the Th2 lineage may be differentiated into IL-9-producing Th9 cells, though this finding remains to be substantiated. The different CD4+ T-cell lineages are driven by lineage-specific transcription factors; T-bet for Th1, GATA-3 for Th2, RORC for Th17, RORC and AHR for Th22, and FOXP3 for iTreg. The various lineages were previously thought of as terminally differentiated phenotypes, yet recent findings indicate a high degree of plasticity and inter-convertibility between the lineages, as indicated by the arrows. Adapted from Zhou *et al.*, 2009 (459).

cells have been observed to convert into Th1 or Th2 cells in the presence of IL-12 or IL-4, respectively (223; 229), whereas iTreg have been perceived to express IL-17 following prolonged exposure to a pro-inflammatory environment (294; 438). In addition, recent observations indicate heterogeneity in the Th17 lineage, for instance by the identification of IL-17+IL-22- cells in the presence of high levels of transforming growth factor- β (TGF- β) (458). Furthermore, Th17 cells differentiated *in vitro* in the absence of IL-23 but in the presence of TGF- β and IL-6 were shown to have reduced pathogenic potential in the murine experimental autoimmune encephalomyelitis (EAE) model, allegedly as a result of upregulation of the regulatory cytokine IL-10 in the Th17 cells and ensuing bystander suppression (257).

The observed instability of Th17 cells may have important consequences for *in vivo* immune responses and immune homeostasis. For instance, IL-10-secreting Th17 cells may confer protection to mucosal tissues even in the presence of the inflammatory cytokines IL-6 and TGF- β . On the other hand, upregulation of IL-23 may increase the pathogenicity of the Th17 cells (257). The apparent reliance of the Th17 lineage on stable expression of pro-inflammatory cytokines in their surroundings points to the importance of commensal bacteria in maintaining a Th17-polarizing environment. In fact, several studies have demonstrated detrimental effects of antibiotic treatment on the differentiation of Th17 cells, followed by complications such as secondary infections and diarrhea (22; 112; 155; 179). Moreover, the observed instability of Treg lineages may have implications for the clinical utilization of Treg in preventing inflammatory or autoimmune diseases, since Treg cells that convert into IL-17-secreting cells may exacerbate the inflammatory condition that they were intended to treat.

In summary, emerging evidence now indicate that the T cell fate is dynamic, rather than irreversible, and that the currently held view that T cells are segregated into lineages insufficiently describes the multitude of possible T-cell fates (459). Rather, a system based on lineages may be limited to crudely describing the properties of T cells based on their functional repertoire (i.e., the cytokines they secrete). Importantly, the newfound understanding of helper T cells as a malleable entity marks the beginning of a new paradigm wherein local cues provided to the T cell by its environment may override the instructions that were initially given to it. Moreover, this new paradigm comes with the ability of modifying and fine-tuning immune responses by the use of external factors, and may revolutionize the way we treat immune-mediated diseases. For instance, provided exhaustive knowledge of the factors balancing an immune response, we may be able to design and administer a cocktail of cytokines invoking not only generalized immune suppression or immune stimulation but a nuanced response that ensures immunity yet prevents immune pathologies.

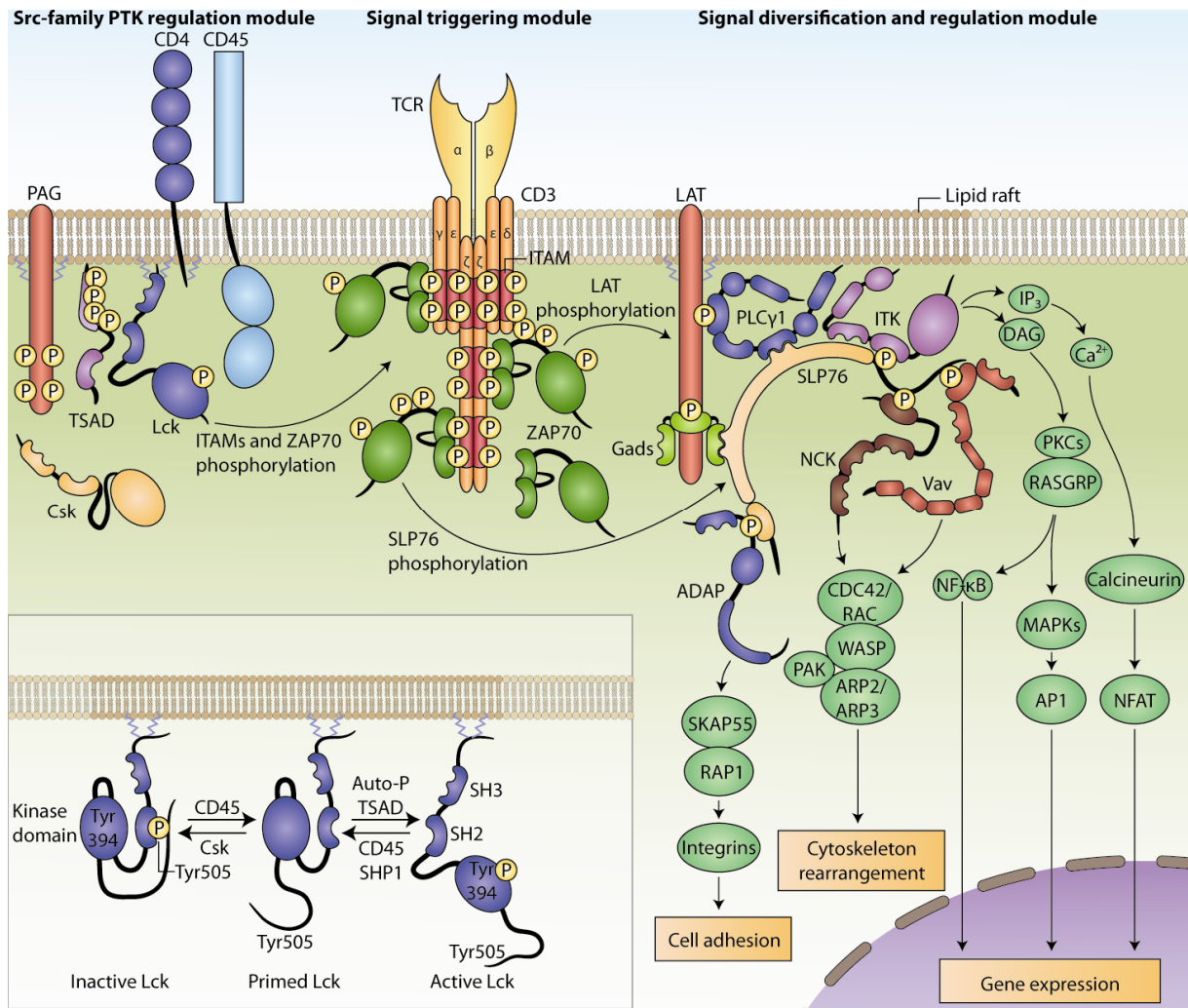
1.3 Immune-cell signaling and activation

The T cells generated in the thymus represent a pool of naive T cells that are, collectively, designed to recognize any structure presented to them in the context of an MHC molecule. The challenge, then, lies in finding and activating the best suited T cell for any given pathogen – and avoiding the activation of T cells that recognize structures related to self. Peptide–MHC complexes binding the TCR with virtually any affinity will be presented to it, and it is the key task of the TCR to distinguish between peptides derived from self and non-self, respectively, based on their affinity and additional cues presented to it through co-stimulatory molecules. The TCR-CD3 complex consists of a clonotypic $\alpha\beta$ TCR dimer that is coupled to six subunits (CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, and CD3 $\zeta\zeta$ pairs), which are responsible for transducing the signal by recruiting cytoplasmic or membrane-bound signaling proteins to a larger multiprotein scaffold capable of diversifying the signal and inducing the expression of multiple target genes (5). Moreover, several regulatory mechanisms serve to influence the TCR signal, intersecting on the level of both proximal and distal signaling molecules to fine-tune the signal and control the outcome.

1.3.1 Proximal TCR signaling

The immediate events following ligation of the TCR by cognate antigen (illustrated in Fig. 7) determine whether or not the T cell will be activated, or if it will become anergic. This depends not only on TCR-ligation but also on the presence of co-stimuli (5). The affinity with which the antigen binds the TCR influences the strength of the ensuing TCR signal and influences the fate of the cell. The first event following engagement of the $\alpha\beta$ TCR is the recruitment of Lck, which phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the CD3 subunits. Notably, the co-receptors CD4 and CD8 are associated with Lck and could possibly serve to co-localize Lck with the CD3 ϵ -chains – although this may also be achieved by targeting to glycosphingolipid-enriched membrane microdomains (GEMs), also known as lipid rafts (109). When Lck has phosphorylated both tyrosines of one ITAM, ζ -chain-associated protein kinase of 70 kDa (ZAP70) is recruited through its tandem SH2 domains (405) and activated by Lck-mediated phosphorylation (6). Activated ZAP70, in turn, phosphorylates the GEM-associated linker for activation of T cells (LAT) on several of its tyrosine residues, inducing the recruitment of various downstream effector proteins.

The SH2-domain-containing leukocyte protein of 76 kDa (SLP76) is a crucial component of the resulting signaling scaffold, binding tightly and cooperatively with multiple kinases and



adaptor proteins. SLP76 binds LAT indirectly through growth-factor-receptor-bound protein 2 (GRB2)-related adaptor protein (Gads), and tethers phospholipase C γ 1 (PLC γ 1) in the other end, acting as a bridge between the two. It also serves to bind other enzymes such as the IL-2-inducible T-cell kinase (ITK), Vav, and son of sevenless homologue (SOS; not shown in Fig. 7) and concentrate them with their substrates so as to facilitate high-specificity interactions of medium-range affinities. The ensuing diversified signaling cascades elicit responses affecting as distinct cellular tasks as cell adhesion, cytoskeleton rearrangement, and gene expression. (5)

Proximal TCR signaling is regulated by a number of refined mechanisms designed to distinguish between self and non-self. For instance, the duration of a TCR-peptide-MHC interaction will vary depending on the nature of the peptide, and will result in different signals depending on its ‘dwell time’. The longer dwell times of foreign peptides will ensure full calcium signaling, which may in turn induce the formation of an immunological synapse (178). A longer dwell time also favors a stronger signal by inducing increased phosphorylation of the cytoplasmic tails of the CD3 subunits (192). In addition, the availability of active (Y394-phosphorylated) Lck influences the signal strength (159). The activation of Lck is, in turn, negatively regulated by a signaling module consisting of CD45, Csk, Csk-binding protein

Figure 7: Modular functions of the TCR signalosome. The lipid raft-associated protein PAG regulates T-cell receptor (TCR) signaling by recruiting the Lck-inhibitory Src-family protein tyrosine kinase (PTK) C-terminal Src kinase (Csk), which phosphorylates Lck at the C-terminal tyrosine residue (Tyr505), thereby maintaining Lck in an inactive state. The activation of Csk is thought to be mediated by anchored PKA (see Chapter 1.5.3), whereas dissociation of Csk upon dephosphorylation of PAG may be responsible for relieving the inhibition of Lck after TCR stimulation. Lck is further activated by the combined efforts of the protein tyrosine phosphatase (PTP) CD45 and the adaptor TSAD, as shown in the insert. This results in equilibrium between the active, primed, and inactive forms of Lck, the position of which determines the excitability of TCR signaling. Upon ligation of the TCR, active Lck may phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 ζ -chain, as well as tyrosine residues of the TCR-proximal kinase ZAP70, which is recruited to the phosphorylated ITAMs and assembles the signal diversification and regulation module through phosphorylation of the scaffold proteins LAT and SLP76. This module encompasses regulators of Ca²⁺ signaling, actin polymerization, and integrin activation, and controls cellular processes like cell adhesion, cytoskeletal rearrangements, and gene expression, which are required for activation, proliferation, and differentiation of the T cell. ADAP, adhesion- and degranulation-promoting adaptor protein; AP1, activator protein 1; ARP, actin-related protein homologue; CDC42, cell-division cycle 42; DAG, diacylglycerol; Gads, growth-factor-receptor-bound-protein-2-related adaptor protein; InsP3, inositol-1,4,5-trisphosphate; ITK, interleukin-2-inducible T-cell kinase; MAPK, mitogen-activated protein kinase; NCK, non-catalytic region of tyrosine kinase; NFAT, nuclear factor of activated T cells; NF- κ B, nuclear factor- κ B; PAG, phosphoprotein associated with glycolipid-enriched membrane domains; PAK, p21-activated kinase; PKC, protein kinase C; PLC γ 1, phospholipase C γ 1; SHP1, SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1; SKAP55, SRC-kinase-associated phosphoprotein of 55 kDa; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; TSAD, T-cell-specific adaptor protein; WASP, Wiskott–Aldrich syndrome protein; ZAP70, ζ -chain-associated protein kinase of 70 kDa. Adapted from Acuto *et al.*, 2008 (5).

(CBP)/phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), T-cell specific adaptor protein (TSAD), lymphoid tyrosine phosphatase (LYP), SH2-containing protein tyrosine phosphatase (SHP)-1, and extracellular signal-regulated kinase (ERK) (5).

SHP-1 is rapidly recruited to the cell membrane upon high doses of antagonist peptide–MHC complexes in naive T cells to dephosphorylate and inactivate Lck, thereby increasing the threshold for T-cell activation and filtering out noise generated by the presence of self-peptide–MHC complexes (197; 233; 370). It is not known precisely how SHP-1 is recruited to the TCR, but it appears to become activated by Lck upon antagonist ligation of the TCR $\alpha\beta$ and upon binding to immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tails of the TCR complex, eventually reducing Lck activity by an, as yet, unknown mechanism (233; 298; 370). The micro-RNA mir181a has also been shown to regulate Lck activity, as it down-regulates the phosphatases LYP, SHP-2, dual-specificity protein phosphatase (DUSP)-5, and DUSP-6 and strongly augments TCR sensitivity (233). These regulatory mechanisms are

expressed at different levels throughout thymic development, suggesting a stage-specific fine-tuning of TCR sensitivity (100). Another immediate negative feedback mechanism involves a complex formed around the adaptor proteins known as downstream of kinase (DOK)-1 and DOK-2, which interact with LAT and control membrane recruitment of pleckstrin homology (PH)-domain-containing signaling effectors, such as Akt, through SH2-domain-containing inositol-5-phosphate 1 (SHIP1)-mediated hydrolysis of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) (108; 164; 445). This mechanism is thought to contribute to reducing signal noise and improving signal specificity. Furthermore, *Dok1^{-/-}Dok2^{-/-}* mice suffer from loss of negative control of cytokine signaling and augmented activities of ZAP70, LAT, and ERK, ultimately causing alterations in myeloid-cell homeostasis and spontaneous lupus-like autoimmunity (108; 281; 445; 446).

In addition to the previously mentioned proximal mechanisms of regulation, other mechanisms act on the TCR signalosome at later time points following the event of TCR triggering. These include the HPK1–SLP76–14-3-3 pathway and the suppressor of TCR signaling (STS) proteins. Phosphorylation of the hematopoietic progenitor kinase (HPK)-1 is induced by Lck or ZAP70 upon TCR triggering, causing it to bind SLP76 or other scaffold proteins (49; 238; 338), upon which HPK1 phosphorylates serine 376 of SLP76 and induces the interaction of SLP76 with 14-3-3 proteins, with the potential to regulate a series of cellular processes (105; 247; 354). The phosphorylation of SLP76 peaks 10-15 minutes after TCR stimulation and is maintained for up to 1 hour, suggesting a delayed onset of the HPK1–SLP76–14-3-3 negative feedback loop (105). Ubiquitylation regulates the degradation of proteins in general (see Chapter 1.3.5), and specifically influences the balance between immune activation and tolerance through the actions of ubiquitin ligases of the Casitas B-lineage lymphoma (CBL) family. The ubiquitin ligases CBL-B, gene related to anergy in lymphocytes (GRAIL), and Itch serve to down-regulate components of the TCR signalosome through activation-dependent degradation, thereby enforcing T-cell anergy (166; 242). Ubiquitylation is positively or negatively regulated by protein tyrosine phosphorylation, and CBL-family E3 ubiquitin ligases are recruited to the TCR signalosome upon activation, resulting in down-modulation of T-cell signaling by a mechanism dependent on STS-1 and STS-2 (118; 208; 436). These suppressor proteins show some redundancy, but their importance is demonstrated in *Sts1^{-/-}Sts2^{-/-}* mice, which have increased activation of ZAP70, SLP76, LAT, and ERK and are more susceptible to induced autoimmunity in EAE models (74; 260).

The numerous regulatory mechanisms mentioned above serve to indicate the level of stringency underlying TCR signaling. In the present Thesis, we have discussed the possible role of the cAMP–PKA–Csk inhibitory pathway in regulating this crucial step of immune activation

(see Chapter 1.5.3), but it should be stressed that this is only one contributing mechanism out of several. It should also be noted that the various mechanisms may operate at distinct stages of development, or under specific conditions, and that they may be partly overlapping or strictly non-redundant.

1.3.2 *Cytokine signaling*

Cytokine signaling is known to play an important role in immune regulation and inflammation, and mutations in important components of the cytokine signaling machinery have been related to a variety of immune pathologies (290). Cytokine receptors are expressed on the surface of all nucleated cells within the human body and serve a variety of functions as parts of the intercellular lines of communication. The conserved family of type I and II cytokine receptors comprise around 40 receptors, including the interleukin receptors as well as hormone and growth-factor receptors (53). Ligation of a receptor by its cognate cytokine results in activation of intracellular signaling cascades that culminate in the generation of specific transcription factors and transcription of target genes. Thus, cytokine signaling constitutes a way of regulating gene expression in the responding cell (290). Depending on the cytokine and the nature of its receptor, genes may be turned on or off, invoking changes that may affect both the phenotype and the functional properties of the target cell. In Paper 1, a population of T cells secreting the cytokine IL-10 was observed to suppress the proliferation of effector T cells. Whether or not IL-10 was part of their suppressive capacity could not be ascertained in this study, yet IL-10 has been established as a cytokine with strong immunomodulatory properties and it is not unlikely that it did play a role in the observed suppression. Indeed, IL-10 has been found responsible for the inhibition of T-cell effector functions mediated by the Tr1-type regulatory T cells (see Section 1.4).

The binding of a cytokine to its receptor causes receptor dimerization and the subsequent activation of Janus kinase (JAK) family tyrosine kinases, which are constitutively associated with the receptor. The activated JAKs phosphorylate specific tyrosine residues in the cytoplasmic tail of the receptor, which in turn recruit SH2-binding latent cytoplasmic transcription factors of the signal transducer and activator of transcription (STAT) family. The STATs dimerize following activation by JAKs, then translocate to the nucleus where they bind DNA and activate gene transcription (99; 225).

Several instances of cytokine signaling are outlined in Figure 8. Thymic development of T cells requires activation of STAT5 through ligation of the IL-7 receptor and signaling through JAK3, and mice negative for key components of the IL-7–JAK3–STAT5 pathway fail to develop T cells (289; 441). Moreover, the different fates of activated helper T cells depend on

the cytokine milieu at the time of activation. Hence, IL-12 directs differentiation into the Th1 lineage whereas IL-4 drives Th2-cell differentiation. Studies in knockout mice have demonstrated these responses to be dependent on STAT4 and STAT6, respectively (286). Furthermore, the main cytokine products of these two lineages – IFN- γ for Th1 cells and IL-4 for Th2 cells – promote the commitment of the differentiated cells to their lineage and inhibit differentiation into the opposing lineage. The generation of Treg is also subject to cytokine regulation, be it the development of nTreg in the thymus or induction of iTreg in the periphery. Both of these regulatory FOXP3-expressing subsets depend on cytokines that bind the γ -chain (γ c), and deficiency of γ c or JAK3 therefore results in a failure to produce FOXP3-positive Treg (254). Correspondingly, a deficiency in STAT5, which is down-stream of JAK3 and directly regulates the transcription of the FOXP3 gene, also results in loss of Treg and inability to induce Treg *in vitro* (65; 442). The Th17 lineage, on the other hand, depends critically on activation of STAT3, which is achieved upon binding of IL-6, IL-21, or IL-23. Activated STAT3 directly regulates the expression of ROR γ t and ROR α , as well as IL-17 and IL-21 (86; 427; 439). Interestingly, IL-2 acting through STAT5 counteracts Th17-cell differentiation (220), and the balance between Treg and Th17-cell differentiation thus appears, in part, to be regulated by STAT5 and STAT3, respectively (290).

Cytokines are potent immune regulators and their actions are therefore tightly regulated (353). Hence, the activation of STATs leads to induction of a family of cytokine-induced inhibitors termed suppressors of cytokine signaling (SOCS), which bind intracellular domains of specific cytokine receptors and block the generation of STAT signals from the receptor (11; 447). Importantly, *SOCS* genes are among the targets of activated STATs, providing a negative feedback mechanism for regulating the output of cytokine signaling. Among the eight SOCS proteins, SOCS1 and SOCS3 mediate most of the immunoregulatory effects in humans, universally affecting the activation, differentiation, and homeostasis of immune cells (11; 447). The SOCS proteins have been suggested to participate in multiprotein complexes that direct substrate proteins to the proteasome for degradation, but their mechanism of action remains to be resolved (196; 290; 447; 453).

According to three recent reports, cytokines and cytokine signaling play a crucial role, as well, in the directional actions of Treg (79; 202; 456). In keeping with these reports, the local cytokine environment not only mediates polarization of the immune response but also determines the effector subsets targeted by Treg for suppression. For instance, the activation of STAT3 by ligation of the Th17-promoting cytokines IL-6 and TGF- β may endow Treg with the ability to suppress Th17 responses. As mentioned in Section 1.4.3, similar mechanisms have been found to regulate Th1 and Th2 responses. Anomalies in the cytokine signaling network

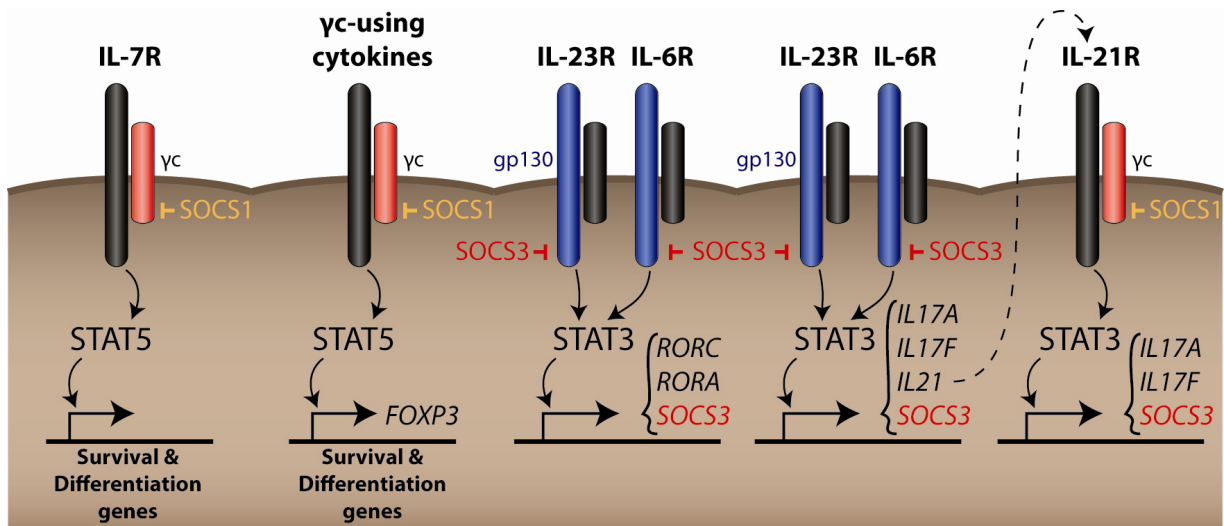


Figure 8: Cytokine signaling as a means of regulation. Cytokines are important mediators of inter-cellular communication. The cytokine signal is conveyed upon binding to specific surface-expressed receptors through activation of JAK/STAT signaling pathways. However, the suppressors of cytokine signaling (SOCS) may specifically block the generation of STAT signals from certain receptors (e.g., SOCS1 blocks induction of STAT5 upon binding of IL-7 to the IL-7 receptor [IL-7R]). The transcription of many genes depends on cytokine signaling – for example, transcription of *FOXP3* in regulatory T cells is positively regulated by STAT5 resulting from activation of γ -chain-associated cytokine receptors (whose ligands are IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). Th17 cells, on the other hand, depend on signaling through STAT3 for the production of lineage-specific transcription factors (*ROR α* and *ROR γ t*), as well as the Th17 cytokines (IL-17A, IL-17F, and IL-21). IL-21, in turn, reinforces the production of IL-17A and IL-17F in an autocrine or paracrine manner by binding to the IL-21 receptor. Conversely, the simultaneous generation of SOCS3 serves to inhibit signaling from the IL-6 and IL-23 receptors in a negative feedback fashion. Adapted from O’Shea *et al.*, 2008 (290).

have been linked to severe immunodeficiency syndromes (290). For example, mutations in *JAK3*, the gene for γ C-associated kinase, or *TYK2*, another cytokine receptor-associated kinase of the Jak family, result in SCID and autosomal-recessive hyperimmunoglobulin E syndrome (AR-HIES), respectively (261; 284; 426). Furthermore, single-nucleotide polymorphisms (SNPs) in *SOCS1* and *SOCS3* were associated with the pathogenesis of asthma (158; 345). *SOCS1* and *SOCS3* may also be important anti-oncogenes, as hyperactivation of STAT1 or STAT3 has been implicated in several malignant disorders (290).

1.3.3 Cross-talk between signaling pathways

In the two previous chapters, a detailed account was given on the signals mediated by ligation of the TCR and the various cytokine receptors, respectively. However, although the recited cascades may appear to proceed in a linear and orderly fashion, recent technological advances have informed us that, in reality, this is rarely the case (123). Rather, the ligation of cell-surface receptors is exceedingly seen to cause the activation of multiple intracellular signaling pathways through extensive networking – a phenomenon known as ‘cross-talk’.

Figure 9 illustrates how our view of signaling has progressed from a linear perspective to a network-based perspective, in which different pathways interact through the use of common constituents. Cross-talk results from the convergence of more than one signaling pathway on the same signaling molecule, and serves to integrate or fine-tune the response to a number of concomitant stimuli, ultimately facilitating the timely induction or repression of gene transcription through the activation or repression of one or more transcription factors. For added complexity, the signaling molecule – most commonly a kinase or a phosphatase – may combine with other proteins to form signaling complexes with manifold targets, or it may be targeted to subcellular domains through the binding of specific anchoring proteins. Importantly, if the signaling molecule is otherwise freely diffusible, this compartmentalization may be an important way of diversifying the effects of a molecule that is involved in multiple signaling pathways.

Cross-talk between signaling pathways constitutes an important mode of regulation within the cell, allowing for fine-tuning and integration of the signals that are received at the cell surface. The activity of a given node in a signaling network may be either positively or negatively regulated by the actions of other nodes, facilitating precise and quantitative responses to a variety of incoming stimuli. In immune-cell signaling, examples of cross-talk are

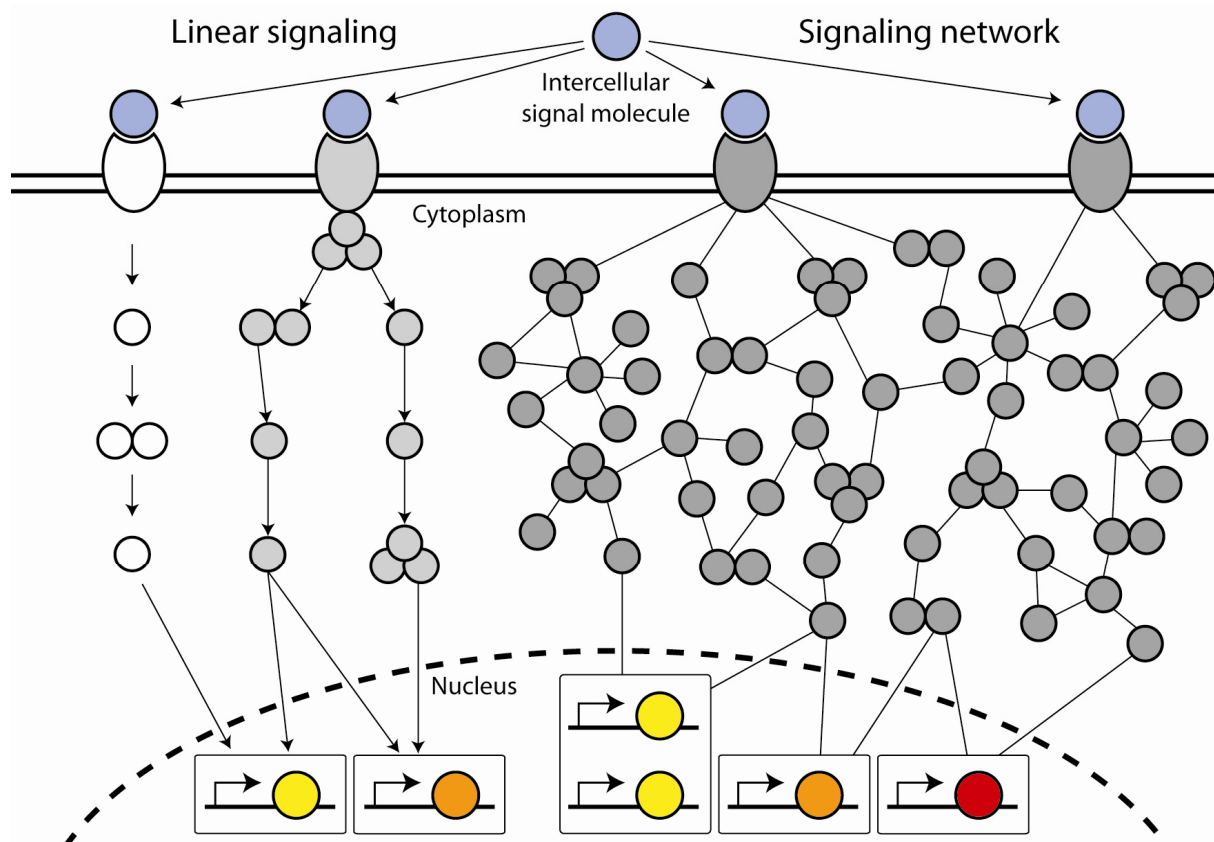


Figure 9: On the perception of intracellular signaling. The diagram illustrates how the dogma on signaling has progressed from a linear sequence of events to highly interactive signaling networks. Adapted from Fraser *et al.*, 2009 (123).

plentiful, and are likely to increase in numbers as the precision of our methods of detection improves. Figure 10 (next page) illustrates some of the signaling pathways that are currently known to be activated upon ligation of the TCR–CD3 complex with peptide–MHC. Notably, a recent study reported using this information to predict the production of IL-2 upon a given stimulus (191). Thus, it is believed that if we achieve accurate knowledge of all the components involved in the signal triggered upon ligation of a receptor, the outcome may be predicted in a quantitative manner. Of course, activation under *in vivo* conditions will be much harder to interpret due to the complexity and variety of the incoming signals, for which the output will represent an integral that involves signals from receptors such as the TCR and its co-receptors, as well as various cytokine receptors and adhesion molecules. The perhaps most prominent instance of cross-talk in T cells results from the ligation of co-stimulatory (e.g., CD28, ICOS) or co-inhibitory (e.g., CTLA-4, PD-1) receptors whose intracellular signaling cascades interfere directly with components of the TCR signaling machinery. A logical model of TCR signaling in conjunction with CD28 co-stimulation in human T cells was proposed recently, involving 94 different signaling molecules and 123 interactions (326). Such comprehensive models are crucial for the detection of new signaling pathways and for elucidating the relationships between different molecules, and may aid in understanding the development, differentiation, and effector functions of T cells – as well as to assess the influence of regulatory cells and mechanisms on these processes.

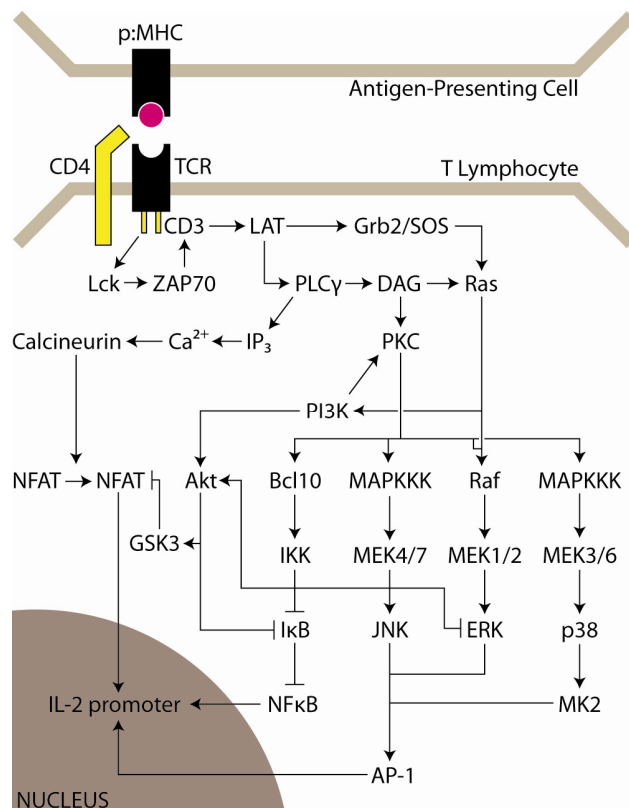
1.3.4 Transcription and the influence of epigenetic control mechanisms

The packaging of DNA has proved an important mechanism for regulating the transcriptional activity of genes and, thereby, controlling the activation status of the host cell. Double-stranded DNA is wrapped around histone clusters consisting of four histone pairs (H2A, H2B, H3, and H4), ~146 base pairs covering one cluster to create a nucleosome. The nucleosomes, in turn, are packed and coiled into dense chromatin fibers, rendering the DNA virtually inaccessible to the transcriptional machinery (181). Thus, in order to allow for the transcription of specific genes, the chromatin must be partly unpacked. This enables selective, tissue-specific regulation of transcription through structural modifications of the gene loci in question. The modifications involved include nucleosome repositioning, posttranslational modification of histone tails, and methylation of CpG dinucleotides, affecting DNA accessibility in a heritable manner without altering the DNA sequence itself (181).

Epigenetic control mechanisms are believed to influence the differentiation of CD4+ T helper cells into either of the committed T-cell lineages, including the Th1, Th2, Th17, and Treg lineages (24; 181). In this context, the *Ifng* locus has been intensely studied and represents

an impressive example of epigenetic regulation. Notably, phylogenetic studies have revealed a stretch of DNA flanking both sides of the human *IFNG* locus that spans more than 150 000 base pairs and contains a high degree of evolutionarily conserved non-coding sequences (NCS). The order, as well as the 5'-3' orientation, of these NCS were absolutely conserved (368). The fact that these sequences are conserved across distant species indicates that they serve important functions in the transcription of the *Ifng* gene. Thus, it was later recognized that NCS widely distributed across the *Ifng* locus were modified by the activating process of histone H4 acetylation, promoting *Ifng* gene expression (461). Histone H4 acetylation across the entire *Ifng* locus was dependent on STAT4 in a Th1 differentiation system driven by IL-12 (77). The transcription factor T-bet is known to bind several of the NCS across the *Ifng* locus, yet has so far only been shown to mediate H4 acetylation in one (77; 165). In addition, this master regulator of Th1 development was found to induce chromatin remodeling in the *Ifng* locus of mice (384) although, apparently, this was not an absolute requirement for Th1 differentiation (402). Hence, with respect to regulating nucleosome organization, STAT4 has actually proven more potent than T-bet (452).

Another set of histone modifications were found in the *Ifng* locus upon differentiation of naive CD4+ T cells along the Th2 pathway. The recruitment of STAT6 and GATA-3 to the *Ifng* locus of murine T cells resulted in the generation of repressive H3K27-dimethylation and trimethylation marks rather than the activating acetylation marks mentioned previously, efficiently silencing *Ifng* in Th2 cells. At the same time, however, STAT6 and GATA-3



mediate transcriptional activation of the *Il4* locus by the widespread introduction of activating histone modifications, leading to transcription of the *Il4* gene. How this selectivity in mediating epigenetic modifications is achieved remains to be

Figure 10: Signal networking in T cells. Ligation of the TCR-CD3 complex in conjunction with CD4 results in activation of an extensive signaling network. Provided exhaustive knowledge of the ensuing processes, models of the network may be used to predict its outcomes. One isolated process that has been tested in this respect is the activation of the IL-2 promoter and transcription of the cytokine IL-2. Adapted from Kemp *et al.*, 2007 (191).

elucidated, but may result from the differential expression of specific binding sites for the respective transcription factors (24).

Very recently it was suggested that, similar to the Th1- and Th2 lineage transcription factors, the master regulator of Treg, FOXP3, could mediate some of its effects through transcriptional repression of target genes (296). Apparently, it does so by recruiting the transcription factor Eos, which induces chromatin modifications in target genes through association with the carboxy (C-) terminal binding protein-1 (CtBP1), a known repressor of gene expression. The FOXP3–Eos–CtBP1 suppression complex was found to target a number of genes – including the *IL-2* gene – which were silenced by the combined effects of histone modifications and methylation of CpG dinucleotides. The putative relevance of Eos for the suppressive function of Treg was confirmed by *in vitro* proliferation assays, as well as in an *in vivo* model of colitis (296). Of note, Eos did not affect the expression of genes that were up-regulated by FOXP3, thus causing only partial conversion of Treg into effector T cells (Teff) when silenced by small interfering RNA (siRNA)-mediated knockdown. Notably, however, the *in vitro* suppressive capacity of Treg was lost in the absence of Eos (296). View Section 1.4 for a more detailed account of Treg.

1.4 Regulatory T cells

1.4.1 The suppressor cell

The suppressor cell was first described by Gershon and Kondo in 1970 (138). They had identified a population of T cells that were different from the helper T cells and served to dampen immune responses. This was an astonishing observation, and in the following years the suppressor cells were intensely studied. Early investigations of the suppressor cells led to a series of findings that seemed to corroborate their existence. For example, as previously mentioned, studies in thymectomized mice revealed that neonatal thymectomy (nTx) between the second and fourth days of life led to T-cell mediated lesions that could be reversed by the administration of T cells from adult euthymic mice (282; 331). These experiments demonstrated that a population of T cells generated after 3 days of life mediated dominant immune tolerance. However, research on suppressor cells stagnated in the 1980s, primarily due to a lack of methodologies sensitive enough to obtain reliable information about these cells, but also due to the absence of any distinctive surface marker that could be used to isolate and purify them (332). Furthermore, it was deemed a serious setback that the I-J chain, which was supposed to be key to the suppressive function of these cells, was found not to exist (210). Only in 1995, with the identification of a suppressive subset of T cells expressing the IL-2 receptor α

chain (CD25), did the suppressor cell resurface – this time under the untainted name “regulatory T cell” (330). The seminal study performed by Sakaguchi and colleagues showed that CD4⁺ T cells depleted of CD25⁺-expressing cells caused autoimmune disease when transferred into nude mice, and that CD4⁺CD25⁺ T cells (i.e., regulatory T cells) could reverse the autoimmune reaction.

Although important for the initial discovery of Treg, CD25 represents a suboptimal marker for Treg as it is expressed on other T-cell subsets as well, including activated effector T cells. In this respect, the discovery of the X chromosome-encoded gene *FOXP3*, which was identified while looking for the underlying cause of the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans and the spontaneous murine mutant *scurfy* (37; 62; 78; 430), has been pivotal in progressing our understanding of the Treg biology. Following its discovery, stable expression of FOXP3 was shown to be restricted to Treg, and the differentiation and suppressive function of Treg was shown to rely on this transcription factor (121; 122; 134; 174; 194; 237). Furthermore, the sustained expression of FOXP3 was required for maintaining the anergic phenotype and suppressive capacity of mature Treg, and the loss of FOXP3 resulted in acquisition of effector T-cell properties such as the production of pro-inflammatory cytokines like IL-2, IL-4, IL-17, and IFN- γ (423; 432). Recently, however, the role of FOXP3 as the “master regulator” responsible for empowering Treg with suppressive functions has been questioned. Firstly, subsets of Treg have been identified that do not express this canonical Treg marker, most importantly the antigen-specific and IL-10-secreting Tr1 cells (412). Secondly, not all T cells that express FOXP3 have suppressive capabilities – in fact, some studies suggest that FOXP3 is up-regulated following normal TCR stimulation, and that the resulting phenotype is neither anergic nor suppressive (135). Even so, FOXP3 remains the most specific Treg marker, yet its intracellular expression limits its uses. A surface-expressed marker, on the other hand, could be used to specifically identify and isolate Treg and would be of substantial importance in promoting further studies of Treg subsets. So far, however, none of the suggested markers – including CTLA-4, GITR, CD38, CD5, low-level CD127, and so on – have been Treg-specific, and the phenotype initially put forth by Sakaguchi and colleagues (CD4⁺CD25⁺) remains the gold standard. Consequently, we still do not have a unique marker that can be used to unambiguously distinguish between Treg and effector T cells.

1.4.2 Natural and inducible subsets of regulatory T cells

The Treg initially described by Sakaguchi and colleagues were generated in the thymus, and have come to be known as ‘natural’ Treg (nTreg). They are recruited during the thymic

selection and maturation process as a result of high-avidity MHC class II-dependent TCR interactions, and play a central role in maintaining tolerance and preventing autoimmunity (329). Only in recent years has the generation of ‘induced’ Treg (iTreg) in peripheral tissues been appreciated. As it was recognized that expression of CD25 was a prerequisite for Treg functionality (131), Curotto de Lafaille and colleagues in 2004 demonstrated peripheral expansion of CD25+FOXP3+ Treg in mice (96). The year after, Knoechel and colleagues formally established that peripherally induced Treg were independent of the thymus, seeing that they could be induced in thymectomized animals (201). In the following years, the minimal requirements for generation of iTreg were shown to include activation through the TCR as well as expression of the cytokines TGF- β and IL-2 (95). Using protocols that include these factors has enabled generation of Foxp3+ iTreg both *in vivo* and *in vitro*, although numerous reports of failed attempts to do so underline our limited understanding of this process so far (95).

In contrast to the highly controlled thymic environment fostering the generation of nTreg, iTreg are generated under the more varied conditions within peripheral tissues. Their primary task appears to be the induction of tolerance to commensal bacteria and food allergens in the gut, thereby maintaining a non-inflammatory environment. In addition, they may interfere with immunity against viruses, bacteria, and parasites, possibly contributing to the development of chronic infections. They have also been shown to accumulate at tumor sites, where they may impair the development of effective anti-tumor immune responses (241). The possibility that nTreg and iTreg may have shared or overlapping tasks has received some attention lately, since a scenario with partially redundant Treg could have therapeutic consequences. Were indeed such redundancies identified, one Treg population could be used to treat diseases caused by deficiencies of another. Thus far, firmly overlapping functions of iTreg and nTreg have not been identified – mainly due to a lack in lineage-specific markers. However, using a colitis transfer model, Haribhai and colleagues (160) demonstrated that iTreg played an important role in protecting against colitis, and that a combination of nTreg and iTreg was necessary to protect from this disease. Hence, nTreg and iTreg may synergize to achieve optimal regulation, at least in some instances. Notably, the distinct origins of nTreg and iTreg and their inherent differences (such as distinct TCR repertoires) support the notion that they also serve different tasks (Fig. 11) (95).

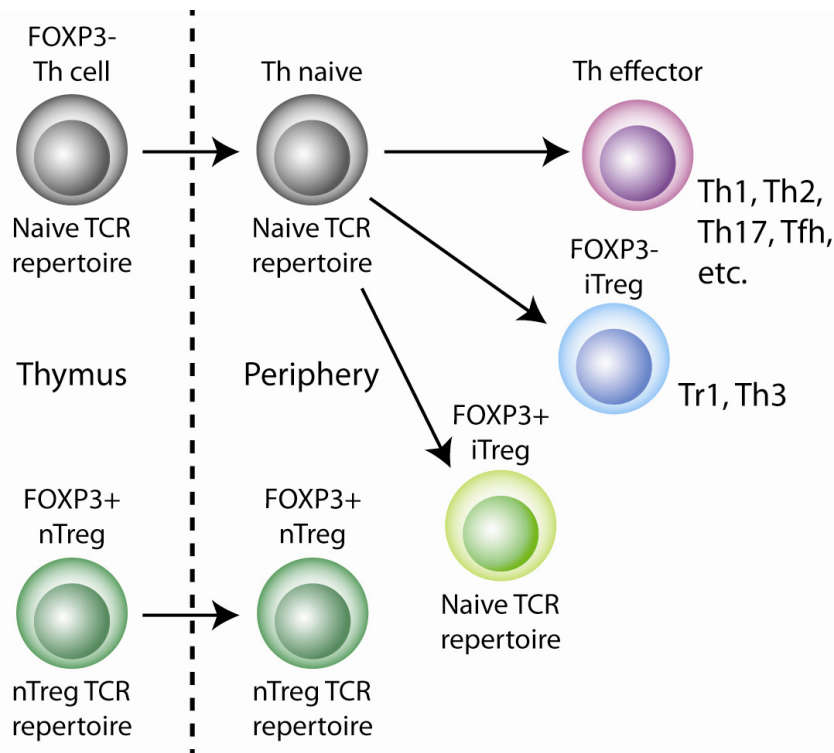


Figure 11: FOXP3+ Treg may be generated in the periphery as well as in the thymus. Whereas natural Treg (nTreg) are generated in the thymus in the course of T-cell maturation, Treg are also induced in the secondary lymphoid tissues of the periphery (including lymph nodes, spleen, and gut-associated lymphoid tissues [GALT]), as well as in inflamed tissues. Thus, the total pool of FOXP3+ Treg includes both nTreg and peripherally induced Treg (iTreg). It is likely that these two subsets have distinct TCR repertoires. In addition, peripheral FOXP3- Treg populations (Tr1 and Th3) have been described. Some activated T cells may express FOXP3 even though they are not suppressive. Adapted from Curotto de Lafaille *et al.*, 2009 (95).

1.4.3 Suggested modes of action

Regulatory T cells are critically involved in maintaining peripheral tolerance, and a number of suppressive mechanisms have been suggested to that end. Typically, these mechanisms have been identified and tested using *in vitro* model systems, and their roles *in vivo* may not yet have been established. One important dichotomy has become clear, though: The Treg may either suppress the effector T cells directly, or they may act on APCs or other accessory cells to indirectly influence the activation status of the effector T cells (Fig. 12).

Modes for the direct suppression of T cells may be either contact-dependent or mediated by soluble factors (see Fig. 12). Hence, four different possible approaches have been identified: 1) The secretion of suppressor cytokines; 2) IL-2 consumption; 3) granzyme-mediated cytotoxicity; and 4) the expression of membrane-bound or soluble inhibitory molecules (350). Only iTreg have thus far been demonstrated to mediate suppression through the secretion of suppressor cytokines, of which IL-10 and TGF- β are the best characterized. In Paper 1, we have demonstrated the suppressive capacity of IL-10-secreting HIV-1-specific T cells. Similar to the previously mentioned Tr1 cells they may suppress target cells through the secretion of IL-10,

though this question was not addressed in the present work. Natural Treg, on the other hand, normally require cell-to-cell contact in order to be suppressive. Recently, however, the inhibitory cytokine IL-35 was found to contribute to nTreg-mediated suppression in mice. Its relevance was substantiated by the finding that IL-35 expression is regulated by Foxp3 (91). However, in human nTreg, the expression level of IL-35 appears to be too low to mediate significant suppression (27). Galectin-1 is a membrane-bound β -galactoside-binding protein that binds to many glycoproteins (e.g., CD7, CD43, and CD45) and may confer nTreg-mediated suppression either as a soluble homodimer, or while presented on the plasma membrane. Interestingly, galectin-1 is up-regulated in nTreg and induced upon TCR activation, and in a recent study the suppressive capacity of both human and murine nTreg was reduced upon galectin-1 blockade (132).

The components of the high-affinity IL-2 receptor – CD25, CD122, and CD132 – are all expressed by Treg, and IL-2 is essential both to Treg homeostasis *in vivo* (448) and for their suppressive function *in vitro* (393). Thus, it has been speculated – and shown in a recent study (297) – that Treg could mediate their suppression through this receptor, possibly by tethering IL-2 and thereby depriving neighboring T cells of important survival cues and imposing cell-cycle arrest. However, other studies rather suggest that Treg inhibit the IL-2 production of responder cells (30; 102; 365). Furthermore, it was recently shown in a hybrid system using human Treg and murine effector T cells that blocking of the high-affinity IL-2 receptor with antibodies specific for human CD25 did not affect suppression (397). Yet another putative suppressive mechanism involves Treg-mediated cytolysis of target cells. This may be achieved by the upregulation of granzymes, as demonstrated in several studies of murine as well as human cells (145; 149; 455). In a recent study in mice, about 5-30 % of tumor-associated Treg were found to express granzyme B, and these were able to kill natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) in a granzyme B- and perforin-dependent manner (71).

The Treg have been shown to act – in the manners mentioned above – on CD4⁺ or CD8⁺ T cells, directly affecting the effector functions and proliferative capacity of these cells (83; 306; 386). However, Treg may also operate indirectly by inhibiting the maturation of DCs or by interfering with antigen presentation (262). Thus, the Treg have demonstrated a wide array of target cells, from the CD4⁺ and CD8⁺ T cells *via* DCs, B cells, NK and natural killer T (NKT) cells to macrophages, osteoblasts, and mast cells (140; 235; 327; 350; 363; 416). Suppressive mechanisms directed against APCs are of considerable interest, since any APC may potentially activate several T cells. In other words, targeting one APC may be the equivalent of targeting multiple T cells. An array of suppressive mechanisms have been suggested that target the APC – here represented by DCs: 1) Modulation of DC maturation stage; 2) DC arrest at the immature

stage; 3) inactivation of extracellular adenosine tri-phosphate (ATP); 4) obstruction of T-cell priming. Several studies have demonstrated the ability of Treg to down-regulate the expression of co-stimulatory molecules on DCs (262; 346). In some studies, Treg-mediated inhibition of DCs could be reversed by anti-CTLA-4 treatment, indicating that ligation of CTLA-4 to the DC-expressed CD80 and CD86 was responsible for the inhibition (293; 350). Correspondingly, treatment of mice with anti-CTLA-4 abrogated Treg-mediated suppression of inflammatory bowel disease (IBD) (316), though perhaps the most convincing piece of evidence so far was obtained in mice that were selectively deleted for Treg-expressed CTLA-4, which developed systemic autoimmunity at 7 weeks of age (433).

A recently identified mechanism of DC-modulation by Treg involves the ligation of Treg-expressed LAG-3 (CD223) to MHC class II molecules expressed by immature DCs, initiating an inhibitory signal that prevents maturation of the DC and inhibits its immunostimulatory

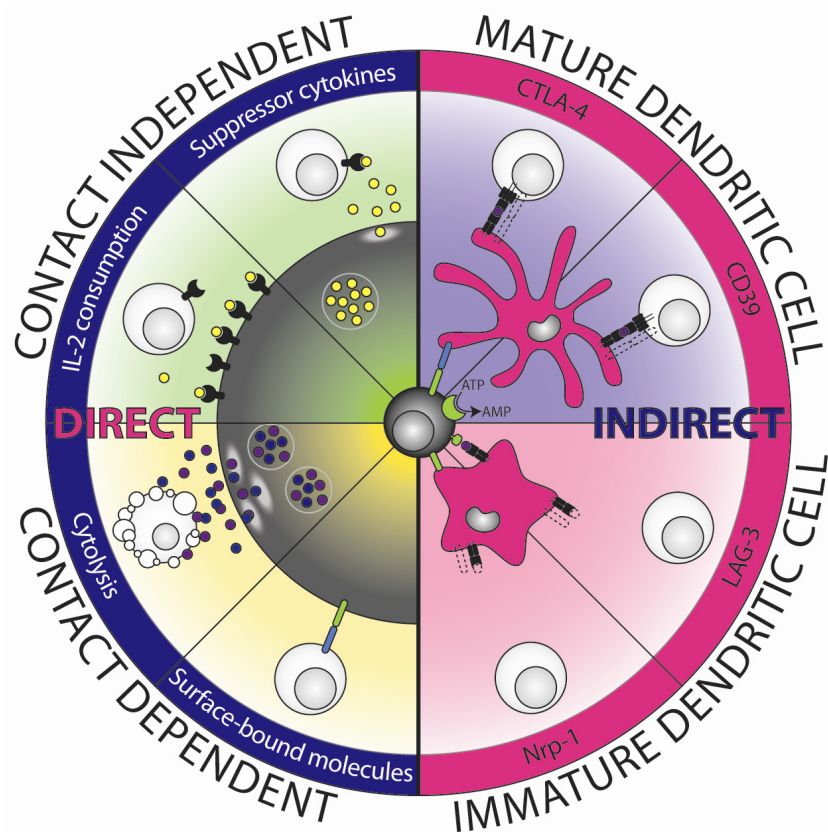


Figure 12: Suggested modes of suppression for regulatory T cells. Regulatory T cells (Treg) may inhibit the activation, effector function, or proliferation of effector T cells through one or several mechanisms of suppression. The different suppressive modes may be separated into direct (left, blue rim) or indirect modes (right, pink rim) of suppression. The direct modes, which may or may not depend on cell-to-cell contact, include the secretion of suppressor cytokines (top left), IL-2 consumption, cytolysis, and inhibition through surface-bound molecules (bottom left). The indirect modes operate through modulation of various accessory cells (typically antigen-presented cells, like the dendritic cells shown here). Indirect mechanisms of suppression that have thus far been described include the actions of CTLA-4 (top right), CD39, LAG-3, and Nrp-1 (bottom right), respectively.

capacity (234). Another putative inhibitory mechanism involves the ectoenzyme CD39, which is expressed by B cells, DCs, all murine Treg, and approximately 50 % of human Treg (52). CD39 serves to hydrolyze extracellular ATP or adenosine diphosphate (ADP) to adenosine monophosphate (AMP), effectively neutralizing ATP-driven inflammation. The subsequent conversion of AMP to adenosine by Treg-expressed CD73 may further amplify the anti-inflammatory effect *via* A2a receptors on effector T cells (103). Furthermore, formation of long-lasting Treg–DC conjugates that interfere with the priming of self-reactive T cells may constitute a suppressive mechanism to prevent autoimmunity (387). In this respect, neuropilin (Nrp-1) was recently identified a Treg-associated molecule that is induced upon Foxp3 expression, and that mediates long interactions between Treg and immature DCs in conditions with limited antigen (337). Hence, Treg may prevent T-cell activation by blocking the interactions between naive T cells and DCs in an obstructive manner.

Very recently, a new paradigm has entered the stage, by which Treg are said to selectively suppress certain T helper cell lineages depending on the cytokine milieu. In essence, the new dogma states that Treg are differentiated into lineages in a manner similar to the T helper cells, and that cytokines directing the differentiation of specific T helper cells may simultaneously induce Treg that specifically suppress the corresponding T helper lineage. In three recent publications, lineage-specific Treg have been identified that target Th1 (202), Th2 (457), and Th17 (79) cells, respectively. In addition to FOXP3, these Treg express lineage-specific transcription factors that reportedly play a central role in their suppressive actions, namely T-bet (Th1), interferon regulatory factor-4 (IRF4; Th2), and STAT3 (Th17). Importantly, the generation of lineage-specific Treg was shown to be instrumental in controlling immune responses and limiting immune pathologies in murine disease models (79; 202; 457). At present, the corresponding mechanisms in humans remain to be established.

1.4.4 The balance between Treg and Th17 cells

The link between Treg and Th17 cells is evident from the transcriptional regulation of these cells. While FOXP3 is the lineage-specific transcription factor of Treg and ROR γ t is required and sufficient for Th17-cell differentiation, double-positive FOXP3⁺ROR γ t⁺ T cells have also been identified (418; 460). The FOXP3⁺ROR γ t⁺ T cells secrete lower levels of IL-17 than the single-positive FOXP3⁺ROR γ t⁺ T cells, indicating that FOXP3 inhibits ROR γ t-induced IL-17 expression in a cell-intrinsic manner (460). Correspondingly, FOXP3 deficiency results in increased IL-17 expression without affecting the levels of ROR γ t (134). Depending on the cytokine environment, cells co-expressing FOXP3 and ROR γ t may differentiate into either Th17 or Treg cells. Differentiation to the Th17 lineage is favored by the presence of pro-

inflammatory cytokines (IL-6, IL-21, or IL-23), whereas low levels of these cytokines and high-level expression of TGF- β shift the balance towards differentiation of Treg (460). The differentiation of Treg is further enhanced by the presence of IL-2 and retinoic acid (RA), both of which inhibit the expression of ROR γ t and augment TGF- β -mediated FOXP3 expression (92; 220; 270; 377).

The recently discovered Th17 cells produce the pro-inflammatory cytokine IL-17 and are thought to be important in the pathogenesis of autoimmune and inflammatory disorders (206; 413). Similarly to the iTreg, Th17 operate predominantly in the mesenteric lymph nodes and Peyer's patches. It was recently shown that the cytokine TGF- β plays a role in regulating the differentiation of Th17 cells (41; 249; 410). This was rather surprising, since the same cytokine controls the generation of iTreg and provides nTreg with survival cues after their exit from the thymus (84; 232; 251). Interestingly, mice that do not express TGF- β lack both FOXP3⁺ Treg and Th17 cells, and suffer severe autoimmunity, largely due to uncontrolled Th1 cell activity (231; 410). Thus, it appears that an intimate relationship exists between Treg and Th17 cells – a notion that is further substantiated by the recent identification of intestinal CD4⁺ T cells in mice as well as humans that simultaneously express both FOXP3 and the Th17 lineage-specific transcription factor ROR γ t, and that produce IL-17, albeit at levels below the *bona fide* FOXP3-ROR γ t⁺ Th17 cells (460). Moreover, two recent studies demonstrated a relationship between the microbiota of the gut and the abundance of Th17 cells – which was shown to be inversely correlated with the abundance of FOXP3⁺ Treg (22; 179). In this respect, the microbial expression of different sets of PAMPs that activate distinct PRRs has been suggested to skew the balance between the regulatory Treg and the pro-inflammatory Th17 cells, and between tolerance and inflammation (312). In a series of knockout studies, a higher Treg frequency was observed in the intestinal tissues of mice deficient for TLR9 (155), whereas TLR2-deficient mice had reduced numbers of Treg (381). The abundance of Treg in TLR9-deficient mice was accompanied by a reduction in the numbers of Th17 cells (155). Thus, activation through TLR9 may serve to shift the balance towards Th17 cell differentiation, which in the presence of TGF- β fosters an inflammatory Th17 response.

1.4.5 *FOXP3-negative Treg*

The IL-10-secreting Tr1 cells are distinct from nTreg as well as iTreg in that they do not express the transcription factor FOXP3 (412). This regulatory subset is otherwise defined by their secretion of high levels of IL-10 and TGF- β in response to cognate antigens, and their ability to suppress antigen-specific activation of effector T cells in a cytokine-dependent manner (320). The suppressive capacity of Tr1 cells was first appreciated by Groux and

colleagues in 1997, as they were able to isolate clones of IL-10-secreting CD4⁺ T cells following chronic allogeneic activation of murine cells in the presence of IL-10 (151). Importantly, the isolated Tr1 cells were shown to suppress a pathological immune response *in vivo* by preventing experimentally induced colitis in severe combined immunodeficiency (SCID) mice (151). Tr1 or Tr1-like cells may be induced in a variety of ways, and the different experimental conditions may generate cells of slightly different phenotypes (8-10; 28; 56; 187; 288; 378; 379; 412). As no specific marker for Tr1 cells has currently been identified, their identification relies on assessment of their suppressive capacity and cytokine profile.

The suppressive actions of Tr1 cells have been ascribed to their secretion of the immunoregulatory cytokines IL-10 and TGF- β (25; 28; 151; 224; 411). This mode of action entails non-specific regulation, termed ‘bystander suppression’, which is in contrast to their antigen-specific TCR-dependent induction (150). The fact that the Tr1 cells may be induced from naive cells upon prolonged exposure to antigen suggests that they are part of the adaptive immune response, and that they contribute to the maintenance of peripheral tolerance upon chronic immune activation (320). The possible implications of Tr1-like cells in HIV-infected individuals were studied in Paper 1.

The Th3 cells constitute another subset of FOXP3-negative Treg, distinguishable by their ability to produce TGF- β . They were initially isolated from the lymphoid tissues of mice that were fed low doses of antigen, and are believed to mediate oral tolerance (85; 127; 429). Similar to Tr1 cells, they mediate their suppression through the secretion of inhibitory cytokines; most notably TGF- β , but also some Th2 cytokines (IL-4 and/or IL-10) that may impede the differentiation and activation of Th1 cells (85; 429). Notably, anti-TGF- β treatment can inhibit several aspects of oral tolerance (157; 277; 428).

1.5 Immunomodulatory roles of the second-messenger cAMP

Cyclic AMP belongs to the cyclic nucleotide family of second messengers, ‘second messenger’ signifying a molecule transmitting an intracellular signal following ligation of the ‘first messenger’ to a cell-surface receptor. In the case of cAMP, the ‘first messenger’ denotes any ligand capable of activating adenylyl cyclase (AC) through binding to a G protein-coupled receptor (GPCR) that is linked to a stimulatory G protein α -subunit ($G\alpha_s$), resulting in the conversion of ATP to cAMP (307). Cyclic AMP regulates a host of cellular processes, and subcellular localization is absolutely crucial for maintaining the specificity of its actions. This is, to a large extent, achieved by the site-specific operations of more than 50 isoforms of cAMP phosphodiesterases (PDEs), which serve to rapidly hydrolyze cAMP, restricting and eliminating local cAMP pools unless a continuous production is maintained by nearby activated ACs. Some

specificity may also be afforded by the nine different isoforms of AC (Fig. 13), all of which have different regulation and expression patterns (308). The stringency of cAMP control explains how cAMP can serve so many different signaling functions simultaneously.

Cyclic AMP has four known cellular effectors; protein kinase A (PKA) type I and II, guanine nucleotide exchange protein directly activated by cAMP (EPAC), and cAMP-gated ion channels (Fig. 14), of which the two PKA isoforms have traditionally played a leading role (34). Over the last decade, however, numerous cAMP-driven cellular processes that are independent of PKA have been identified, several of which are regulated by EPAC (334). Cyclic AMP positively regulates the transcription of cAMP-responsive genes through the activation of the transcription factors cAMP response-element (CRE)-binding protein (CREB), cAMP response-element modulator (CREM), and activator transcription factor (ATF)-1, all of which belong to the basic-leucine zipper class of transcription factors (334). CREB recruits CREB-binding protein (CBP) upon phosphorylation on serine 133 by PKA, thereby enabling the binding to CREs. The alternative splicing of primary transcripts from the CREB and CREM genes serves to generate factors capable of repressing target gene expression, as opposed to the activating properties of the full-length proteins (263; 323; 420). Of note, one of the splice variants of CREM, known as inducible cAMP early repressor (ICER), was demonstrated to be of particular importance to the negative regulation of CRE-mediated transcription (263). The expression of ICER is regulated by an alternative, intronic CREM promoter containing four closely spaced CREs in tandem that is strongly induced by cAMP. *De novo* synthesized ICER represses the transcription of genes by binding to CREs in their promoter, and, similarly, autoregulates itself by tethering to the clustered CREs of its own promoter, which are therefore called cAMP autoregulatory elements (CARE) (263).

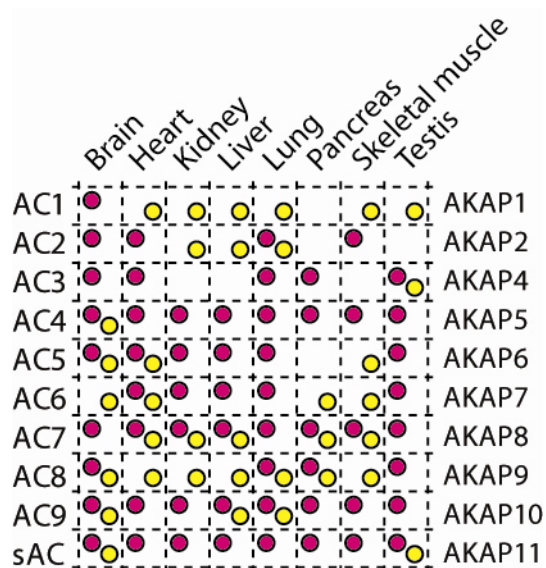


Figure 13: Restricted expression of components of the cAMP-PKA signaling machinery. Both adenylyl cyclases (ACs; pink circles) and A-kinase anchoring proteins (AKAPs; yellow circles) have restricted expression in humans. In addition, the tissue distribution of the cAMP phosphodiesterases (PDEs) and the different isoforms of PKA further contribute to the specificity in cAMP signaling.

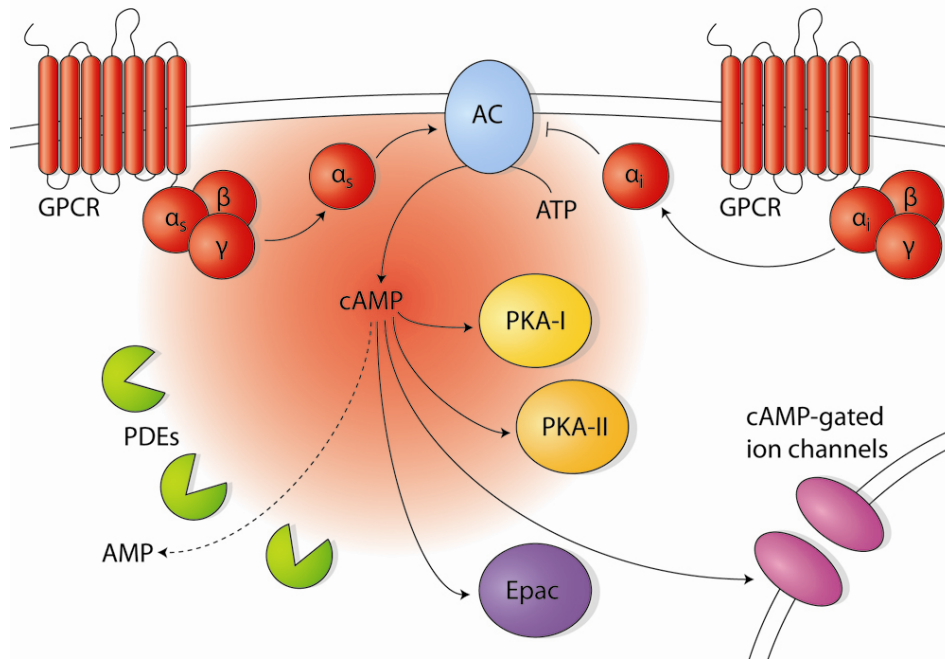


Figure 14: The second-messenger cAMP. Cyclic AMP (cAMP) is generated by adenylyl cyclases (ACs), which are regulated by inhibitory and stimulatory G-protein-coupled receptors (GPCRs). The cAMP phosphodiesterases (PDEs) negatively regulate cAMP-levels and serve to maintain distinct cAMP pools. The effects of cAMP are mediated through four cAMP-sensitive effector proteins; PKA type I and II, Epac, and cAMP-gated ion channels.

Presently, most of the immunoregulatory effects of cAMP appear to be mediated through PKA, which will be discussed below. The cAMP-gated ion channels have no known function in the immune system, but should not be disregarded at this point since the field of cAMP signaling is far from exhausted, and new modes of action are likely to be discovered. In this respect, the recent development of EPAC-selective cAMP analogs (173) will certainly expand our knowledge on the non-redundant functions of EPAC, of which some could also be of relevance to immune function. As a matter of fact, EPAC has already proven itself an immunoregulator in macrophages and dendritic cells by inhibiting phagocytosis, microbicidal activity, and the generation of inflammatory mediators upon activation by cAMP (19; 20). Finally, the recent development of a method for real-time detection of cAMP (40) should reinforce the many ongoing investigations regarding the pluripotency of this second messenger.

1.5.1 The cAMP-dependent protein kinase

Protein kinase A (PKA) – also known as the cAMP-dependent protein kinase – is a ubiquitously expressed serine/threonine kinase of broad substrate specificity. The tetrameric holoenzyme consists of two catalytic subunits that are maintained in an inactive conformation by a regulatory subunit dimer. The kinase is activated by the consecutive binding of two cAMP molecules to each of the regulatory subunits, inducing conformational changes that serve to release the enzymatically active catalytic subunits. Three isoforms of the catalytic subunit exist

(C α , C β , C γ), as well as four different isoforms of the regulatory subunit (RI α , RI β , RII α , RII β). The resulting PKA isoforms (designated type I or type II PKA depending on the nature of their regulatory subunit dimer) perform non-redundant actions and are distinctly distributed throughout the cell by specifically tethering to certain A-kinase anchoring proteins (AKAPs) *via* the dimerization and docking (D/D) interface of their regulatory subunit dimer (104; 106). Thus, the AKAPs afford an important degree of specificity to cAMP-dependent signaling by the docking of PKA (and EPAC) to localized subcellular domains. In addition, the scaffolding nature of AKAPs facilitates co-localization of PKA with its substrates, as well as with phosphatases and PDEs that may serve to terminate the signal (391). Notably, the fact that PKA signaling relies on sub-cellular targeting has been exploited while studying the relevance of the respective isoforms in various cellular systems by employing anchoring disruptor peptides like the RI anchoring disruptor (RIAD) (72) and SuperAKAP-*in silico* (SuperAKAP-IS) (143) that specifically disrupt the anchoring of PKA type I and PKA type II, respectively. Moreover, the wish to study PKA functions *in vivo* has now led to the development of anchoring disruptor peptidomimetics that are resistant to enzymatic proteolysis (see Paper 2 of this Thesis).

PKA, and in particular PKA type I, has been implicated at several levels in immune regulation and is known to regulate various aspects of T-cell activation (341; 396), as illustrated in Figure 15. A transient increase in cAMP levels has been reported in lymphocytes upon antigen receptor stimulation, which could signify activation of the intracellular cAMP receptors (215; 216; 222). Several reports have indeed suggested a role for PKA in the modulation of proximal TCR signaling (80; 200; 300; 322), and PKA type I has been shown to co-localize with the capped TCR following its activation (359; 462). In addition, CREs have been identified in the *TCR* and *CD3* genes, as well as in several other genes associated with lymphocyte activation, which could potentially be regulated by PKA (16; 152). These and other studies point to a physiological role of PKA in T-cell activation. PKA type I is known to inhibit T- and B-cell proliferation (226; 358), and may also affect NK-cell cytotoxicity (395). Moreover, PKA has been deemed an important regulator of innate immunity by affecting multiple functions of macrophages as well as dendritic cells (19; 342; 421). So far, *in vivo* data on the immunological effects of PKA is limited to observations in the RII α -/- knockout mouse, in which no marked effects on immune function were seen. Corresponding studies in RI α -/- knockout mice were hindered by the embryonic lethality of this genotype (340). We do know, however, that ablation of the C α subunit causes immune cell hyperresponsiveness, indicating non-redundant immune-regulatory functions of PKA isozymes carrying the C α subunit (130).

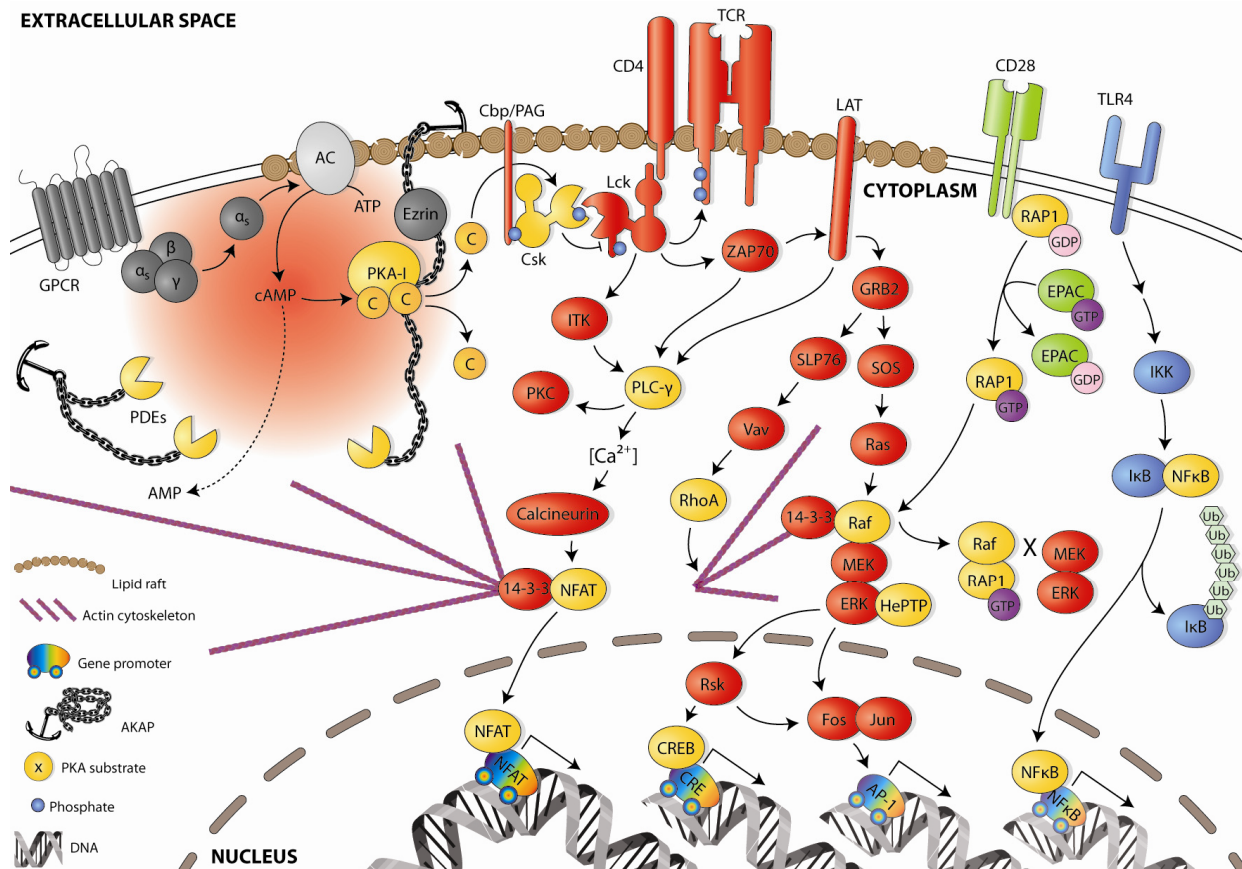


Figure 15: Multiple substrates of PKA in T cells. PKA has been shown to phosphorylate a number of proteins involved in TCR signaling, which points to a potential role in modulating the activation, differentiation, and/or proliferation of T cells. Adapted from Torgersen *et al.*, 2002, and Shillace *et al.*, 2006 (341; 396).

1.5.2 A-kinase anchoring proteins

The ubiquitous expression of cAMP and the abundance of PKA substrates invoke assistance from extrinsic mechanisms for subcellular targeting and signal specificity. Thus, although PKA (as well as the ACs and PDEs) exists in several isoforms, and the diffusion of cAMP may be limited by cellular structures, one has increasingly appreciated the role of the A-kinase anchoring proteins (AKAPs) for maintaining fidelity in the actions of cAMP and PKA (40; 119; 361). Indeed, the more than 30 known mammalian members of the AKAP family are themselves distinctly distributed in a tissue-dependent manner (73). However, the role of AKAPs in mediating specific cAMP signaling is tightly linked to their ability of co-localizing the cAMP effectors (most prominently PKA) along with their respective substrate molecules. Recent findings indicate that AKAPs are also capable of recruiting the cAMP-generating ACs to the molecular scaffold – as well as GPCRs that may activate the ACs – and PDEs that degrade cAMP into AMP, thereby providing a precise constellation for the control of local cAMP levels. The activity of associated ACs may be further regulated through phosphorylation or dephosphorylation by kinases and phosphatases present in the scaffold. Similarly, the activity of PKA may be regulated in a feedback fashion through dephosphorylation by associated

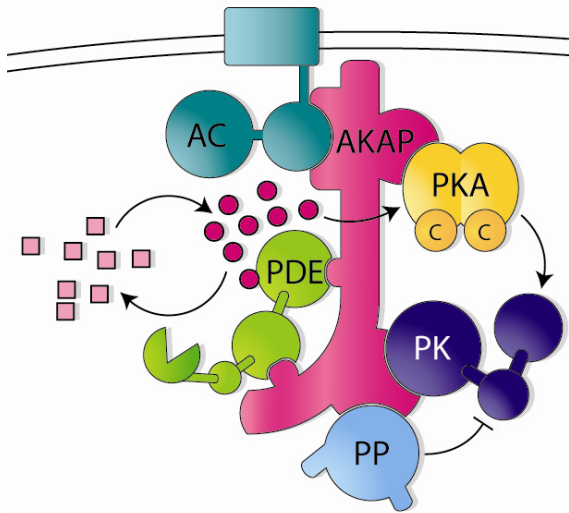


Figure 16: The different AKAPs tether specifically a certain combination of proteins, including one or more adenylyl cyclases (ACs); cAMP receptors such as PKA and/or EPAC; cAMP-degrading phosphodiesterases (PDEs); PKA substrates including different protein kinases (PKs); and signal inhibitory molecules such as protein phosphatases (PPs). The AC is normally activated by an associated G-protein coupled receptor (e.g., adrenergic receptors). The AKAP may also tether other membrane-bound receptors or ion channels, and constituents of the scaffold may influence AC activity (325; 391).

phosphatases or as a result of PDE-mediated cAMP depletion. Some of the attributes of AKAP-mediated scaffolding complexes are illustrated in Figure 16.

The AKAPs bind PKA through a helical A-kinase-binding (AKB) domain that tethers the dimerization/docking (D/D) domain of the regulatory subunit dimer of PKA (66). The D/D domain forms a groove between the two R subunits, the conformation of which differs between PKA type I and PKA type II and determines the specificity of the AKAP for either of the two PKA isozymes (26; 278). This forms the basis for isotype-specific anchoring disruption, which has successfully been achieved with the RIAD (for PKA type I) and SuperAKAP-*IS* (for PKA type II) peptides. In Paper 2, we have developed stabilized analogs of the RIAD peptide that may facilitate pharmacological studies of PKA anchoring disruption *in vivo*.

1.5.3 The cAMP–PKA–Csk inhibitory pathway

As outlined in the previous chapter, several of the signaling pathways that facilitate T-cell activation may be influenced by PKA, including the signaling of the TCR itself and its various co-receptors. This chapter will focus mainly on the effects of PKA on proximal TCR signaling, which has attracted considerable interest over the past years, and which constitutes an important part of our lab’s work. Notably, when it comes to regulating T-cell activation, the TCR signaling cascade is pivotal and perhaps the most relevant to target.

In T cells, the majority of PKA (70-80 %) are of the type I isoform (RI α 2C $_2$), whereas about 20 % constitute type II (RII α 2C $_2$) PKA (104). While most of the type II PKA is particulate (i.e., is associated with intracellular organelles), PKA type I is found predominantly in cytosol, and has the propensity to translocate to lipid rafts and co-localize with the TCR upon T-cell activation (104; 409; 462). The targeting of PKA type I to lipid rafts was originally thought to depend on the AKAP ezrin (4; 322), though recent evidence indicate a certain degree of functional redundancy conferred by moesin, the other member of the ezrin, radixin, and

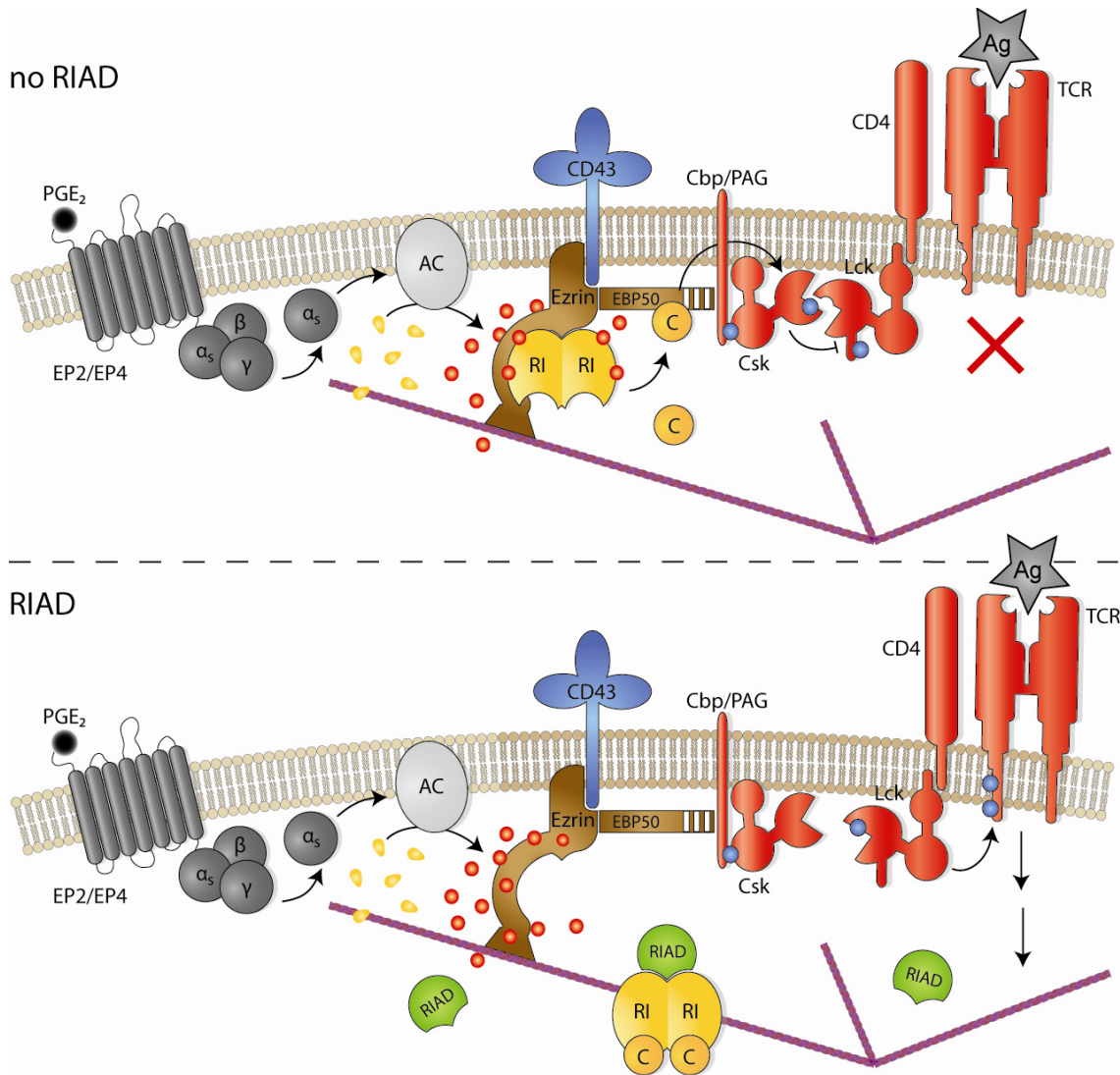


Figure 17: Specific displacement of PKA type I relieves the inhibitory tonus imposed on proximal TCR signaling by the cAMP–PKA–Csk pathway. PKA type I is targeted to the immunological synapse by the A-kinase anchoring protein (AKAP) ezrin (upper panel). In the presence of cAMP, activated PKA type I phosphorylates and activates Csk, which in turn inhibits Lck by phosphorylating its inhibitory Tyr505 residue. This results in inhibition of proximal T-cell receptor (TCR) signaling. The RI anchoring disruptor (RIAD) peptide may restore TCR signaling by specifically displacing PKA type I from the immunological synapse by blocking its interaction with Ezrin.

moesin (ERM) family present in T cells (177; 347). Ezrin also serves to co-localize anchored PKA with its substrate Csk through binding of Cbp/PAG (322). Once in lipid rafts, PKA activates Csk by phosphorylating its serine 364 residue, whereupon Csk phosphorylates the C-terminal inhibitory residue (tyrosine 505) of Lck (409). This renders Lck inactive, thereby inhibiting the proximal step of TCR-signaling, which involves Lck-mediated phosphorylation of the TCR ζ -chains (374). Thus, PKA type I is involved in the regulation of proximal TCR signaling (see Fig. 17, top panel).

Different approaches were employed in establishing PKA type I as a mediator of TCR-proximal regulation, including the isoform-specific cAMP analogs, which were central to the

discovery of the cAMP–PKA–Csk pathway (358). Recently, RIAD was introduced as another tool for delineating the isoform-specific effects of PKA in T cells (322). However, the original RIAD peptide was not sufficiently stable to be used *in vivo*. Therefore, in the present Thesis (Paper 2), a substitution strategy using unnatural amino acids was employed to improve the stability of RIAD through the introduction of unnatural amino acids. The resulting peptidomimetics were shown to have a 50-fold increase in serum stability compared to RIAD, which should be sufficient for their use *in vivo*. Figure 17 (bottom panel) illustrates how the newly designed anchoring disruptors (represented by RIAD) are envisioned to interrupt proximal T-cell signaling by disrupting the anchoring of PKA type I.

1.5.4 Prostaglandin E2

The prostanoids, including the biologically active prostaglandins PGD₂, PGE₂, PGF₂, and PGI₂, as well as thromboxane A₂, are all diverted from the membrane lipid arachidonic acid (357; 406; 407). The rate-limiting step of their synthesis is governed by the cyclooxygenases (COX), of which two isozymes exist: COX-1 is constitutively expressed in most tissues, and the output of this isozyme is considered to fulfill mostly homeostatic functions. In contrast, the expression of Cox-2 is inducible upon a variety of stimuli, including inflammation, injury, and growth factor stimuli (357; 406; 407). Notably, it is the inducible Cox-2 isozyme that is responsible for most of the symptomatic PG production, causing inflammation, pain, and fever.

PGE₂ is the most prominent prostanoid with respect to immunomodulatory functions, and has been known for a long time to suppress Th1 differentiation by increasing the intracellular cAMP concentrations (3; 42; 142; 146; 163). A study assessing the immunosuppressive actions of PGE₂ in knockout mice for each of the four PGE₂ receptors (EP1-EP4) identified EP2 and EP4 as the receptors responsible for PGE₂-mediated immune suppression (275). However, two recent studies found treatment with PGE₂ to promote inflammation through Th1 differentiation and expansion of Th17 cells (48; 440). The effect on Th1 differentiation was assigned activation of PI3K, whereas the Th17 cells were expanded in a PKA-dependent manner. These were the first studies assessing *in vivo* effects of PGE₂ and the effects they demonstrated were indeed convincing, yet surprising. It should be noted, however, that the PGE₂-treatment was performed in highly stimulatory conditions and in the presence of polarizing cytokines that may have affected the effects of the treatment, and which could partly explain the discrepancy with the previous observations.

1.6 HIV-1 infection and acquired immunodeficiency syndrome (AIDS)

The human immunodeficiency virus (HIV) was first identified in 1983 as the cause of an outbreak of malignant disease in previously healthy, young, homosexual men in North America (29). More than 25 years of intense research has provided extensive knowledge on the virus and the immune pathologies associated with infection, along with a number of drugs efficient in keeping the virus at bay, though the prospects of a cure remain distant. Following years with steady progress and the development of several drugs with potent anti-viral properties, the field was practically swept off its feet by the disappointing outcome of the 2008 STEP trial, which was testing the efficaciousness and safety of what was intended to be the first HIV vaccine (63; 256). The Merck HIV vaccine included in the trial consisted of a recombinant replication-incompetent adenovirus type 5 (Ad5) expressing the HIV Gag, Pol, and Nef genes from subtype B strains of HIV. The vaccine was designed to induce T-cell immunity, and was hoped to prevent infection or reduce early viral burden in HIV-exposed individuals. Sadly – and surprisingly – it failed in both instances, and the trial was stopped following the first interim analysis. Thus, in spite of promising results in non-human primates (343; 351), the vaccine did not protect against infection, and did not reduce viral load in infected individuals. Instead, the data suggested an increase in the risk of HIV infection among the vaccinated male participants.

In the wake of the disastrous results of the STEP trial, immunologists are now attempting other strategies for tempering and eliminating the HIV virus, turning to pristine viral targets in the development of new drugs and, furthermore, investigating the possibility of wielding endogenous immune mechanisms against the virus in newfound immunomodulatory therapies.

1.6.1 The virus

HIV is believed to have evolved from the simian immunodeficiency virus (SIV), which is known to infect primates, following transmission to humans sometime in the 19th or 20th century (301). Of the two viral strains that are pathogenic in humans HIV-1 is the strain responsible for the HIV pandemic that has now spread to every corner of the globe, whereas HIV-2 is confined to areas of West-Africa and considered less virulent (302). HIV (see Fig. 18 for schematic structure) is transmitted through intimate sexual contact, through transmission of blood and blood products, and from mother to child during childbirth (180). The several cross-species transmissions that must have occurred to generate the different subtypes of HIV-2 and the three HIV-1 groups are believed to result from the killing and handling of infected animals (154; 302). In humans, clade C is becoming the most prevalent of the HIV-1 clades, possibly as a result of the typically high viral set point in clade C infections and the abundance of virus found in genital fluids of infected patients (186). Of note, the clade C virus contains one or two

extra NF- κ B sites compared to the other clades, which makes it more responsive to positive cytokine stimuli (182). Another factor contributing to the great variability of HIV is the rapid mutation rate, which implies that viruses with different genetic and biological properties may be found within the same individual as a result of independent evolution of viruses in distinct bodily compartments (128; 193; 434). Furthermore, co-infection with several viral strains in the same patient is not uncommon, and may lead to the emergence of recombinant viruses that are immunoevasive and resistant to certain drugs (75; 129; 360).

HIV primarily infects immune cells (T cells and macrophages) that express the surface marker CD4. In addition, several other surface-bound molecules have been found to facilitate infection, including dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN; on dendritic cells), the LFAs, and the ICAMs (55; 167). However, viral tropism is mainly determined by the chemokine receptor(s), CCR5 and/or CXCR4, being used by the virus as co-receptor for viral entry. Viruses targeting CCR5 (M-tropic) typically infect macrophages, whereas CXCR4-binding viruses (T-tropic) target established T-cell lines and generally appear only later in the infection (39). The virus tethers firmly to the target cell through binding to CD4 and co-receptors (see Fig. 19, page 50), and eventually injects its content into the cell by fusing with the plasma membrane (450). As the virus enters the cell the capsule is lost, resulting in the release of the reverse transcription complex into the cytosol. Components of this complex (in particular reverse transcriptase, RT) serve to convert the single-stranded viral RNA into double-stranded DNA. Subsequently, the newly synthesized DNA is translocated into the nucleus where the associated viral integrase and cellular repair enzymes serve to permanently integrate the viral DNA into the host genome (124).

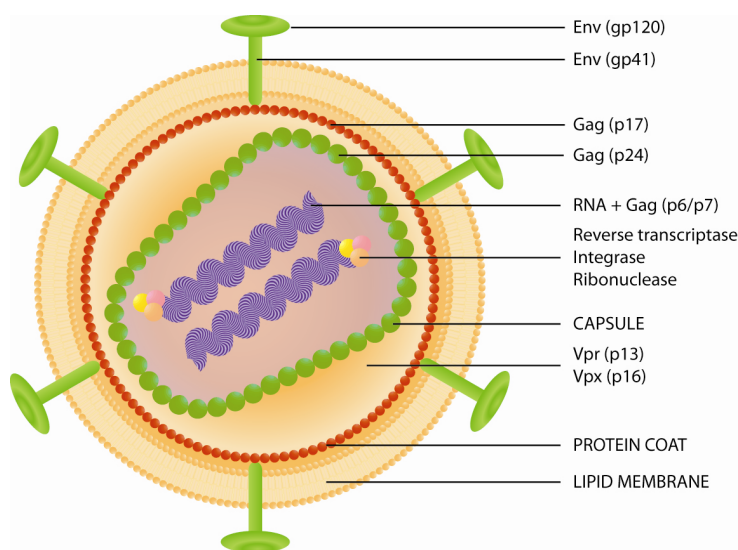


Figure 18: The human immuno-deficiency virus (HIV) consists of a capsular core, which contains the RNA genome and the machinery required for host-genome integration; a protein coat; and a lipid membrane containing envelope-protein spikes. Adapted from Collier and Oxford, 2006 (90).

As long as the infected cell remains quiescent, the integrated provirion will linger non-symptomatically in the cell's genome. However, the transcriptional activity of the cell increases upon activation, as does the availability of transcription factors and co-activator molecules (such as NF- κ B) required for transcription of the viral DNA, leading to the generation of multiple copies of viral genomic RNA and the production of viral proteins. When all the required components of the virus are present, new virions reassemble in the cytoplasm and, eventually, bud off through the plasma membrane to create functional viruses. (124; 227)

1.6.2 Why chronic?

What primarily distinguishes HIV infection from most other infections is the fact that, in the vast majority of cases, the immune system is unable to clear the infection. The virus persists in the infected individual in spite of ongoing immune responses, and is capable of escaping the immune system by integrating as a provirion into the genome of infected cells. Importantly, many different somatic cell types, and not solely immune cells, may be infected by HIV and can serve as reservoirs for the virus during chronic infection (227). Once a virus has successfully integrated into the host genome it remains in the body as a latent infection and, even though the body is able to mount an effective immune response and all the circulating virus particles are eliminated, may reinvigorate the infection at a later time point. Another crucial point in HIV infection is that the virus predominantly infects immune cells. Unless the virus is transmitted directly through blood, the original infective unit is an M-tropic virus infecting CCR5-expressing macrophages that are present in mucosal tissues, most commonly at urogenital or rectal sites. However, HIV mutates frequently and already during the acute infection will T-tropic viruses emerge that are able to infect CXCR4-expressing activated T cells. Moreover, even though the virus may initially attain the latent state – during which it remains integrated in the genome of the infected cell – as soon as the T cell is activated, it will start producing multiple copies of the virion, as well as inflammatory mediators that facilitate its differentiation and proliferation. Soon, the newly synthesized HIV virions will escape the T cell by budding through the cell membrane, and the T cell will eventually succumb to the lytic pressure and undergo apoptosis (227). Thus, even though the immune system initially is capable of keeping the virus in check, the depletion of virus-specific T cells severely weakens anti-viral immunity over time.

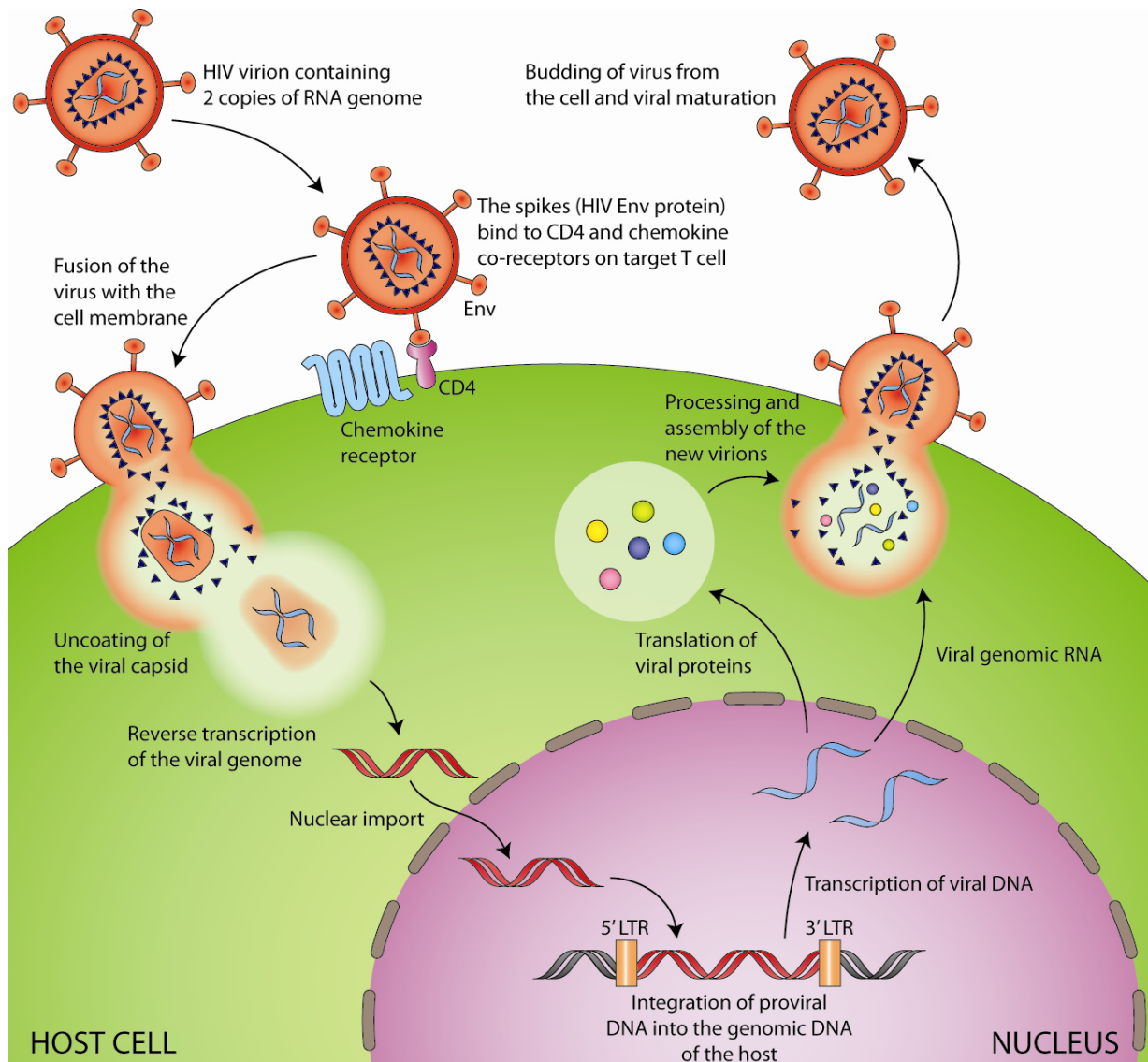


Figure 19: Key aspects of the HIV life cycle. Although the human immunodeficiency virus (HIV) is able to infect a variety of cell types, acquired immunodeficiency syndrome (AIDS) results from the depletion of CD4+ T-helper lymphocyte cells, a key component of the human immune system. HIV enters the cell by docking to CD4 and using a chemokine receptor (either CCR5 or CXCR4) and other surface proteins to adhere firmly to the cell and induce fusion with the plasma membrane. The viral capsid is released inside the cell, and the viral genome integrates into the host genome by the actions of viral integrases. As viral genes are transcribed in activated CD4+ T cells, new viruses are formed that eventually lyse the cell as they bud off and are released into the surrounding tissues. Adapted from Rambaut *et al.*, 2004 (313).

1.6.3 AIDS – an exhausting affair

Weakened HIV-specific immunity alone does not explain the progression to AIDS (acquired immunodeficiency syndrome), which is characterized by a broadly acting immune deficiency that equally affects both HIV-specific immune responses and responses to other pathogens and malignancies. Several mechanisms are believed to contribute to the overall weakening of the immune system, one of which includes exhaustion of immune cells as a result

of chronic activation. The observed exhaustion has been associated with loss of proliferative capacity and limited functionality of a given immune cell, which relates to the expression of a set of inhibitory co-receptors, notably PD-1 and CTLA-4 (101; 398). In Paper 3, the simultaneous expression of PD-1 and CD38 was used to assess chronic activation. Furthermore, cellular exhaustion has been shown to correlate with prognostic markers such as viral load and CD4+ T-cell count in HIV patients (101; 172; 398). The observation that loss of PD-1 and CTLA-4 leads to autoimmunity in the respective knockout mice indicates that these molecules play important roles in the maintenance of immune homeostasis (82; 147). Normally, the balance between positive and negative regulation determines the outcome of TCR stimulation and weighs the T-cell response between activation and anergy. This may still be the case in chronic infection, but the balance has been severely skewed towards anergy by the abnormal hyperactivity of negative regulatory mechanisms. In the event of TCR stimulation, the expression of PD-1 and CTLA-4 on the cell surface is induced – in a manner dependent on nuclear factor of activated T cells (NFAT)-c1 – as part of a negative feedback mechanism (292). However, in face of chronic activation, the expression of these receptors are induced to such levels that the ability of the T cell to initiate a response becomes severely crippled. Moreover, should the T cell become activated, its functionality in terms of production of effector molecules is gravely limited. Thus, rather than generating polyfunctional responses, in which the T cell produces several cytokines and inflammatory factors, the response may be restricted to producing only one or two cytokines (13).

The interaction of T-cell expressed PD-1 and CTLA-4 with their cognate receptors, B7-H1 (PD-L1, where PD-L is PD ligand) or B7-DC (PD-L2) for PD-1 and B7-1 (CD80) or B7-2 (CD86) for CTLA-4, negatively influences TCR signaling but may also convey ‘reverse signals’ through the B7 molecules and provide potentially important feedback to the APC. However, PD-L1, CD80, and CD86 may also be expressed on T cells and it has recently been shown that the interaction between PD-L1 and CD80 may confer inhibition of T-cell activation (67). CTLA-4 is also constitutively expressed on regulatory T cells and assumed to play a role in Treg-cell mediated suppression (125; 433). PD-1 is highly expressed on both CD4+ and CD8+ HIV-specific T cells (101; 304; 398). Interestingly, the expression of PD-1 on T cells specific for epitopes that had undergone a mutational escape was lower than on T cells recognizing conserved epitopes (375). The reduction in PD-1 expression following mutational escape was accompanied by regained polyfunctionality in these T cells, indicating that the exhaustion inflicted by high-level expression of PD-1 is reversible. Moreover, the findings in this longitudinal study suggested a link between modulation of PD-1 expression and the repeated exposure to cognate antigen (375). PD-1 levels may also be modulated in response to

TCR-independent stimuli, such as cytokine stimulation (199) or exposure to the accessory HIV protein Nef (273). Studies of CTLA-4 in HIV infection demonstrated a moderate overexpression of CTLA-4 in HIV-specific CTLs, which conferred suppression of HIV-specific CD4⁺ T-cell proliferation independent of the CTLA-4 expressed on Treg (188). In murine models of chronic infection, blockade of the PD-1 pathway has had promising effects in improving the response to therapeutic vaccination (153). Moreover, systemic administration of blocking antibodies to CTLA-4 was able to induce tumor regression in patients with metastatic melanoma or ovarian cancer, although at the cost of systemic inflammation (168; 305). One concern with therapeutic strategies directed against these inhibitory mechanisms, therefore, is the possibility of immune hyperactivity and autoimmunity.

Other immunoregulatory mechanisms that have been implicated in HIV-1-associated immune suppression include IL-10 and cAMP, both of which have been addressed in the work presented in this Thesis (see Paper 1 and Paper 3, respectively). Interleukin-10 is a predominantly immunosuppressive cytokine known to suppress a wide variety of cells, including most, if not all, of the hematopoietic lineages, as well as a number of somatic cell types (264). This cytokine is known to potently inhibit the cytokine- and chemokine production of responder cells, as well as the expression of other inflammatory molecules and receptors, resulting in wide-spread immune suppression and the induction of anergic T cells. Notably, IL-10 has been shown to be crucial to the induction and regulatory function of inducible regulatory T cells in mice. Furthermore, IL-10 has been involved in a number of immune pathologies, of which various cancers and persistent infections may be the most prominent (264). Moreover, IL-10 was linked to the pathogenesis of HIV, since the potencies of anti-HIV immune responses were inversely related to the concurrent levels of IL-10, and poor HIV-specific T-cell responses were restored by the addition of anti-IL-10 antibodies (88; 89; 218). Interestingly, IL-10 has also been shown to inhibit the production of PGE₂ through down-regulation of Cox-2, which was, in turn, shown to down-regulate the expression of matrix metalloproteinases in a mechanism dependent on cAMP (259; 279; 280). Moreover, the elevated levels of cAMP in T cells of HIV-infected patients was, in another study, shown to result in suppression of T-cell-dependent immune responses through the activation of PKA type I (1). Notably, several other mechanisms of HIV-associated immune suppression have also been suggested, though the relative importance of the different mechanisms remains to be established.

1.6.4 Tale of a survivor

In February 2009 a team of medical doctors and scientists at Charité Universitätsmedizin Berlin published their sensational report on the first case ever to have been cured from HIV-1

infection (176). A 40-year old male HIV-1-infected patient underwent chemotherapy and stem-cell therapy for acute myeloid leukemia. Following myeloablation and T-cell ablation of the patient, CD34+ peripheral-blood stem cells were transferred from a human leukocyte antigen (HLA)-identical donor that had previously been screened and found homozygous for the *CCR5* delta32 allele. Following two consecutive transplantations, complete remission of the acute myeloid leukemia was achieved. Twenty months after the first transplantation (and six months after the second), the patient remained healthy from his leukemia and free of plasma HIV-1 RNA, even in the absence of HAART. (176)

One important prerequisite for the success of this HIV-1-ablative strategy was the identification of an allogeneic stem-cell donor with identical HLA genotypes. Furthermore, the fact that the viral strains of the recipient were predominantly (97.1 %) CCR5-tropic, combined with the inability of the virus to infect residual long-lived CCR5-expressing macrophages, crucially contributed to the success of the endeavor (176). Overall, this report underscores the importance of the CCR5 co-receptor during HIV-1 infection and disease progression, and advocates further investigation into therapies targeting CCR5-mediated viral entry. Perhaps still more important, however, is the hope communicated by this sensational report to infected people and health-care professionals world-wide, nurturing the belief that HIV may, in fact, be cured.

2. Aims of the study

The human immune system is extensively regulated and the modulation of immunoregulatory mechanisms represents a promising avenue for future immunotherapeutics. Regulatory T cells constitute one such mechanism that may be capable of controlling both autoimmunity and responses against infectious agents or malignancies. In addition, a number of molecular controls serve to direct and fine-tune immune responses on the subcellular level. These include the second messenger cAMP, which is known to control T-cell activation by inhibiting proximal TCR signaling in a cAMP–PKA–Csk inhibitory pathway. Cyclic AMP, in turn, is generated by adenylyl cyclases in response to a variety of different stimuli, of which some, like the prostaglandin PGE₂, are produced by the inducible Cox-2 enzyme.

The work presented in this Thesis has explored mechanisms of immune regulation at both cellular and sub-cellular levels, with the overall objective of elucidating the regulatory processes involved in HIV-associated immune dysfunction. Specifically, our aims were to:

1. Investigate the suppressive properties and phenotype of HIV-1 antigen-specific T cells secreting IL-10 in chronic HIV.
2. Design RI-specific PKA anchoring disruptor peptides of sufficient serum stability to be used in studies investigating the immunoregulatory role of PKA *in vivo* and, possibly, with the ability of alleviating immune dysfunction.
3. Improve our basic understanding of the interaction between the regulatory subunit of PKA and its corresponding AKAP.
4. Evaluate the potential of using the selective Cox-2 inhibitor celecoxib as immunomodulatory treatment in chronically HIV-infected, treatment-naive individuals.
5. Examine the effect of treatment with Cox-2 inhibitors on the activation status and responsiveness of peripheral T cells.
6. Investigate the possible influence of Cox-2 on the function of regulatory T cells.

3. Summary of Results

Paper 1: *Interleukin-10-secreting T cells define a suppressive subset within the HIV-1-specific T-cell population.*

In this paper, we investigated the possibility that a subset of HIV-1-specific T cells found in the peripheral blood of viremic HIV-positive individuals could harbor immunoregulatory properties, possibly contributing to the state of immune deficiency seen in individuals with progressive HIV-1 infection and AIDS. An immunosuppressive subset of HIV-1-specific T cells was indeed identified based on the capacity of this subset to produce and secrete the immunoregulatory cytokine IL-10. We isolated live, cytokine-secreting cells through the capture of IL-10 (or IFN- γ , in the control cells) on the surface of the respective cells using specific antibodies and targeting surface-bound IL-10 with immunomagnetic beads. The IL-10-secreting cells were ultimately enriched on magnetic columns. They were subsequently phenotyped by FACS, and their ability to suppress the proliferation of normal T cells was determined in co-cultures using the CFSE dilution assay. We demonstrated that the suppressive capacity of the HIV-1-specific IL-10-secreting T cells was independent of FOXP3-expression, by which they resemble Tr1 cells. The number of these cells in peripheral blood turned out to be very low, which eventually hampered our attempts at determining their mechanism of action.

Paper 2: *Design of proteolytically stable RI anchoring disruptor peptidomimetics for in vivo studies of anchored type I protein kinase A-mediated signaling.*

This paper concerns the development of proteolytically stable peptidomimetics for disrupting the intracellular targeting of type I PKA. Starting with the sequence of the RIAD peptide recently published by our group (72), we introduced unnatural amino acids in strategic positions to increase the stability of the peptide. First, residues of the RIAD peptide that were particularly susceptible to proteolytic cleavage were identified in stability studies monitored by HPLC and mass spectrometry. Enzymatically protected RIAD analogs were designed by substituting labile residues with unnatural amino acids and, importantly, capping of the amino (N-) terminal leucine residue. The resulting peptidomimetics were tested with respect to specificity and affinity for PKA type I by membrane overlay with radio-labeled RI or RII. The stability of the analogs was determined by HPLC peak analysis following incubation in diluted human serum for varying amounts of time. The identity of the analogs and resulting metabolites was validated by mass-spectrometry. Finally, the newly designed peptidomimetics were tested *in vitro* regarding their capacity to reverse cAMP-mediated suppression of T-cell activation (in terms of IL-2-production; as assessed by enzyme-linked immunosorbent assay [ELISA] of culture supernatants).

PKA type I is known to regulate T-cell activation, and dysfunction in the cAMP–PKA–Csk regulatory pathway has been implicated in the state of immune deficiency seen in progressive HIV-1-infection and AIDS. In this paper, RIAD analogs of improved enzymatic stability were designed that will aid in studying the involvement of PKA in immune function *in vivo*. We report a 50-fold improvement of the stability of these peptides compared to RIAD when tested in 30 % human serum *in vitro*.

Paper 3: *Cyclooxygenase type 2 inhibitor downregulates chronic immune activation, CD38, and PD-1 in patients with untreated HIV-1 infection.*

The third study included in this Thesis aimed to address the effects of treating healthy, treatment-naive, HIV-infected individuals with the selective Cox-2-inhibitor celecoxib. The study was performed as a prospective, open, exploratory trial and included 31 HIV-infected patients in total. The primary endpoints were the expression of CD38 and PD-1 on various T-cell subsets as an indicator of immune activation and clinical progression. Effects of the celecoxib treatment were evident on CD38 and PD-1 levels, as well as on Treg numbers and function (as determined by CD25 depletion). Furthermore, the treatment induced changes in the susceptibility of T cells to indomethacin-mediated immune activation, possibly due to an up-regulation of Cox-2 in T cells. Given the reduction in markers of chronic immune activation and T-cell exhaustion, in addition to an up-regulation of cytokine-secreting effector T cells, the treatment can be said to have had a positive effect on immune function. It would be reasonable to expect that at least some of these effects were the result of reduced intracellular levels of cAMP. Thus, it is conceivable that celecoxib and other Cox-2 inhibitors may be useful immune modulating additions to the treatment of HIV.

4. Discussion

In the introduction of this Thesis, the central principles of immunity and immune regulation were laid down, to provide an understanding of the tightly regulated processes of an immune response. From this information we may, perhaps, begin to grasp the complexity of the systems we rely on for our good health and admire the fact that, most of the time, the numerous intricate systems that constitute our immune system manage to keep us healthy and strong. However, faulty immune responses do occur and, in cases such as autoimmune diseases and cancer, have been known to cause disease. The present Thesis has focused on the regulatory mechanisms that are put in place to avoid aberrant immune activation and limit immune pathology, addressing the potential of manipulating these mechanisms in a therapeutic context. Emphasis has been put on the emerging idea that exaggerated immune regulation may contribute to immune deficiencies, as seen in HIV infection, or suppress immunity against malignant cells. As far as the treatment of autoimmune diseases is concerned, this has traditionally relied on “shotgun therapies”, i.e. drug regimens that tend to knock out the entire immune system, or at least large parts of it, in order to keep the pathogenic processes at bay. This type of treatment comes with severe side effects and a high risk of attracting serious infections (see Table 1). On the other hand, treatments directed at improving immune responses in sub-immunogenic individuals are few and with limited applicability (Table 2). These, as well, suffer from side effects and are frequently inefficient at restoring immunity. Hence, by identifying new and more specific targets for immune modulation, we hope to improve the treatment of immune-mediated diseases.

Table 1: The immunosuppressive drugs currently marketed in Norway. The table lists the immunosuppressive drugs that are currently on the market, indicating their mechanism of action and including a list of their most common adverse effects. The most important therapeutic uses are noted, as well. IBD, inflammatory bowel disease; ALL, acute lymphocytic leukemia; MS, multiple sclerosis; RA, rheumatoid arthritis; CLL, chronic lymphocytic leukemia; PA, psoriatic arthritis; GVHD, graft-versus-host disease; SLE, systemic lupus erythematosus; WG, Wegener’s granulomatosis. Sources: Felleskatalogen, 2009 (97); Legemiddelhåndboken, 2007 (126).

Compound	Therapeutic uses	Site of action	Immunological effects	Adverse effects
<i>Glucocorticoids</i> (Hydrocortisone; prednisone; triamcinolone; dexamethasone)	Rheumatic disorders; allergic disease; bronchial asthma; IBD; ALL and lymphomas; MS; organ transplantation	Regulate gene transcription by acting on glucocorticoid response elements in DNA; may also activate membrane-bound receptors	Decreased release of vasoactive and chemoattractive factors; diminished secretion of lipolytic and proteolytic enzymes; decreased leukocyte extravasation; reduced fibrosis; diminished expression of Cox-2 and NOS	Affect glucose and lipid metabolism; perturb electrolyte and water balance; induce redistribution of body fat; may precipitate diabetes in predisposed individuals; muscle wasting and fatigue upon prolonged treatment; behavioral changes
<i>mTOR inhibitors</i> (Sirolimus; everolimus)	Organ transplantation;	Inhibit the protein kinase mTOR downstream of the IL-2 receptor and other T-cell growth-factors	Blocks cell-cycle progression at the G1 → S phase transition; prevents T-cell proliferation	Anti-proliferative effects on blood cells resulting in anemia, leukopenia, thrombocytopenia; increased risk of infections and neoplasms
<i>Antiproliferative agents</i> Mycophenolic acid	Organ transplantation	Inhibits <i>de novo</i> guanine nucleotide synthesis.	Blocks proliferation and effector functions of, in particular, B and T lymphocytes	Leukopenia; diarrhea; vomiting; increased incidence of some infections
<i>Calcineurin inhibitors</i> (Cyclosporine;	Organ transplantation; RA; psoriasis; IBD	Inhibit the phosphatase-activity of calcineurin	Inhibition of antigen-triggered signal transduction in T lymphocytes; increased expression of TGF-β	May cause renal dysfunction; tremor, motor disturbances, seizures; hypertension; hyperlipidemia; gum hyperplasia
<i>Immunoglobulins</i> Antithymocyte globulin (polyclonal)	Acute renal transplant rejection	Bind proteins exposed on the surface of T lymphocytes	Deplete circulating T lymphocytes by direct cytotoxicity (complement or cell-mediated) and block lymphocyte function	Fever and chills; potential for hypotension; serum sickness; glomerulonephritis; leukopenia; thrombocytopenia; increased risk of infection and neoplasms
Monoclonal - anti-CD3 (murmonab-CD3)	Acute transplant rejection	Binds the ε-chain of the T-cell receptor complex, inducing rapid internalization and down-regulation	Preventing antigen recognition by the internalization of TCR complexes; causing redistribution of T cells into non-lymphoid organs	Cytokine release syndrome, including high fever, chills, headache, tremor, nausea, diarrhea, malaise (these are relieved by glucocorticoids); increased risk of infection and neoplasms
- anti-CD25 (daclizumab, - anti-CD52 (alemtuzumab)	Organ transplantation CLL; renal transplantation	Presumably binds IL-2Rα on activated T cells Induces apoptosis in CD52-expressing cells	Causes reduced expression of IL-2Rα Depletes lymphocytes, monocytes, macrophages, and natural killer cells from circulation by inducing apoptosis in the targeted cells	Low-frequency adverse effects include anaphylaxis, lymphoproliferative disorders, and opportunistic infections Nausea; hypotension; rash; opportunistic infections; fever; chills; leukopenia; anemia; myalgia; tremor; headache
- anti-TNF (infliximab, etanercept, adalimumab) - anti-LFA-1 (efalizumab) - CTLA-4-IgG1 f.p. (abatacept)	Crohn's disease; RA Psoriasis RA	Binds to TNF-α and prevents the cytokine from binding to its receptors Binds the CD11a chain of LFA-1 Binds the CD80/CD86 on APCs	Sequesters TNF-α and alleviates TNF-α-mediated immune pathologies Prevents the interaction of LFA-1 with ICAM and blocks T-cell adhesion, trafficking, and activation Blocks CD28 co-stimulation of T cells, suppressing activation, proliferation and cytokine production	Infusion reaction (fever, urticaria, hypotension, dyspnea); serious infections; lupus-like syndrome Flu-like symptoms (fever, chills, headache); leukocytosis; lymphocytosis; psoriasis; connective tissue disorders Increased risk of infections; dermatitis; nausea, hypertension; may affect liver function
<i>Cytokine antagonists</i> Anakinra Etanercept	RA RA ; PA; psoriasis; Behkterev's disease	Interleukin-1 receptor antagonist TNF- α receptor antagonist	Neutralizes inflammatory actions of IL-1α and IL-1β Blocks TNF-α-mediated immune pathologies	Neutropenia; headache; serious infections Serious infections; fever; thrombocytopenia; rash; skin lesions
<i>Cytotoxic agents</i> Methotrexate Cyclophosphamide	GVHD; RA ; psoriasis Highly active MS; lymphomas/leukemias; SLE; WG	Inhibits <i>de novo</i> purine synthesis; promote adenosine release Cross-links DNA strands	Anti-inflammatory effects Cytotoxic effects on rapidly dividing cells; differential effects on T-cell subsets and regulatory T cells. Blocks both cellular and humoral immune responses.	Nausea; dyspepsia; stomatitis; headache; alopecia. Bone marrow suppression and liver function abnormalities can occur Hemorrhagic cystitis; vomiting; infertility
Chlorambucil Leflunomide	CLL RA ; PA	Cross-links DNA strands Inhibits DHODH, an enzyme involved in mitosis of lymphocytes	Blocks cell proliferation Blocks cell proliferation of lymphocytes	Bone marrow suppression; nausea; diarrhea Mild leukopenia; diarrhea; nausea; liver function abnormalities; alopecia; headache

Compound	Therapeutic uses	Site of action	Immunological effects	Adverse effects
<i>Cytokines</i> Interferons - IFN- α -2b - IFN- γ -1b - IFN- β -1a/b	α : Malignancies; chronic hepatitis B/C infection; condylomata acuminata; β : multiple sclerosis; γ : chronic granulomatous disease. Also used against a variety of viral diseases	Bind to specific cell-surface receptors	Multiple and diverse effects, including induction of certain enzymes, inhibition of cell proliferation, and enhancement of immune activities (increased phagocytosis by macrophages and augmentation of specific cytotoxicity by T lymphocytes)	Flu-like symptoms (fever, chills, headache); hypotension; arrhythmias; myalgia; weight loss; depression; confusion
Interleukin-2	Metastatic renal cell carcinoma; melanoma	Binds to specific cell-surface receptors	Activation of cellular immunity, including lymphocytosis, eosinophilia, thrombocytopenia. Release of cytokines.	Capillary leak syndrome (hypotension, reduced organ perfusion); impaired neutrophil function may cause increased risk of disseminated infection
<i>Levamisole</i>	Colon cancer	Unknown	Restores depressed immune function of B cells, T cells, monocytes, and macrophages	Agranulocytosis (occasionally fatal)
<i>Thalidomide</i>	Erythema nodosum leprosum; multiple myeloma	Unknown	Immunomodulatory, anti-inflammatory, and potential anti-neoplastic activities	Neutropenia; leukopenia; anemia; lymphopenia; thrombocytopenia; tremor; vomiting; rash; depression
<i>BCG</i>	Urinary bladder carcinoma; papillary tumors	Unknown	Inhibits or prevents tumors	Hypersensitivity, shock, chills, fever, malaise; immune complex disease
<i>Vaccines and immunoglobulins</i>	Infectious diseases; cancer; autoimmunity	Induces specific immune response	Prevents or treats infectious diseases, cancer, or autoimmunity	Flu-like symptoms (fever, chills, headache); myalgia; lethargy; anaphylactic shock

Table 2: The immunostimulatory agents currently marketed in Norway. The table lists the immunostimulatory drugs that are currently on the market, indicating their mechanism of action and including a list of their most common adverse effects. The most important therapeutic uses are noted, as well. Sources: Felleskatalogen, 2009 (97); Legemiddelhåndboken, 2007 (126).

4.1 A global view of immune regulation

‘Immune regulation’ as a phenomenon spans a wide range of mechanisms, ranging from regulation of homeostatic output of immune cells in the bone marrow to modulation of the signaling of peripheral effector cells. As suggested above, the modalities currently available for treating immune-mediated diseases suffer a number of shortcomings, of which a lack of efficacy and frequent serious adverse events are among the major concerns. Improved therapies are expected to spring from the identification of molecular mechanisms that may be specifically targeted. In this respect, efficacy is maintained only to the extent that the targeted entity effectively controls the pathogenic mechanism. Moreover, specificity – and, thereby, the desired reduction in adverse effects – requires limited influence of the targeted mechanism over other immunological and physiological processes.

In the present Thesis, modulation of T-cell activation through the inhibition of PKA type I has been presented as a putative treatment strategy for various immune deficiencies, including AIDS (Paper 2). Indeed, the peptidomimetics that we have developed selectively target PKA type I over type II PKA, and are able to disrupt the functions of PKA type I without affecting processes dependent on PKA type II (Paper 2). However, this does not address the fact that PKA type I is also expressed in other cell types than T cells, and that administration of the peptidomimetics *in vivo* may cause side effects that are unrelated to the immunoregulatory mechanism we wish to target. Yet it remains conceivable that intravenous administration, combined with low dosage, allows for selective treatment of circulating blood cells and sufficiently discriminates between these and tissue cells to avoid adverse effects. It is also possible that any effect imposed on other cell types by transient inhibition of PKA type I is clinically irrelevant. Thus, in planning for improved immunomodulatory therapies, not only is the specificity of the chosen target mechanism crucial; the drug itself will also have to be highly specific in its actions.

From what was outlined in the introductory chapters of this Thesis, we may gather that regulation is the rule rather than the exception in the realm of immunity. Figure 20 summarizes the most important checkpoints for a given immune response. Importantly, all of these checkpoints represent putative targets for immunotherapy. For example, it could be of interest to administer an agent that would shift the thymic T-cell output towards more nTreg in individuals suffering from autoimmune diseases. Of note, induction of iTreg in the periphery could serve the same purpose, and even be more efficient if antigen specificity was required for optimal suppression. Such non-invasive modulation of Treg remains largely untested, although some reports have claimed Treg-modulating effects of some registered drugs (see Section 4.3).

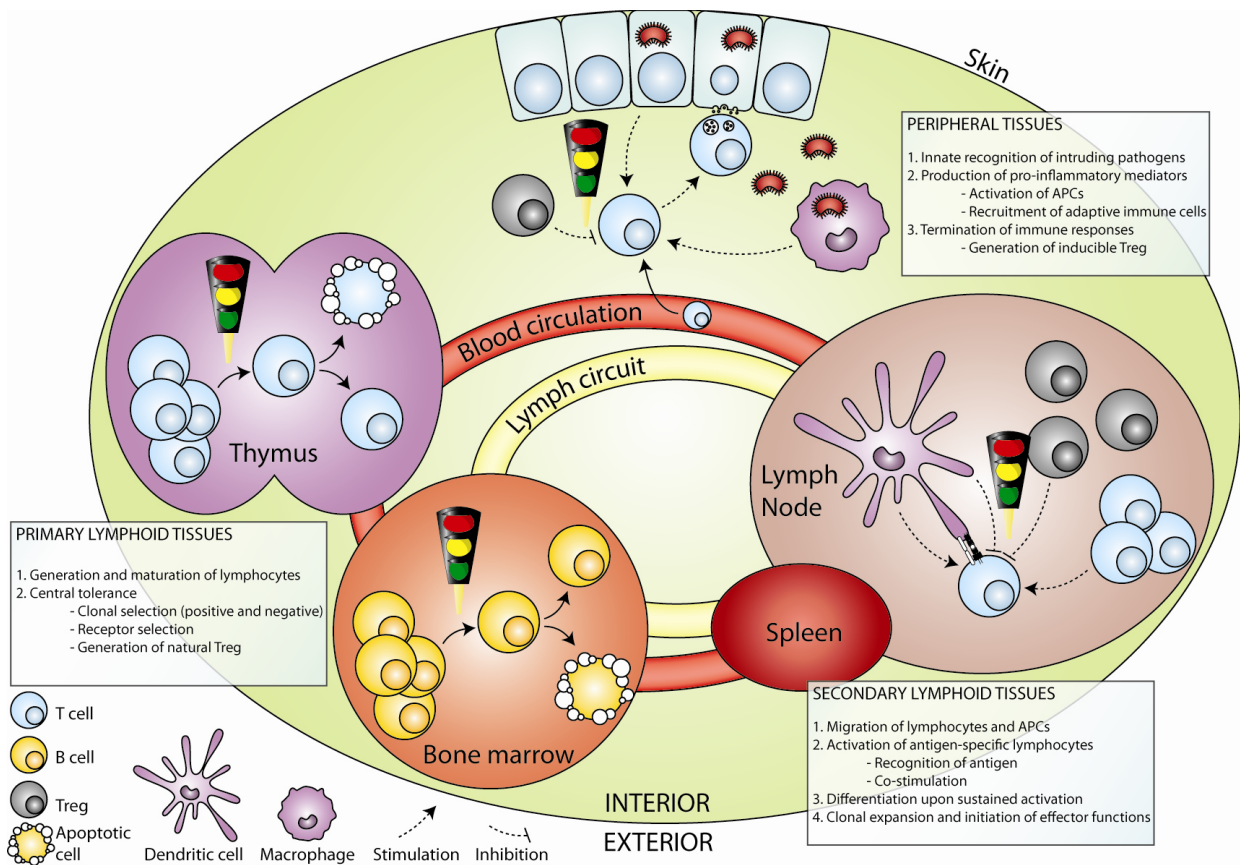


Figure 20: Regulation abounds. Every critical step of the immune response is subject to regulation. Key check points include lymphopoiesis and clonal selection in the primary lymphoid tissues; activation and maturation in secondary lymphoid tissues; and generation of effector responses in the periphery. Both extrinsic and intrinsic mechanisms are put in place to ensure timely responses against potential threats based on the relative abundance of incoming stimulatory and inhibitory signals, respectively. These may result from cell contact or the release of soluble signaling molecules.

On the other hand, the adoptive transfer of *ex vivo* isolated and/or expanded Treg could represent a more imminent feat (see Section 4.2). The homing of immune cells to specific tissues may be utilized in immunotherapy, as may the requirement for inflammatory factors and co-stimulation for activation of these cells when appropriately positioned in the respective tissues. Moreover, the antigen presentation itself could constitute another interceptive focus. As we have seen, the DC may, for instance, attain either an inflammatory or a tolerizing state, which directly affects the response of peptide–MHC-cognate lymphocytes by inducing either activation or tolerance. Furthermore, the intracellular signaling that ensues in the T cell following interaction with an APC may pose another range of possible targets, as even subtle changes in the different signaling pathways have been seen to abruptly modify the functions of a lymphocyte. The question is: At which level do we achieve the highest specificity? Or, in terms of developing drugs that can be used to treat a range of pathologies, which level should be targeted to achieve the greatest versatility?

Regardless of what level is chosen, it will always remain important to maintain a global view of the perturbations we introduce to modulate immunity: Although we see the desired effects in T cells, adverse effects may occur elsewhere. Hence, if the aim is to administer an immunomodulatory drug systemically, we will have to consider what other systems will be affected and, if necessary, provide means to ensure targeted delivery of the drug or otherwise limit its adverse effects.

4.2 Harnessing the regulatory properties of Treg for therapeutic purposes

The leading principle of modern medicine says that any pharmacological intervention should aim to maintain a healthy balance between the expected benefit of the treatment, versus the potential risk. However, it does preclude a significant number of patients with immune-mediated diseases from getting the most effective treatment, due to a high risk of side effects. Thus, providing new, targeted therapies with a smaller risk of side effects could result in improved quality of life for a great number of patients. Therefore, the identification of targets with potential value for the treatment of immune-mediated diseases has been a major focal point over the recent years of medical research. In this respect, the identification of regulatory T cells has been particularly intriguing.

Since the discovery of regulatory T cells, the possibility of utilizing these cells in immune therapy has attracted substantial interest. Their therapeutic potential was recognized as early as in 1975 (137), yet decades later we are still awaiting the results from the first clinical studies (319). However, evidence of the potential effects of such a therapy has, since then, been demonstrated in a series of studies looking at the effects of removing and adoptively transferring specific T-cell subsets in mice (258; 311; 328; 362; 376), and the first report identifying the suppressive subset as CD4+CD25+ T cells was published in 1995 (330). Importantly, in these studies, tolerance could be restored by the adoptive transfer of the T-cell populations that had originally been removed, further substantiating the possible link to a new form of cell-mediated immunotherapy. One important lesson learnt from these studies was that loss of regulatory T cells (Treg) by itself can be sufficient to induce autoimmunity. This notion has been compellingly corroborated by the finding that loss-of-function mutations in the *FOXP3* gene, encoding the Treg ‘master-switch’ transcription factor FOXP3, results in a syndrome characterized by multiple autoimmune diseases in humans, known as the IPEX syndrome (37; 430). Correspondingly, mutations in the murine ortholog of FOXP3, *scurfy*, were found to be responsible for the lethal scurfy phenotype in mice (62).

Several potential issues have held back the process of translating adoptive Treg therapy from the pre-clinical studies to clinical practice. First of all, there is the issue of safety, which

puts stringent demands on the quality of clinical Treg isolates. According to the U.S. Food and Drug Administration (FDA) these have to be sterile, identifiable, pure, and with a proven potency (319). However, the preparation of Treg isolates is a complex process, especially when it comes to preparing large-scale isolates that are to be used in human beings: First, there is a limited availability of Treg in human donors, as Treg donated from adults will have to come from peripheral blood or from bone marrow. Alternatively, Treg may be isolated from frozen umbilical cords (141). Regardless, the large number of cells required for therapeutic purposes necessitates *ex vivo* expansion of the isolated Treg. This, however, is complicated by the fact that effector T cells (Teff) are more easily expanded than Treg, implying a risk of amplifying Teff impurities that were present in the original isolate. One recent advance that may facilitate selective expansion of Treg *in vitro* involves treating the cultures with the immunosuppressant rapamycin, which preferentially preserves Treg over Teff due to the FOXP3-induced expression of a kinase (Pim2) that confers rapamycin resistance (31; 33).

Another major hurdle to the introduction of adoptive Treg therapy lies in demonstrating the potency of the product prior to administration to the patient, since standardized *in vitro* procedures for this task has yet to be defined. Quite significant to this end is the fact that, although multiple mechanisms have been suggested, we lack a clear understanding of how Treg-mediated suppression comes about *in vivo* (388). Moreover, the suppressive capacity observed *in vitro* does not necessarily correlate well with the ability to suppress *in vivo* (144). The recent development of humanized animal models carrying human immune systems may aid in the assessment of *in vivo* suppressive responses with human Treg (355; 417), though it remains to be seen whether they will be able to predict therapeutic value in humans.

The adoptive transfer of Treg has been tested in a variety of autoimmune diseases in mice, of which the results from models of type-1 diabetes (T1D) and graft-versus-host disease (GVHD) related to hematopoietic stem cell transplantation (HSCT) have shown the greatest promise: Several studies have demonstrated the potential benefit of adoptively transferring either polyclonal or antigen-specific Treg into non-obese diabetic (NOD) mice, which are otherwise prone to acquiring T1D (385; 389; 390). Similarly, the adoptive transfer of freshly isolated or expanded Treg into recently grafted recipients prevented acute as well as chronic GVHD (392; 454). Furthermore, *in vivo* activated Treg have been shown capable of ameliorating ongoing chronic GVHD (15; 454). These successes collectively constitute a strong advocacy for the continued efforts in bringing adoptive immunotherapy into clinical practice, although several challenges remain.

There is, presently, a considerable body of evidence indicating a contribution of Treg in infections (2; 36; 212), during tumor progression (32; 94), or following allogeneic

transplantations (399). Other examples of Treg-related autoimmunity exist, like type I diabetes (T1D), systemic lupus erythematosus (SLE), and Sjögren's syndrome, yet the precise links of these disorders to Treg dysfunction are less clear. Thus, in all of these diseases there is a potential benefit of employing adoptive Treg therapy. In other autoimmune disorders, like multiple sclerosis (MS), parts of the available data point to increased resistance of the T_H17 to suppression rather than reduced function of the Treg per se, possibly resulting from an inflammatory environment (228). Related to this, it has been suggested that the high secretion by T_H17 of the cytokines IL-6 and TNF- α may have reinforced their inflammatory activities and protected them from inhibition (207).

The HIV-1-specific IL-10-secreting Treg described in Paper 1 constitute a population of potent regulatory T cells that could have potential use in alleviating hyper-activation of HIV-1-specific T effector cells. Notably, it has been suggested that chronic activation may result in exhaustion of the T cells, and that a measured reduction in the response could improve immune competence (64). Also, such a strategy might reduce the number of activated T cells expressing the HIV co-receptors (CCR5 or CXCR4), thereby limiting the availability of T cells susceptible to HIV infection and, thus, the propagation of the virus.

4.3 Targeting Treg as a means of improving immune function

Given the suppressive nature of Treg and their alleged involvement in the promotion of malignancies and chronic infection, there has been some interest in wielding this regulatory T-cell subset in favor of human health by pharmacological means. The feasibility of this approach was indicated in studies reporting inhibitory effects of traditional drugs on the number and activities of Treg. These include several cancer drugs, such as cyclophosphamide (139), gemcitabine (382), and fludarabine (43), as well as the NSAID indomethacin (443) and the selective Cox-2 inhibitor celecoxib (Paper 3, indirectly). In recent years, a number of reagents have been developed that specifically target Treg, most of which take advantage of surface proteins that are preferentially Treg-expressed. One example is the depletion of Treg *in vivo* by administration of anti-CD25 antibodies, which has been achieved in mice but remains to be tested in humans. Other Treg-depleting agents include chimeric fusion proteins that consist of anti-CD25 in one end and a recombinant toxic protein in the other, one example being LMB-2, which combines the single-chain Fv fragment of anti-CD25 and a 38 kDa fragment of *Pseudomonas* exotoxin A. LMB-2 has shown Treg-depleting properties in a phase I study among patients with CD25⁺ T-cell malignancies (310). Another fusion protein called denileukin diftitox combines recombinant human IL-2 with the enzymatically active domain of diphtheria toxin. Upon binding to the high-affinity IL-2 receptor (consisting of CD25, CD122,

and CD123) the toxin is internalized, leading to cell lysis. Diftitox has been tested in several phase I studies, of which some have indicated a benefit of depleting Treg in terms of improved tumor-specific immune responses (248; 315). Correspondingly, the depletion of Treg has been shown to improve vaccine-induced responses as well, and one emerging strategy for improving immune responses in cancer therapy constitutes the combined use of Treg-depletion and vaccination or other immunomodulatory therapy (98; 239; 267; 324). Some controversy persists, though, with respect to the robustness of the Treg depletion, and regarding the clinical value of the procedure (23; 283). Rapid reversal of the Treg levels after depletion has been observed in some studies, suggesting that repeated therapy may be required. Notably, however, up to 75 % of the Treg generated after the first treatment were treatment refractory (98; 309).

CTLA-4 is another putative target for therapies modulating Treg activity and function. It is constitutively expressed on Treg and has been suggested to mediate Treg suppression through binding to the CD28 ligands CD80 and CD86 expressed on APCs (317; 335). Thus, CTLA-4 prevents the interaction of CD80 and CD86 with T-cell expressed CD28, which is a prerequisite for potent T-cell activation. The important role of CTLA-4 in immune homeostasis has been demonstrated using CTLA-4-deficient mice, which suffer death at an early age following massive lymphoproliferation and multi-organ failure (394; 425). The use of anti-CTLA-4 therapy has been evaluated in humans, and two humanized anti-CTLA-4 blocking antibodies (ipilimumab and tremelimumab) have been tested in phase I/II trials (68; 336). Ipilimumab – which is the more extensively studied of these two – has demonstrated efficacy in several phase II trials, involving more than 300 patients with advanced melanoma (336). Between 10 and 15 per cent of the patients experienced complete or partial therapeutic responses. Furthermore, the study participants had a median survival of around 15 months, as opposed to a median range of 6 to 9 months without treatment. Unfortunately, there appeared to be a correlation between clinical efficaciousness and severe immune-related adverse events, which affected up to 40 % of the patients. However, although the immune-related adverse events may have been serious, they were manageable and generally reversible if treated promptly (336).

The hope is that, with the use of these new agents, pathogen-specific immunity and anti-tumor immune responses may be improved by the specific depletion or modulation of Treg.

4.4 PKA as a putative target for immunotherapy

Cyclic AMP serves a variety of functions and is expressed in every cell of the human body. In T cells, this second messenger is known to inhibit growth, differentiation, and proliferation (47). Precisely how these suppressive effects are exerted remains largely unknown, however. Treg express high levels of cAMP even in the resting state, whereas in

CD4⁺ effector T cells only negligible levels are found (50). This may be explained by the strongly reduced levels of the cAMP phosphodiesterase PDE3B in Treg (50; 133), which would otherwise keep the cAMP-levels in check. Thus, high levels of endogenous cAMP may be responsible for the anergic phenotype of Treg, as well as central to their suppressive actions. Interestingly, pharmacological interventions that seek to normalize the elevated cAMP levels or block cAMP function have been shown to prevent nTreg-mediated suppression. Examples include co-stimulation through CD28 – which recruit PDE4 (4) – and the selective antagonism of cAMP and its receptors (364).

The most prominent cAMP receptor in T cells, the cAMP-dependent kinase PKA, has been suggested by several studies to harbor immunoregulatory properties (341; 396). Moreover, if nothing else, the death at the embryonic stage of RI α ^{-/-} knockout mice suggests that PKA type I plays an important role in the developing embryo (14). Furthermore, the finding that RII α ^{-/-} knockout mice survive and display normal immune function (340) effectively dismisses type II PKA from the current discussion of potential targets for immunotherapy, as this isozyme appears not to be necessary for a well-functioning immune system. The involvement in immune regulation of PKA type I, on the other hand, has been corroborated by a number of studies: Using isoform-specific cAMP analogs, Skålhegg and colleagues were the first to identify PKA type I as the cAMP receptor regulating T-cell activation upon ligation of the TCR (358). In a follow-up study, they went on to demonstrate that PKA type I translocates and interacts with the TCR-CD3 complex (359). More recently, it was shown that PKA type I inhibits T-cell signaling through the activation of Csk, thereby inactivating the tyrosine kinase Lck and preventing the phosphorylation of CD3 ζ -chains, resulting in blockade of the TCR-proximal events that would normally lead to T-cell activation (409). Since then, several other studies have substantiated the initial findings, also demonstrating the involvement of the cAMP-degrading PDEs and the importance of PKA type I anchoring and targeting to lipid rafts for cAMP-mediated regulation of T-cell responses (3; 4; 244; 314; 322; 408; 462). Thus, substantial data advocates the pharmacological targeting of PKA type I for immunoregulatory purposes. In this context, the design of stable anchoring disruptor peptidomimetics for the displacement of PKA from lipid rafts (Paper 2) or isoform-specific cAMP analogs that block the activation of PKA type I may prove important steps in developing treatment modalities that specifically target the PKA type I-mediated inhibition of T-cell activation.

Very recently, a report by Becker and colleagues (35) suggested that upregulation of cAMP was responsible for mediating tolerance in response to ligation of HIV-1 gp120 to cell-surface expressed CD4 on CD4⁺CD25⁺ nTreg. They found that gp120-mediated activation of CD4 on Treg, but not Teff, resulted in activation of ACs and production of cAMP (35).

However, whether or not PKA type I was involved in this mechanism was not specifically assessed. Previously, Bopp and colleagues have identified intercellular transfer of cAMP through gap-junctions as a mechanism for Treg-mediated suppression (50; 51), which may also account for the tolerizing effects of gp120. Correspondingly, an upregulation of intracellular CTLA-4-levels upon ligation of gp120 may signify Treg activation (35). Thus, the selective activation of Treg with gp120 could constitute a new way of inducing immunological tolerance *in vivo*. Consequently, recombinant gp120 may represent a promising agent for *in vivo* immunomodulatory therapy, activating Treg in a cAMP-dependent manner. This observation further underlines the importance of cAMP as immune regulator.

4.5 Immunomodulatory roles of IL-10 and IL-10-producing Treg

Numerous studies in mice have demonstrated the important role of IL-10 in curtailing autoimmunity and its potentially detrimental effect of suppressing desirable immune responses. This duality of IL-10 indicates the need for tight regulation of its expression, but also points to its potential uses in immunotherapy (269). IL-10-deficient mice have been shown to succumb to what are normally sublethal doses of lipopolysaccharide (LPS) (38), and to develop lethal autoimmunity while failing to eliminate what would normally be self-contained bacterial and parasitic infections (136; 175). Moreover, mice lacking IL-10 typically experience exacerbated disease when exposed to murine models of autoimmunity, such as EAE, IBD, and rheumatoid arthritis (RA) (269). In contrast, overexpression of IL-10 results in powerful immune suppression, which may allow for tumor growth and the occurrence of lethal infections (269).

Recombinant IL-10 has been tested in several clinical trials over the last decade, in general being well tolerated yet inadequately controlling autoimmune symptoms – except for some trials, in which IL-10 was administered locally, for instance directly beneath psoriatic lesions of the skin (344). In Crohn's disease, administration of IL-10 through ingestion of genetically modified IL-10-secreting bacteria seems to have some potential (58; 76; 371). Moreover, ingested gelatin nanoparticles containing copies of the IL-10 gene were shown to accumulate in the large intestine and provide local expression of IL-10 and relief of inflammatory symptoms in a murine model of acute colitis (44). Conversely, antibodies to IL-10 or its receptor may be used to prevent or reverse IL-10-mediated immune suppression, or enhance immune responses against infection or upon vaccination. For example, blocking IL-10 while administering a DNA vaccine against lymphocytic choriomeningitis virus (LCMV) improved vaccine responses and resulted in subsequent protection against infection (61). Furthermore, IL-10-receptor blockade in persistent LCMV infection resulted in a rapid

resolution of the infection (114). A similar effect was found in bacterial infections, exemplified by infections with *Mycobacterium avian* (321; 356).

The overall effect of IL-10 is to shift an immune response towards a Th2-type humoral response, whereas treatment with anti-IL-10 will favor a Th1 response with cellular immunity (287). Also, the capacity of DCs to present tumor antigens is diminished by IL-10 (255). Thus, as many solid tumors secrete IL-10 themselves (264; 431), or induce the secretion of IL-10 by bystander cells or tumor-associated IL-10-producing Treg (94; 252), cellular immunity is impeded. Hence, therapeutic blockade of IL-10 through administration of monoclonal antibodies restores immune function and, when administered along with cytotoxic drugs and/or immunostimulatory agents (e.g., TLR ligands), may facilitate elimination of the tumor (269).

The IL-10-secreting suppressive cells described in Paper 1 are, admittedly, unlikely to make it to the bedside, mainly because of their scarcity in peripheral blood. However, based on recent advances in the procedures developed for adoptive Treg therapy, it is conceivable that future progress might enable the generation of highly specific suppressor-cell clones from one cell alone. Methodologies might also evolve to allow for generation of Treg with increased suppressive capacity, thus reducing the number of cells required for an efficacious adoptive transfer. In this respect, Treg suppressing through soluble mediators or capable of inducing bystander suppression may prove more potent than Treg relying on contact-dependent suppressive mechanisms. The HIV-1-specific IL-10-secreting T cells identified in Paper 1 were indeed rather potent in their suppression of the proliferative responses of Teff *in vitro*. Unfortunately, their suppressive mechanism of action could not be elucidated in the present work, but should be pursued in future studies. It seems likely that their site of action *in vivo* would be secondary lymphoid tissues rather than peripheral blood, enabling them to interact more closely with their activated target cells, and probably improving their numbers relative to the number of target cells. An assessment of the homing receptors expressed on the surface of the cells could serve to indicate their site of action. In addition, analyzing the release of soluble inflammatory or anti-inflammatory mediators such as cytokines and chemokines could reveal some of their functional properties. The recent advent of multiplexing techniques has allowed the concurrent analysis of up to several hundreds of different factors from one sample. Moreover, the genome-wide chip (microarray) analyses constitute a powerful strategy for assessing the transcriptional processes within a cell, simultaneously detecting mRNA transcripts from every gene expressed. Finally, the epigenetic status of the cell, as judged by the presence of various non-coding modifications of the chromatin structure or the DNA itself, indicate the segments of the genome that are transcriptionally active and may provide additional info on the properties of a cell. With the recent advances in molecular biology one cell can

indeed be enough – given robust expression of the molecule in question – although improved precision of the analysis generally correlates with higher cell numbers. Other procedures that should be attempted include the blockade of putative or known secreted factors, as well as the cloning and expansion of single cells to see if their suppressive tendencies are heritable, and if they could be reversed in any way – for instance by the application of polarizing conditions favoring other T-cell lineages, such as Th1, Th2, or Th17.

4.6 Current efforts in the development of new immunotherapies

Academic and corporate scientists worldwide are currently racing to develop new candidate immunotherapeutics, and various strategies are employed in targeting ‘anything that moves’ among numerous promising targets. It would be well beyond the scope of this Thesis to present all of these efforts comprehensively, though the following chapters will deal with some of the candidates that aim to regulate immunity by modulating cytokine responses. This is a field that has seen substantial progress over the last couple of years, and the strategies used for targeting cytokines in immune cells may also be used in targeting other regulatory mechanisms.

4.6.1 Putative new targets for small-molecular compounds

Small-molecular synthetic compounds represent the mainstay of conventional drug therapy. However, a large portion of the currently marketed drugs are based on compounds that were discovered empirically, for which we still lack a plausible mechanism of action. The design of small-molecule mimetics constitutes a more refined approach, employing state-of-the-art technologies to predict structures that will have a certain pharmacological action. The tyrosine-kinase inhibitor peptide (TKIP) is one example of a recently developed small-molecule mimetic that has been tested *in vivo*. TKIP is a SOCS-1 mimic designed to bind the autophosphorylation site of JAK-2, thereby inhibiting the phosphorylation and activation of STAT1 α as well as STAT3. This peptide has been shown to inhibit both constitutive and IL-6-induced STAT3 activation in several human prostate cancer cell lines, and may represent a promising therapeutic for STAT1/STAT3-dysregulated malignancies (120). In addition, the TKIP peptide was shown to protect against EAE in mice, indicating that it could also be developed into drugs targeting neurodegenerative autoimmune disorders such as multiple sclerosis (271).

JAK-3 may be another target for a new line of immunotherapeutic drugs. It is expressed only in hematopoietic cells, and inducibly so in T cells, B cells, and myeloid cells, providing an interesting drug target for inflammatory diseases (245; 299). Loss-of-function mutations in the *JAK-3* gene results in immunodeficiency, albeit without the pleiotropic effects seen with current

immunosuppressive treatments like cyclosporine A, tacrolimus, and steroids (291). Therefore, specific targeting of JAK-3 with a small-molecule inhibitor may alleviate autoimmune symptoms with a smaller degree of side effects than the traditional therapies. Notably, the CP-690,550 compound from Pfizer has already been tested in a phase 1 trial following promising results in the pre-clinical studies, and was shown to be safe in humans (404).

4.6.2 *Treatment with recombinant proteins or expression systems*

Cytokines pose an interesting target for immunotherapeutics due to their importance in regulating immune responses, and to the fact that a number of inflammatory diseases are caused by aberrant cytokine signaling (447). An important regulator of cytokine signaling is the SOCS family of proteins, which bind to intracellular moieties of cytokine receptors to block the initiation of STAT signaling, as outlined in Section 1.3.2 of the introduction. Normally, SOCS proteins are induced upon cytokine signaling to provide negative feedback, and this may be exploited therapeutically by overexpressing specific SOCS proteins. Thus, in a recent study, the development of rheumatoid arthritis in murine models was prevented by the overexpression of SOCS3 (352). Furthermore, the administration of SOCS3 fusion proteins was shown to be effective in curtailing inflammation in mice challenged with superantigen and other pathogen-derived inflammatory molecules (185). Conversely, the expression of dominant-negative SOCS proteins might be used to enhance cytokine signaling and improve anti-tumor or anti-viral immunity, though the feasibility of this approach has so far not been tested *in vivo* (447).

The use of monoclonal antibodies to block the effects of inflammatory cytokines has already achieved clinical approval in a number of inflammatory diseases (54; 205; 243; 372; 373). This strategy has proven effective in alleviating chronic inflammation in patients with Crohn's disease (250) and psoriasis (211), as well as rheumatoid arthritis (115). Specifically blocking the inflammatory actions of cytokines using antibodies directed against the cytokines themselves or against subunits of their receptors thus constitutes a promising and well-tested mode of anti-inflammatory therapy. Recently, an antibody against the common p40 subunit of IL-12 and IL-23 receptors was developed that has the potential to broadly alleviate inflammation caused by the two inflammatory cytokines IL-12 and IL-23. A phase III study is currently underway to test the clinical efficacy of the ABT-874 antibody (also known as briakinumab), after successful phase I and phase II studies (198; 236).

4.6.3 *RNA silencing*

Several studies have used siRNA to reduce the levels of SOCS proteins in different systems, demonstrating a great diversity in the possible applications for such a therapeutic

strategy. One promising application would be in DC-based tumor vaccines, as treatment of DCs with *Socs1* siRNA was shown to significantly improve the ability to break tolerance and induce anti-tumor immunity, primarily as a result of improved antigen presentation (116; 348). Furthermore, systemic administration of *Socs1* siRNA using single-walled nanotubes as carriers to target phagocytic APCs could slow the growth of established tumors in a mouse model with peripherally injected B16 tumor cells (437). Interestingly, this approach could also be used to enhance the efficiency of DNA- or DC-based HIV vaccines in mice, as assessed by *in vitro* HIV-specific immune responses following vaccination (367). Moreover, antisense RNA therapy targeting *Socs-1* and *Socs-3* as a means of reducing SOCS expression and improve cytokine responsiveness in obese diabetic mice improved insulin sensitivity and ameliorated hepatic steatosis and hypertriglyceridemia, which are hall-marks of diabetic or pre-diabetic patients suffering metabolic syndrome (401).

5. Conclusions

In the present Thesis, cellular as well as molecular regulatory mechanisms were evaluated in the interest of paving the way for new immunomodulatory therapies.

1. A subset of IL-10-secreting HIV-1-specific T cells was identified that was capable of inhibiting T-cell proliferative responses *in vitro*. Based on these data, we find it likely that HIV-1-specific T cells contribute to the immune deficiency observed in progressive HIV infection and AIDS. Akin to Tr1 regulatory T cells, these cells did not express the canonical transcription factor FOXP3.
2. RI-specific anchoring disruptor peptidomimetics of improved stability were successfully designed, and should be able to withstand the proteolytic enzymes of serum to an extent that allows their use in live animals. For the first time, the implications of anchored PKA type I in the regulation of T-cell responses may now be studied through pharmacological intervention *in vivo*.
3. Through site-specific substitutions in the RIAD sequence we have extended our understanding of the interaction between the regulatory subunit dimer of PKA type I and its AKAP ligand, and identified residues that may be substituted without compromising the affinity or the specificity of the peptide for RI.
4. We observed a possible immunoregulatory effect of the selective Cox-2 inhibitor celecoxib in chronically infected HIV-positive individuals: Patients treated with celecoxib for 12 weeks experienced a reduction in the levels of T-cell expressed CD38 and PD-1, which represent markers of immune hyperactivation and exhaustion that have prognostic value in HIV.
5. Through the depletion of CD25-expressing PBMC we showed that a higher proportion of the CD25+ cells appeared to produce effector T-cell cytokines (IFN- γ and TNF- α) after celecoxib treatment, indicating diminished influence of Treg upon treatment with celecoxib. This points to a possible effect of Cox-2 inhibitors on Treg.

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