Analysis of Fcγ receptor cross-linking and anti-CD4-specific monoclonal antibody function

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Erklärung

Hiermit erkläre ich, dass ich die Dissertation „Analysis of Fcγ receptor cross-linking and anti-CD4-specific monoclonal antibody function“ selbständig angefertigt und keine anderen als die von mir angegebenen Hilfsmittel und Quellen benutzt habe. Die vorliegende Arbeit wurde im Zeitraum von November 2008 bis Mai 2013 am Twincore, Zentrum für Experimentelle und Klinische Infektionsforschung, in Hannover unter der Anleitung von Herrn Prof. Dr. Ulrich Kalinke angefertigt. Ich erkläre, dass diese Dissertation weder in gleicher noch in anderer Form in einem anderen Prüfungsverfahren vorgelegt wurde. Außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden habe ich früher keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Hannover, 13.06.2013

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Stephanie Vogel

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Index

Abbreviations and acronyms

°C	degree celsius
µ	micro
7-AAD	7-aminoactinomycin
Ab	antibody
ADCC	antibody dependent cell mediated cytotoxicity
ANKRD34A	ankyrin repeat domain-containing protein 34A
ANOVA	analysis of variance
AP	activation protein
APC	antigen presenting cell
APC	allophycocyanin
ATAC	chemokine-related cytokine
ATP	adenosine-5'-triphosphate
Bcl-6	B cell lymphoma 6 protein
BCR	B cell receptor
BD	Becton, Dickinson
BFA	Brefeldin A
BSA	bovine serum albumin
C	constant
Ca	calcium
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
cDNA	complementary DNA
CDR	complementary determing region
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHX	cycloheximide
CLR	c-type lectin receptor
CRAC	Ca^{2+} release-activated Ca^{2+}
CRS	cytokine release syndrome
CSV	comma-separated values
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen 4; CD154
Cy	cyanin
d	day
DAG	diacylglycerol
DAI	DNA-dependent activator of IFN regulatory factors
DC	dendritic cell
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
e.g.	*exempli gratia*
EDTA	ethylenediaminetetraacetic acid
EGR	early growth response protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase
et al.	*et alii*

F	phenylalanine
Fab	fragment antigen-binding
FACS	fluorescence activated cell sorting
FasL	fas-ligand
Fc	fragment, crystallizable
FcR	Fc receptor
FcRn	neonatal FcR
FCS	fetal calf serum
FDA	Food and Drug Administration
Fig.	figure
FITC	fluorescein isothiocyanate
Foxp3	forkhead box p3
FSC	forward scatter
g	gram
Gads	growth factor receptor-bound protein 2-related adapter protein 2
GATA-3	GATA binding protein 3
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
h	hours
h	human
HACA	human anti-chimeric antibody
HCA	hierarchical cluster analysis
HEY2	hairy/enhancer-of-split related with YRPW motif protein 2
HLA	human leucocyte antigen
HR	high responder
HRP	horseradish peroxidase
HSA	human serum albumin
i.e.	*id est*
i.v.	intravenously
IC50	half maximal inhibitory concentration
ICAM	intercellular adhesion molecule
ICOS	inducible T cell co-stimulator
ICS	intracellular cytokine staining
IFI16	IFN-γ-inducible protein 16
IFN	interferon
Ig	immunglobulin
IL	interleukin
im	immature
IP_3	inositol trisphosphate
IP_3R	Ca^{2+}-permeable ion channel receptor
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
Itk	IL-2-induced tyrosin kinase
iTreg cell	inducible regulatory T cell
l	liter
LAT	linker for the activation of T cells

Lck	lymphocyte-specific protein tyrosine kinase
LFA1	lymphocyte function-associated antigen 1
LN	lymph node
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
LR	low responder
Lrrfip 1	leucin-rich repeat interacting protein-1
LSIRF	lymphocyte specific IRF
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
m	milli
M	molar
m	murin
mAb	monoclonal antibody
MAC	membrane attack complex
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompabitility complex
min	minute
MLR	mixed leukocyte reaction
mRNA	messenger RNA
MS	multiple sclerosis
n	nano
NA	neutrophil specific antigen
NaCl	sodium chloride
NaN_3	sodium azide
Nck	non-catalytic region of tyrosine kinase adaptor protein
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κ-light-chain-enhancer of activated B-cells
NK cell	natural killer cell
NOD	nucleotide binding oligomerization domains
nTreg cell	natural regulatory T cell
PAMP	pathogen associated molecular pattern
PASI	psoriasis area and severity index
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed-death
PD-L1	programmed death-ligand 1
PE	phycoerythrin
PerCP	peridinin-chlorophyll-proteine complex
PFA	paraformaldehyde
Pip	PU.1-interaction partner
PIP_2	phosphatidylinositol 4,5-bisphosphate (Itk)
PK	protein kinase
PKCθ	protein kinase C θ
PLC-γ	phospholipase C-γ
PMA	phorbol-12-myristate-13-acetate

PMN	polymorphonuclear leukocytes
Pol III	polymerase III
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PROC	protein C
PRR	pathogen recognition receptor
RA	rheumatoid arthritis
RAS	rat sarcoma
RBC	red blood cell
RIG-I	retinoic acid inducible gene I
RNA	ribonucleic acid
ROR	retinoic orphan receptor
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute (cell culture medium)
RT	room temperature
s.c.	*sub cutaneous*
SCM	single cysteine motif
SEM	standard error of mean
SF	synovial fluid
SH2	Src homology 2
SLP-76	SH2 domain–containing leukocyte phosphoprotein of 76 kDa
SNP	single nucleotide polymorphism
Src	sarcoma
SSC	side scatter
STING	stimulator of IFN genes
Tab.	table
T-bet	T-box transcription factor
TCR	T cell receptor
Tfh cell	follicular T helper cell
TGF	transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TMC	tonsillar mononuclear cell
Tmem cell	memory T cell
TNF	tumor necrosis factor
Tr1 cell	regulatory type 1 cell
TRAIL	TNF-related apoptosis inducing ligand
Treg cell	regulatory T cell
tRNA	transfer RNA
U	unit
V	variable region
V	valine
V	variable
vs	versus
XCL	chemokine (C motif) ligand
xg	x gravity
Zap-70	ζ-chain-associated protein kinase 70

1. Abstract

1.1. Abstract

Autoimmune diseases occur in up to 3–5% of the general population by now. Currently the standard treatment involves long-term administration with e.g. steroids, azathioprine and cyclosporine. This therapeutic approach can cause severe adverse effects e.g. development of opportunistic infections or organ toxicity. Therefore the need for more specific and long lasting therapeutics arises. The treatment of autoimmunity with monoclonal antibodies (mAb) promise to be an alternative approach, because of the unique target specificity. CD4-targeted therapy has the potential to modulate peripheral T cell tolerance, which often is dysregulated in autoimmune diseases. Amongst CD4-specific mAb, some induce T cells depletion, others down-modulation of CD4 upon specific T cell binding. Here we studied *in vitro* the mechanism of CD4 down-modulation on T cells mediated by BT-061, a CD4-specific humanized IgG1κ mAb that is currently in clinical trials. BT-061 is suspected to activate regulatory T cells (Treg cells) upon binding of a specific epitope of CD4. Therefore the influence of BT-061 treatment on T cell activation and differentiation was investigated. Interestingly, BT-061 needed to be cross-linked to induce CD4 down-modulation of T cells. This cross-linking was mediated within PBMC by the fragment, crystallizable γ receptor (FcγR) CD64 expressed by monocytes. Secreted factors were not crucial to down-modulate CD4. However, monocytes pre-treated with serum showed significant abrogation of BT-061 mediated CD4 down-modulation. The experiments revealed that upon BT-061 treatment of T cells no cytokine responses such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α or interleukin (IL)-2 were induced. Furthermore, T cells showed no up-regulation of typical activation markers upon administration of BT-061, whereas a down-modulation of CD3, CD25, CD28 and CD69 was observed. Additionally the obtained data revealed that BT-061 possessed neither proliferative nor anti-proliferative effects on T cell proliferation under the tested conditions. The pharmaceutical potential of these mechanisms needs to be further explored in clinical trials.

Keywords: Immunology, T cells, monoclonal antibodies

1.2. Zusammenfassung

Autoimmunerkrankungen treten heutzutage in bis zu 3–5% der Gesamtbevölkerung auf. Derzeit besteht die Standardbehandlung aus einer Langzeittherapie mit z.B. Steroiden, Azathioprin oder Zyklosporin. Da diese Therapie schwere Nebenwirkungen verursachen kann, besteht ein Bedarf an spezifischeren Therapeutika und Medikamenten, mit einer länger andauernden Wirksamkeit. Die Behandlung von Autoimmunerkrankungen mit monoklonalen Antikörpern (mAk) verspricht durch die einzigartige Zielspezifität dieser Medikamentenklasse eine Alternative darzustellen. Therapieansätze, die sich gegen das Oberflächenmolekül CD4 richten, besitzen das Potential, auf die periphere T-Zell-Toleranz Einfluss zu nehmen. Dieser Teil des Immunsystems ist im Rahmen von Autoimmunerkrankungen häufig dysreguliert. Unter der Vielzahl CD4-spezifischer mAk induzieren einige die Depletion von T-Zellen, andere die Herunterregulation von CD4 nach deren Bindung an T-Zellen. Im Rahmen dieser Arbeit wurde der CD4-bindene humanisierte mAk BT-061 untersucht. Der Antikörper wurde als Immmunglobulin (Ig) der Subklasse IgG1κ exprimiert und befindet sich derzeit in der klinischen Erprobung. Es ist beschrieben, dass BT-061 in der Lage ist durch die Bindung an ein spezifisches Epitop von CD4, regulatorische T-Zellen zu aktivieren. In *in-vitro*-Studien wurde der Mechanismus analysiert, der einer BT-061 vermittelten CD4-Herunterregulation auf T-Zellen zu Grunde liegt. Des Weiteren wurde dessen Einfluss auf die Aktivierung von T-Zellen und deren Differenzierung untersucht. Die Versuche ergaben, dass eine Kreuzvernetzung von BT-061 notwendig ist, um eine Herunterregulation von CD4 auf T-Zellen zu induzieren. Diese Kreuzvernetzung von BT-061 wurde bei Versuchen mit PBMC durch den von Monozyten exprimierten Fcγ-Rezeptor (FcγR) CD64 mediiert. Allerdings setzt die Vorbehandlung von Monozyten mit Serum deutlich den Effekt einer BT-061-vermittelten CD4 Herunterregulation herab. Die Untersuchungen ergaben, dass in Folge einer Behandlung von T-Zellen mit BT-061 keine Zytokine wie Interferon (IFN)-γ, Tumornekrosefaktor (TNF)-α oder Interleukin (IL)-2 ausgeschüttet wurden. Weiterhin konnte keine Aufregulation von T-Zell-typischen Aktivierungsmarkern festgestellt werden, wohingegen eine Herunterregulation von CD2, CD25, CD28 und CD69 beobachtet wurde. Die Versuche zeigten, dass BT-061 unter den getesteten Bedingungen weder die Proliferation von T-Zellen induziert noch die Proliferation von T-Zellen supprimiert. Die pharmazeutische Wirkung dieser Mechanismen wird zur Zeit im Rahmen von klinischen Studien untersucht.

Schlagwörter: Immunologie, T-Zellen, monoklonale Antikörper

2. Introduction

2.1. The role of T cells in the immune system

The human immune system is a highly complex system that evolved over millions of years. It defends the host against potentially harmful toxins and infectious agents, and consists of a multifaceted network of soluble factors and cells. These components belong either to the innate or the adaptive part of the immune system [1, 2]. A hallmark of the immune system is its ability to discriminate between self-molecules of the host organism and foreign substances, so-called antigens, which need to be eliminated. Because of the virtually unlimited diversity of pathogens, the immune system requires mechanisms to specifically respond to individual pathogens.

2.1.1. Adaptive and innate immunity

The immune system is composed of the non-specific, immediately reacting innate immune system, along with the specific adaptive immune system that may confer long-lasting protection. The innate immunity depends on a limited number of germline-encoded molecules, which comprise an invariant repertoire of soluble proteins (e.g. the complement proteins, cytokines), receptors and cellular components. The cellular compartment is composed of the dendritic cells (DC), granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages and natural killer (NK) cells. These cell types eliminate pathogens or infected host cells by phagocytosis or lysis, therefore they need mechanisms to recognize pathogenic structures. These cells express pathogen recognition receptors (PRR), which recognize molecular patterns shared by many microbes and toxins [3]. These structures are called pathogen associated molecular patterns (PAMP) [4]. PRR include Toll-like receptors (TLR) [5, 6], retinoic acid inducible gene I (RIG-I)-like receptors [7], c-type lectin receptors (CLR) [8], nucleotide-binding oligomerization domains (NOD)-like receptors [7, 9, 10] and at least seven intracellular deoxyribonucleic acid (DNA) sensors. To date the DNA-dependent activator of interferon (IFN) regulatory factors (DAI) [11], ribonucleic acid (RNA) polymerase III (Pol III) [12, 13], leucin-rich repeat interacting protein-1 (Lrrfip 1), DExD/H box helicases DHX9 and DHX36, stimulator of IFN genes (STING) [14, 15], and IFN-γ-inducible protein 16 (IFI16) [16] have been identified. The recognition of PAMP by cells of the innate immune

system induces variable signal pathways. After activation cells can up-regulate co-stimulatory receptors, secrete cytokines or increase major histocompatibility complex (MHC) surface expression [17-19]. Furthermore, innate immune recognition is critical for activation of the adaptive immune system. Specific cells of the innate immune system are able to degrade pathogenic structures into peptide fragments, process and present these to cells of the adaptive immune system. Those cells are named antigen presenting cells (APC) and they display antigens in the context of MHC to cells of the adaptive immune system.

In contrast to the innate component, the adaptive immune system possesses specificity for its target antigens and may develop an immune memory against the pathogens encountered. The enormous repertoire of antigen-specific receptors is based on somatic recombination of gene fragments. This mechanism allows the generation of specific binding regions directed against nearly every antigen-specificity conceivable, including specificity for self-antigens. To avoid cell or tissue damage due to self-reactive lymphocytes two mechanisms to ensure self-tolerance are known. On mechanism is named central tolerance by clonal deletion. This way to prevent self-reactivity acts during lymphocyte development, which takes place in the thymus and in the bone marrow. In these compartments self-reactive T cells and B cells are deleted when exposed to self-antigens. The other mechanism is known as peripheral tolerance, which acts by deletion and inactivation of self-reactive lymphocytes. In contrast to central tolerance the peripheral tolerance affects mature lymphocytes after they have left the primary lymphoid organs. Peripheral tolerance could be mediated by suppression of auto-reactive cells by other immune cells or due to the induction of anergy. Lymphocytes become anergic when they encounter an antigen – independently whether the antigen is pathogen- or self-originated - in the absence of co-stimulatory signals.

The adaptive immune system can be divided into a cellular and a humoral part. The cellular part consists of the T and B lymphocytes. Whereas B cells recognize intact antigens, T cells recognize antigens that are presented in the context of a MHC-complex [20, 21]. B cells as well as T cells express hyper variable surface receptors – the B cell receptor (BCR) and the T cells receptor (TCR). Ligation of these receptors with antigens induces immune responses [1].

All immune cells originate from a haematopoetic progenitor. These haematopoetic progenitor cells differentiate either to myeloid or lymphoid progenitor cells in the bone

marrow, with the latter giving rise to B and T cells [22]. The T cell progenitors migrate from the bone marrow into the thymus, where the cells further differentiate and rearrange their specific TCR. Finally, after positive and negative selection, the T cell migrates into the secondary lymphatic organs [23].

2.1.2. T cell subsets

The majority of T cells express a TCR composed of a αβ-heterodimer. These cells are predominant in the lymphoid organs and respond to peptide antigens. A minor population of T cells bears a TCR that consists of a γ and a δ chain able to recognize lipid antigens. The cells are mainly located in the mucosal and epithelial barrier. TCR are associated with the glycoprotein cluster of differentiation (CD)3, which is expressed by all T cell subsets. Additionally to CD3, T cells express another co-receptor on the surface. This co-receptor is also a cell surface marker that distinguishes the main T cell subsets – $CD4^+$ and $CD8^+$ T cells – and determines the corresponding T cell interacting MHC type. These two T cell subsets reveal different functions and can be further subdivided according to their localization, cytokine production and functional properties. In general, an antigenic peptide bound to MHC class I molecules activates T cells expressing the co-receptor CD8 – also named cytotoxic T lymphocyes (CTL). CTL have the ability to kill infected or tumor cells. The co-receptor CD4 on T helper cells (Th cells) normally recognizes extracellular antigens, which are presented after endocytosis on MHC class II. The name Th cells derives from the cells ability to modulate activation and differentiation of other cell types such as B cells via production of specific cytokines. Aside from CD4 or CD8, T cells express additional co-receptors. The expression patterns of the co-receptors to some extent indicate the associated T cell subset, but the released cytokines are the determining factor. The first classification of effector $CD4^+$ T cells was thought to be limited to Th1 and Th2 cells [24] based on the selective production of the two cytokines, IFN-γ and interleukin (IL)-4. However, it became clear, that T cells possess a plasticity and a degree of flexibility in lineage commitment during their lifespan [25], which complicates the definite classification. Th1 cells are also identified by the expression of the transcription factor T-cell-specific T-box transcription factor (T-bet) [26-28] and are able to defend the host against intracellular pathogens, such as bacteria and viruses. The main transcription factor of Th2 cells is named

GATA binding protein 3 (GATA-3) and further studies demonstrate that these cells secrete IL-5 and IL-13, thereby protecting against extracellular pathogens such as nematodes, and contributing to allergic responses [29-31]. More recently the Th17 cells were discovered, and though these cells are mainly expressing IL-17, additional data revealed production of other cytokines including tumor necrosis factor (TNF)-α, IL-9, IL-10, IL-21, IL-22 and, in humans, IL-26. Those cells are defined through expression of the transcription factor retinoic orphan receptor (ROR)γt and RORα, are known to protect against extracellular bacteria and fungi, and promote pro-inflammatory processes *in vivo* as well as *in vitro* [30-33]. However, more recent studies indicate that in the human system RORC2 is a master switch that initiates a wide range of phenotypic and functional programming during Th17 cell differentiation [34].
Other putative lineages were discovered in the last years including Th9 and Th22, which produce their namesake cytokines IL-9 and IL-22, respectively. Th9 cells seem to be involved in airway hypersensitivity reactions and immune responses against helminthes [35, 36], whereas it is still not clear whether these Th9 cells represent a distinct lineage, because it was reported that human memory $CD4^+$ cells can switch to IL-9-secreting cells in response to transforming growth factor (TGF)-β [37, 38]. The IL-22 producing subset is part of the immunity at mucosal surfaces such as the skin [39-41]. Recently another T cell subset was described named follicular T helper cells (Tfh cells). This T cell subset is defined by the expression of the transcriptional factor B cell lymphoma 6 protein (Bcl-6). Tfh possess specialized function in helping B cells with the organization of germinal centers and thereby direct the humoral immune response [42-46]. The regulatory type 1 cells (Tr1 cells) express T-bet much like Th1 cells, but express IL-10 instead of IFN-γ and are therefore associated with immune-modulatory functions [47, 48]. Another $CD4^+$ T cell lineage with immune modulatory function is named regulatory T cells (Treg cells). These cells constitutively express the surface marker CD25 and the transcriptional factor forkhead box p3 (Foxp3), whereas these markers are up-regulated on and in activated human T cells as well [49, 50]. Consequently, CD25 as well as Foxp3 do not serve as exclusive markers for Treg cells in the human system, whereas in the murine system they are used to phenotypically characterize Treg cells [51-55]. Treg cells possess the ability to regulate self-tolerance and prevent autoimmunity, by suppressing adaptive T cell responses [30, 31, 56, 57]. Most of the Treg cells mature within the thymus and are referred to as natural Treg (nTreg cells) cells [50], but

their differentiation is also inducible in the periphery, which gives rise to the name inducible Treg (iTreg cells) cells. iTreg as well as nTreg cells secrete TGF-β, IL-10, and IL-25 [58].

2.1.3. T cell signaling

Naïve T cells generally lack characteristics, which enable a categorization in different T cell subsets despite from the surface expression of CD4 and CD8. In order to develop features restricted to a certain T cell subtype, such as the expression of surface markers, transcriptional factors, or cytokines characteristic for a selected T cell subset, T cells need to be activated. The T cell activation is a multistep process that generally is initiated by the interaction of the TCR with a peptide-MHC-complex. APC take up pathogens in the periphery, process and present the pathogens, and then migrate to the draining lymph node. Upon pathogen engulfment the APC starts to mature, which includes the induction of MHC class II expression and of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86). The APC presents the processed antigen to T cells within the lymph node T cell zone, and antigen-specific T cells recognize their respective antigen in the context of the MHC-complex. Nevertheless, additional signals are necessary to induce T cells activation [59-61]. The second signal is the binding of one of several co-stimulatory receptors, which is critical for the induction of T cell proliferation and effector differentiation. The constitutively expressed surface molecule CD28 is thought to be the physiologically most relevant co-stimulatory receptor on T cells [62]. It binds to B7-1 and B7-2, which are displayed on APC. The third signal is provided by cytokines. The interplay of the signals initiates specific but integrated signaling cascades of particular strength and duration. Upon TCR binding to the corresponding peptide-MHC-complex, elements of immuno-receptor tyrosine-based activation motifs (ITAM) are phosphorylated by the tyrosin kinases lymphocyte-specific protein tyrosine kinase (Lck) and v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (Lyn) [63]. Each ITAM element contains two phosphorylation sites located in the four polypeptides of the TCR complex (CD3γ, CD3δ, CD3ε and TCRζ). But TCR engagement does not necessarily result in the phosphorylation of all possible ten ITAM [64], thereby enabling precise adjustment of signaling. The CD45 receptor displayed on the T cell surface modulates phosphorylation and activation of Lck and Lyn. The ζ-chain-associated protein kinase 70 (ZAP-70) is recruited to the phosphorylated CD3-ζ-chain, thereby inducing its

activation. This event changes the TCR, which possesses no intrinsic enzymatic function, into an active protein tyrosin kinase. After this modulation the TCR is able to phosphorylate other substrates that then induce variable down-stream signals. Activated ZAP-70 supports the phosphorylation of transmembrane adapter protein linker for the activation of T cells (LAT) and the cytosolic adapter protein Src homology 2 (SH2) domain–containing leukocyte phosphoprotein of 76 kDa (SLP-76) [65, 66]. This event leads to the recruitment of Vav1 and other adaptor proteins like non-catalytic region of tyrosine kinase adaptor protein (Nck) and growth factor receptor-bound protein 2 -related adapter protein 2 (Gads), and the IL-2-induced tyrosin kinase (Itk) [67]. Itk phosphorylates the phospholipase Cγ1 (PLCγ1), which in turn results in the hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and the second messenger diacylglycerol (DAG) [63]. DAG activates the protein kinase C θ (PKCθ) and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) signaling pathway; both events induce activation of the transcription factor nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB). The IP_3 generated by PLCγ1 activity stimulates calcium $(Ca)^{2+}$-permeable ion channel receptors (IP_3R) that are located in the endoplasmic reticulum (ER) membrane. This event enables the release of stored ER Ca^{2+} into the cytoplasm. The second messenger Ca^{+2} triggers a sustained influx of extracellular Ca^{2+} through the activation of plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Ca^{2+}-bound calmodulin activates the phosphatase calcineurin, which in turn induces the transcription factor that mediates IL-2 production: nuclear factor of activated T cells (NFAT) (Figure 2.1).

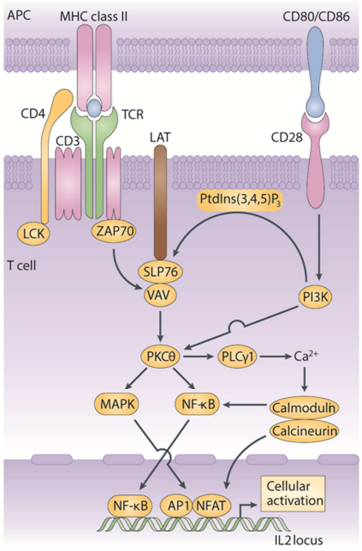

Figure 2.1: Signal transduction pathways involved in T cell activation (adapted from [68]. The first signal is provided by the engagement of the TCR. The second signal is induced by the triggering of co-stimulatory receptors (such as the binding of CD28 to B7). The first and the second signal mediate different signaling pathways that result in the activation of multiple transcription factors. The binding of the TCR to its cognate peptide presented by MHC triggers the recruitment of signaling molecules. Amongst other events PLCγ1 is recruited, which affects Ca^{2+} influx, which furthermore activates NFAT. Additionally PKCθ is activated, which regulates NFκB and AP-1 pathways, respectively. Within the nucleus, NFAT acts together with AP1 and other transcription factors to induce gene expression that mediates IL-2 production.

These events activate the T cells and induce production of IL-2, which affects a positive feedback loop to enhance T cell proliferation [69, 70]. As mentioned above, a second signal is accomplished by the binding of the co-stimulatory molecules on T cells CD28 to B7-1 or B7-2 on the APC. Upon interaction of CD28 with B7, a specific signal is transmitted that results in an enhanced production of NFκB, which induces a 3-fold increase of IL-2 mRNA expression (Figure 2.1). To regulate T cell activity T cells additionally express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is very similar to CD28 and also binds to B7 displayed on APC, though with a higher affinity. Interestingly, CTLA-4 contains an immune-receptor tyrosine-based inhibition motif (ITIM), which leads to the induction of inhibiting signals upon binding [71]. However, data obtained in the last decades revealed that TCR signaling is not a linear process. It rather appears to be a complex network of feedback and feedforward regulations at each step.

2.2. Fc receptors as a link between humoral and cellular immunity

In the human system, several different types of receptors have been identified that interact with the fragment crystallizable (Fc) of antibodies such as Fc receptors (FcR). These receptors bind the Fc fragment of an immunglobulin (Ig) and are classified based on the type of antibody they recognize. Upon binding the Fc fragment of an antibody to its epitope, a signal is transmitted into the FcR expressing cell. This signal triggers inhibitory or activating pathways and mediates effector functions like induction of phagocytosis, production of inflammatory cytokines, chemokine release, antibody dependent cell mediated cytotoxicity (ADCC) or modulates antibody production. Consequently, FcR play a key role in linking humoral and cell-mediated immunity.

2.2.1. The family of FcγR and their structural properties

The natural ligand of FcR is the Fc portion of Ig. In humans five major Ig subclasses are known: IgM, IgD, IgG, IgA and IgE, and specifically bound by the corresponding FcR , FcμR, FcδR, FcγR, FcαR and FcεR [72]. The subfamily of FcγR belongs to the Ig-superfamily and is further divided into FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16).

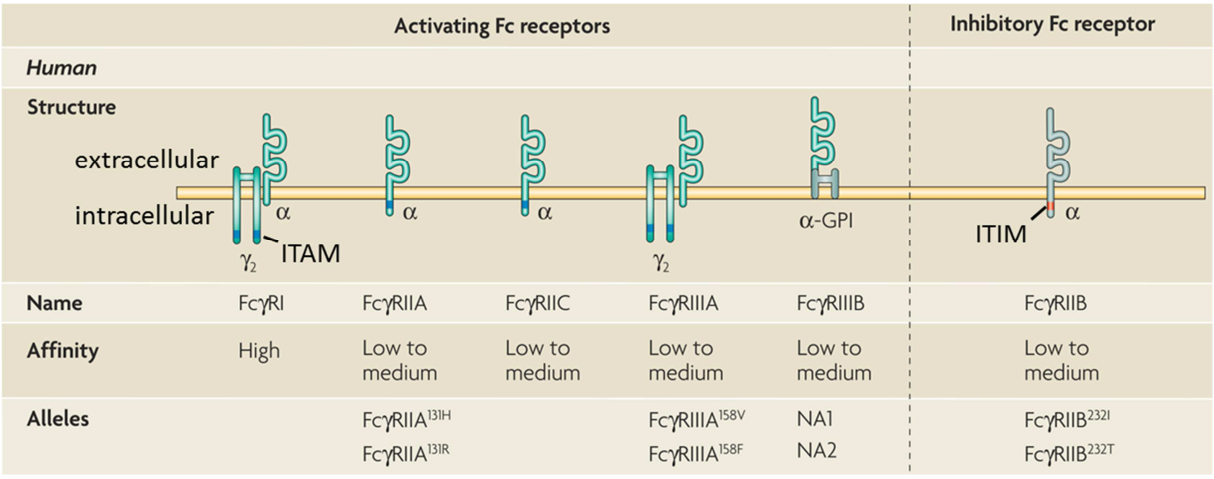

Figure 2.2: Schematic depiction of human FcγR (adapted from [73]). The human FcγR are divided in three classes, with the classes differing in terms of binding affinity for the variable Fc fragments and their structure. The ligand binding is provided by an α-chain. In case of CD32 (A, B and C), the signal is transduced via the a-chain as well. The FcγRI and III possess g-chains to transmit the signal into the cell. The activating FcγR contain an ITAM in the signal transducing domain and the inhibitory FcγR CD32B instead an ITIM domain. The FcγR CD16B is connected with cell surface via a glycosylphosphatidylinositol (GPI)-anchor. The most important allelic variants for CD32A, CD16A/B and CD32B are also indicated in the figure.

Each receptor has distinct structural and functional properties. In the human system CD64 represents the high-affinity receptor, while the other molecules possess low to medium affinity for their ligand. The group of FcγR is additionally separated based on their signaling properties. In the human system one inhibitory receptor, FcγRIIB, is described, which contains an ITIM in its cytoplasmic domain; the other FcγR transduce an activating signal into the cell [74]. Generally, activating FcγR are composed of an Ig-binding α-chain and a signal-transmitting homodimeric γ−chain, which carries an ITAM. FcγRIIA and FcγRIIC are the exception because the transmembranic α-chain already includes the ITAM and no additional γ-chain is needed. In the human system neutrophils display a specific variant of CD16 on their surface, which is characterized by the linkage to a glycosylphosphatidylinositol GPI-anchor (Figure 2.2) [73]. Upon binding of FcR expressing cells to an antibody:antigen-complex, antigen specificity is provided to a cell type that would normally lack antigen recognition structures. This mode of action enables this receptor type to link humoral and cell-mediated immunity [75].

2.2.2. Expression patterns and polymorphism of FcγR

The groups of activating and inhibitory receptors function in concert. Several of them are co-expressed on the cell surface. Therefore, signaling pathways of single FcγR are often simultaneously triggered thus shaping the multitude of different effector responses mounted by different cell subsets [76]. FcγR are broadly expressed on cells of the hematopoetic system. The high affinity receptor CD64 is constitutively expressed on granulocytes, macrophages, monocytes and DC [77], with expression levels in the latter being highly dependent on the DC subtype and its stimulation status [78]. CD32A is displayed on almost all myeloid cells and platelets, whereas its inhibitory counterpart CD32B is found on phagocytes, DC and B cells. CD32C is exclusively expressed on NK cells [79, 80]. CD16A is expressed on monocytes, macrophages, DC, Langerhans cells, NK cells, while CD16B is constitutively expressed on neutrophils, and can be induced on eosinophils [81, 82]. Since Lanier *et al.* described the function and properties of $CD16^+$ T cells for the first time in 1985 [83], the expression of FcγR on T cells is part of an ongoing controversial discussion [73]. A more recent study suggested that a minor population of T cells expresses CD16 [84], whereas in the review of Toshiyuki Takai no expression of any FcγR on T cells was

summarized [80]. Notably, the expression profile of the various different FcγR may vary depending on immune cells, depending on where the cells derive from and which activation status they show. In addition to the variable expression patterns of the different FcγR, the multitude of allelic variants enhances the diversity of this receptor family. The most frequently found polymorphic forms of FcγR show modifications in the extracellular domains, which contain the ligand binding sites and thereby affecting the affinity for binding of IgG. Thus, different human FcγR alleles exist which may show a variety of different functions. The frequency and distribution of these allelic FcγR variants differs among ethnic groups [85]. One of the first functional genetic polymorphisms identified in the class of FcγR was the allelic variant for the low-affinity receptor FcγRIIa at the amino acid sequence position 131. The point mutation results in an amino acid substitution from arginine to histidine. This modification is located in the extracellular region and directly affects the Fc binding to the respective antibody. The variant with an arginine at position 131 is also known as high responder (HR) and shows low affinity for all IgG subclasses, particularly IgG2. Its counterpart, which is also named low responder (LR), has a histidine at the corresponding site and possesses a higher affinity to IgG2 and IgG3 [86] (Figure 2.2). Interestingly, the terms HR and LR arise from the capacity of T cells co-cultured with monocytes, which express either the HR or the LR allelic variant, to proliferate upon stimulation with a murine IgG1 anti-CD3 monoclonal antibody (mAb) [87]. FcγRIIIB is characterized by a GPI-anchor and is exclusively expressed on neutrophils. This FcγR exists in three allelic forms. These variants are known as neutrophil specific antigen (NA)1, NA2 (Figure 2.2) and SH. The NA1 and NA2 polymorphism is composed of four non-synonymous and one synonymous mutation within the membrane distal Ig-like domain. The four unequal amino acids influence the N-linked glycosylation of the receptor, which also affects the affinity for IgG subclasses. The NA1 allotype exhibits a stronger binding affinity and phagocytosis potential of IgG1- and IgG3-immune complexes than the NA2 allotype [88]. The substitution of alanine at position 78 to aspartic acid determines the SH allele, whereas the consequence of the single nucleotide polymorphism to antibody binding is unknown [89]. The amino acid variations at position 158 of FcγRIIIa directly regulate NK cell activity. The single amino acid substitution from a valine (V) into a phenylalanine (F) results in a stronger affinity of the V158 allotype for IgG1 and IgG3 compared to F158, and furthermore enables IgG4 binding [90, 91].

2.2.3. Antibody-FcγR-interactions

Most effector functions triggered by mAb, such as induction of phagocytosis, production of inflammatory cytokines, chemokine release and ADCC, are mediated by the interaction of the Fc part of the mAb with FcγR expressed by immune cells [92], [93]. To understand the interaction between the FcγR and its ligands, the structural properties need to be considered in more detail. As the structure of FcγR is closely related, a description of the low-affinity receptors serves as model. The molecule consists of two extracellular Ig like domains (D1 and D2) that are connected via a hinge region. In contrast to the low affinity receptor, CD64 contains an additional Ig-like domain (D3), which has been suggested to be responsible for its strong binding-affinity [94]. The antibody's Fc fragment, which represents the ligand for the FcγR, is a homodimer and consists of the CH2 and CH3 domains. These domains form a horse-shoe-like structure with sugar moieties linked to the hinge proximal amino acid asparagine at position 297. This glycosylated residue is located in a region known to potentially interact with the corresponding FcγR. Therefore, it is suggested that the sugar moiety is critical for FcγR recognition [95]. Interestingly, in spite of the impact of the oligosaccharide on the interaction between the FcγR and Ig, it is known that more than 30 different variations of oligosaccharides can be found in human serum [73]. Even though the sequence of the various FcγR possesses similarities, the molecular structure differs, causing the variable binding affinities of the receptors to their respective Ig. In the human system, CD64 binds the Fc fragment with high affinity and is additionally able to interact with monomeric IgG [73, 96, 97]. Studies of Bruhns *et al.* indicate that the low affinity receptor CD16A has the capacity to interact with the constant region of an antibody but on a lower level than CD64 [97]. CD64 strongly interacts with the subclass IgG1, to a lesser extent with IgG3 and IgG4 and shows nearly no affinity for IgG2. Amongst the FcγR the low affinity receptor CD32 is also able to bind to the complexed version of IgG4. The Ig subclasses IgG1 and IgG3 are potential interaction partners for all described FcγR, although they differ in the level of binding affinity. In contrast to this, IgG2 exclusively interacts with CD32A and to a lesser extend with CD32B [80, 97]. Finally the binding affinity of the antibodies Fc fragment to the receptor and the interplay of the FcγR expressed on one immune cell orchestrates the resulting effector function.

FcγR furthermore play a crucial role in controlling immune responses upon interaction with immune complexes. The triggering of the ITAM in the receptor and the following activation

cascade results in cell activation, which potentially induces phagocytosis, ADCC, superoxide generation, production and release of cytokines. In contrast, the stimulation of the ITIM in the transmembrane domain of CD32B mediates inhibition of ITAM induced cell activation [97]. Therefore, CD32B on B cells is also known as the tolerance checkpoint for humoral immunity due to its capacity to down-modulate B cell activity [98, 99].

2.3. MAb in clinical use

An antibody (Ab) is a large roughly Y-shaped protein of approximately 150 kDa. All Abs are summarized as Ig, whereas they can differ regarding their specificity, structure, biological activity and distribution in the body. Ab are generally produced by B cells and in the immune system they are responsible for identifying and neutralizing toxins and pathogens. The Ab recognizes a unique part of a foreign target, called an antigen. In contrast to polyclonal Abs, mAb are monospecific and therefore possess specificity for a single epitope of an antigen.

2.3.1. Structure, types and mode of action of immunglobulins

Antibodies are glycoproteins and belong to the Ig superfamily. Ig molecules comprise a pair of identical heavy and light chains linked by covalent disulphide bonds [100]. Ig are divided into two regions: The fragment antigen binding (Fab), which contains the antigen specific variable regions, and the Fc fragment, which mediates effector functions [101]. The epitope specific variable (V) domain at the N terminus of the heavy chain (V_H domain) and three constant (C) domains, the C_H1 domain, C_H2 domain and C_H3 domain, build up the heavy chains of a soluble IgG. The light chains also contain a V domain (V_L domain) and a single C domain (C_L domain). Thus, the V_H domain of the heavy chain as well as the V_L domain of the light chain contributes to the antigen-binding side of an Ig. The highly specific antigen-binding side contains six loops, known as complementarity-determining regions (CDR), with three loops being contributed by the V_H and V_L domains, each (Figure 2.3). The light chains in the mammalian system reveal two variants, a kappa (κ) or a lambda (ι) chain, whereas only one type is represented in one Ig molecule. In contrast, five variants are known for the heavy chains in mammals that also determine the Ig class. The types are denoted by the Greek letters: α, δ, ε, γ, and μ, resulting in the classes IgA, IgD, IgE, IgG and IgM [102]. Generally a B cells expresses IgM and IgD in the form of a BCR. Upon activation the B cell responds by

secreting antibodies. IgM and IgD are simultaneously displayed on the B cell surface, whereas the secretion of IgD is not as common as the one of IgM. IgM and IgA are predominantly represented in a monomeric structure, but in serum they exist as a pentamer. IgG is the most abundant type of Ig in human serum and provides the majority of antibody-based immunity against invading pathogens. In humans 4 different subclasses of IgG are known: IgG1, IgG2, IgG3 and IgG4. Ig molecules belong to the group of glycoproteins because they possess an N-linked sugar moiety linked to the conserved asparagine residue at position 297 within the Fc portion. The Fc part of an Ig is responsible for the development of an appropriate immune response towards the respective antigen by binding to specific receptors, and or other immune molecules such as complement proteins.

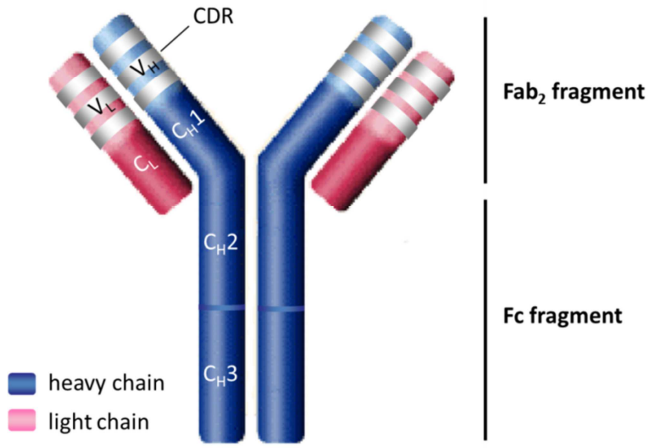

Figure 2.3: Schematic depiction of an antibody (adapted from [102]). An antibody consists of two equal heavy chains and two equal light chains. Each Fab fragment is made out of a V_L, V_H, C_L and C_H1 domain, with the V parts including the antigen binding CDR. The constant Fc fragment contains two C_H2 and two C_H3 domains.

By binding to an antigen and initiating effector functions via the Fc fragment, antibodies are the connection between the cellular and the humoral part of the immune system. IgG and IgA antibodies can neutralize bacterial toxins by sterically inhibiting a toxin from binding its respective receptor on the target cell. This is accomplished by the antibody interacting with the toxin's receptor binding domain. Because Ig need to easily pass through extracellular fluid in order to neutralize toxins, IgG is the common Ig type for toxins within the tissue and IgA for toxins at the mucosal side. Viruses act in a similar way as toxins, by binding to the cell surface receptors. Therefore similar types of antibodies are able to prevent either. IgA as well as IgG could interact with the virus, thus preventing the binding to the cell surface or fusion of the virus membrane with the cell. The previously described neutralizing effects are

the simplest way of antibodies to respond to pathogens; in addition, Ig possess the ability to mediate effector functions via their Fc portion upon binding to a pathogen. One option is the activation of the complement system via the classical pathway to eliminate pathogens, also known as complement-dependent cytotoxicity (CDC). Upon turning on the complement cascade, a series of proteolytic cleavage reactions starts. Within the cascade inactive enzymes are cleaved and turned into active forms, finally leading to the attachment of components to the pathogen surface that mediates its destruction. The mechanism of enhancing phagocytosis by coating the surface of the pathogen is known as opsonization. As most antibodies do not neutralize the pathogen itself, they require the help of effector cells to defend the host. Therefore, the Ig are able to interact via their Fc portion with cells that possess the ability to destroy pathogens. This receptor type is described in detail in chapter 2.2. FcR initiate neutralization by triggering macrophage, DC and neutrophil activation. On the contrary non-phagocytic cells, such as NK cells, eosinophils, basophils and mast cells are induced to secrete mediators, e.g. cytokines or granzymes, upon ligation of their FcR. When an antibodies´ Fc fragment is bound to the FcR of NK cells which then mediates the destruction of the coated target this is called antibody dependent cell mediated cytotoxicity (ADCC) [101, 102]. The mentioned characteristics of antibodies and the knowledge that injecting an antigen into a mammal induces the production of specific antibodies, initiated the concept of using this molecule class in different scientific approaches. The serum isolated from the treated animal contains polyclonal antibodies specific for many epitopes of the same antigen. A further step in the development of the manufacturing process of mAb was essential for the establishment of a commercial use: the hybridoma technology invented by Cesar Milstein and Georges J. F. Köhler in 1975 [103]. This method allows the production of antibodies specific for a single epitope of an antigen, so-called mAb. Hybridomas are immortalized cells, which are generated by the fusion of a cancer cell line with antibody-secreting lymphocytes, from an antigen challenged animal. Paul Ehrlich already realized the potential therapeutic properties of antibodies in 1891. In his manuscript named “Experimentelle Untersuchungen über Immunität. I. Über Ricin” on the immunization of animals with ricin he reasoned that if a compound could be engineered to selectively target a disease causing organism, then a toxin for that organism could be delivered to the organism by the selective compound [104].

2.3.2. Therapeutic mAb

The application of mAb used as pharmacotherapeutics started in 1986 when the fully murine CD3-specific mAb OKT3 (also known as Muromab) was licensed for the prevention of acute organ rejection [105-108]. Since that time, the field of monoclonal antibody drugs has flourished. At the end of 2010, a total of 30 of these types of drugs (meaning 25 mAb and five Fc fusion proteins) were in Phase 2/3 or Phase 3 clinical studies, whereas 9 mAb were in phase 3 studies as treatments for immunological indications [109].

Sponsoring company	International non-proprietary name	Target and type	Indication of Phase 3 study
Millennium/Takeda	Vedolizumab	α4 β7 integrin; IgG1	Moderate-to-severe Crohn disease; ulcerative colitis
Tolerx	Otelixizumab	CD3; IgG1	Type 1 diabetes mellitus
Macrogenics/Eli Lilly	Teplizumab*	CD3; IgG1	Type 1 diabetes mellitus
Biocon/CIMAB SA	T1h	CD6; humanized IgG1	Psoriasis
Immunomedics/UCB	Epratuzumab	CD22; IgG1	Systemic lupus erythematosus
Cephalon	Reslizumab	IL-5; IgG4	Eosinophilic esophagitis
Regeneron	REGN88	IL-6R; human IgG1	Ankylosing spondylitis, rheumatoid arthritis
Abbott	Briakinumab**	IL-12/23; IgG1	Plaque psoriasis
Novartis	AIN-457	IL-17A; human IgG1	Uveitis

Figure 2.4: mAb in phase 3 studies as treatments for immunological indications (adapted from [109]). Information current as of September 1, 2010. *Note added in proof: In a press release issued in October 2010, Lilly announced that the primary endpoint in the PROTÉGÉ study (NCT00385697) had not been met and enrollment and dosing in the PROTÉGÉ and PROTÉGÉ Encore (NCT00920582) studies were suspended. **Note added in proof: In a press release issued in October 2010, Abbott announced that marketing applications for Briakinumab were filed in the US and Europe during the third quarter of 2010.

Data from 2009 revealed that global sales exceeded US $1 billion [110]. The properties of mAb offer new therapy strategies for the treatment of cancer, autoimmunity and inflammatory disease. To use mAb as a new therapeutic approach, the development of techniques to manufacture these molecules was required. For example, the invention of the hybridoma technology by Cesar Milstein and Georges J. F. Köhler in 1975 allowed the production of mAb that were used as therapeutic drugs. However, the manufacturing of these drugs in non-human mammals resulted in the development of non-human proteins designed for the application in humans. The administration of non-human mAb caused the occurrence of a new class of side effects in the recipients. Upon application, the patients developed immune responses against the non-human amino acid sequences of the drugs.

The repeated administration of these compounds leads to the induction of severe side effects mediated by the immune system, like allergic reactions or hypersensitivity, partially caused by human anti-chimeric antibodies (HACA) [111-113]. Additionally the weak interactions of mouse antibodies with human complement and FcγRs proved to be problematic when the anticipated efficacy is dependent on these interactions. Furthermore, the murine Fc fragment does not bind to the human neonatal FcR (FcRn) [114] which is responsible for an antibody-half-life of typically less than 20 hours [115, 116]. The FcRn is not related to the previously described types of FcR. The receptor binds a different region of the antibodies´ Fc fragment and is structurally related to the family of MHC class I molecules. The option to produce chimeric antibodies decreased the emergence of immune-mediated toxicities and improved effector functions and mAb stability in the patient. The technique was published in 1984 and is based on the principle that the antigen-binding variable domains of a murine mAb are fused to the constant domains of a human mAb [117, 118]. This method however resulted in a molecule still containing non-human fragments with the potential to provoke inadvertent immune responses. The development of a protocol to create humanized antibodies led to a significant reduction of side-effects. The CD52 targeting drug Campath-1 was the first reshaped mAb by humanization [119]. The simplest method to humanize a mAb is by grafting the antigen-binding loops, also called (CDRs), from a murine mAb into a human IgG [119-121]. The structural differences between murine, chimeric, humanized and human mAb are shown in (Figure 2.5).

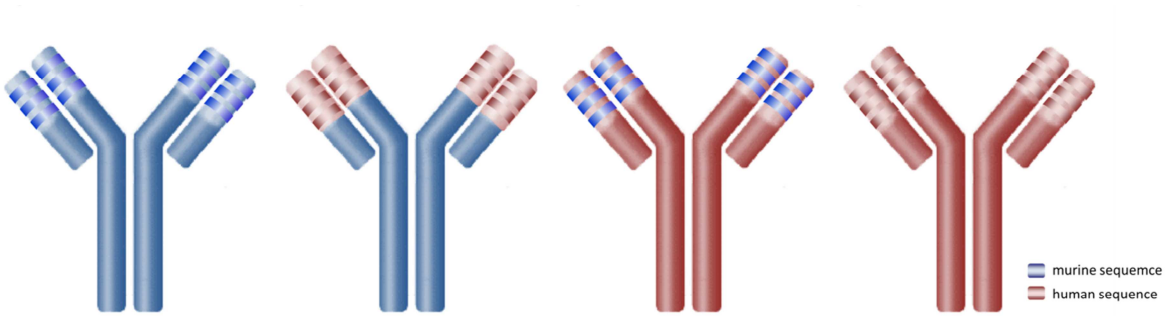

Figure 2.5: Structure of a murine, a chimeric, a humanized and a human monoclonal antibody. A murine mAb is completely based on a murine sequence, whereas a chimeric mAb consists of human V domains fused to murine C domains. A humanized mAb is composed of murine CDRs integrated in a human IgG. The human mAb is fully derived from a human sequence.

By now most of the therapeutic mAb entering clinical trials are completely human [122]. In general these drugs are produced by phage-display technology [123] or transgenic mice expressing human immunoglobulin genes [124]. Furthermore, the generation of human antibodies using ribosome-, mRNA- and yeast-display libraries [123] or patient- derived human hybridomas [125, 126], or the cloning of antibody-cDNA from single lymphocytes selected on an antigen [127, 128] are used but not common. Aside from the method to generate the mAb, the choice of the anticipated effector-function profile of a mAb is important. Five mechanisms for the function of mAb have been described to date. These mechanisms need to be considered in the phase of developing the mAb because they are determined by the Fab as well as the Fc fragment. One mechanism is the blockage of a specific target by a mAb, which requires no mediation of effector functions via the Fc part. Therefore, instead of full length Ig, antibody-like molecules might also prove to be effective. MAb sharing blocking properties bind to a specific molecule but do not change the molecule itself. Consequently, the drug can prevent ligands from activating their cognate receptors by interacting with the ligand or the cognate receptor. The previously described mechanisms to block receptor-ligand-interactions are clearly not dependent on the constant regions of a mAb, as the molecule exclusively binds to the target and sterically inhibits the interaction. In the following examples of down modulation and signaling-induction this precise classification is not feasible, because drugs are known to exhibit their function merely with Fc mediated interactions that others do not depend on. Internalization or down-regulation offers another option to interfere with ligand-receptor-interactions by limiting cell surface receptors that can be activated by the ligand. Some mAb need to be taken up by the target cell upon binding to the antigen, therefore internalization is an additional mechanism of mAb to develop their effector function. The direct arming by covalent linkage of mAb to toxins or radionuclides causes selective killing of the antigen displaying cell upon mAb binding and internalization of the cytotoxin. So far this approach is exclusively used in cancer therapy [129, 130]. Therapeutic antibodies can also induce activating signals that influence the activation status and the differentiation of the cell. Generally the mAb mimics the ligand and triggers a signal; nevertheless, the strength of the signal and the outcome can differ to the natural ligand. An agonistic drug is capable of binding to a cell surface receptor causing a response similar to the endogenous receptor binding partner. Except from the previously described covalent linkage of mAb to toxins, the utilization of effector functions mediated by

the Fc part of a mAb offers an alternative way to deplete the target cell. In this case designed mAb take advantage of the effector functions also mediated by natural serum Ig, with efficacy varying by IgG subgroup. Upon binding of the mAb to its respective antigen-bearing cell or other immune cells, the membrane attack complex of the complement system is attracted to destroy or engulf the labeled target. The CD20 targeting chimeric IgG1 mAb induces the depletion of mature B lymphocytes and not Ab-producing plasma cells, due to the expression profile of the target. The efficacy of the drug is partially due to intrinsic cytotoxic activity but is for the most part mediated by the Fc induced ADCC [131] and to a lesser extent CDC [132, 133] (Figure 2.6).

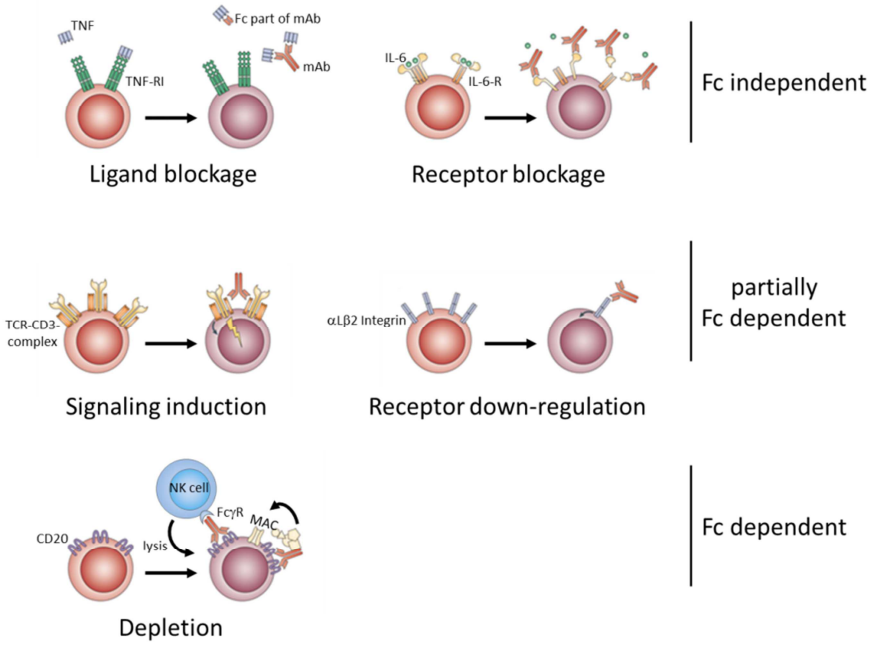

Figure 2.6: Therapeutic mAb mediate their function via different mechanisms (adapted from [112]). The mode of action of mAb is separated in five main mechanisms, whereas they differ concerning the dependence on Fc mediated interaction. Mab, which act by the blockage of the ligand or the receptor, do not depend on the binding of the Fc fragment. For example the fusion protein Etanercept neutralizes TNF activity by binding soluble and transmembrane TNF, and inhibiting binding to the TNF receptor [134]. In contrast, Tocilizumab is able to inhibit ligand-receptor interaction by binding to the IL-6 receptor, which prevents the interaction with soluble IL-6[135, 136]. The signal induced by receptor down-modulating mAb, are partially dependent on the interaction of the Fc fragment with the FcγR. The murine mAb OKT3 was designed to specifically target CD3. Upon binding to the target the TCR is cross-linked and transient cytokine release is induced. The mAb Efalizumab specifically binds to αL integrin (also known as CD11a and lymphocyte function-associated antigen 1 (LFA1)) and was originally designed for the treatment of autoimmune diseases. On the one hand this drug blocks the interactions between αL integrin and intercellular adhesion molecule 1 (iCAM1), but on the other hand Efalizumab down-regulates αL integrin expression by T cells as well as the expression of other T cell co-stimulatory molecules. The

decrease of αL integrin surface expression together with other molecules on T cells causes disrupted T cell homing by interfering with the αL integrin–iCAM1 interactions and disturbing T cell activation by missing co-stimulatory molecules [137]. Rituximab is a CD20 targeting mAb, which functions by depleting the target-bearing cell [138]. The Fc fragment of Rituximab mediates the depletion by ADCC or the formation of a membrane attack complex, which induces CDC [133, 139].

The Fc fragment of a mAb determines whether the molecule interacts with FcγR or the complement system and furthermore regulates the kind and strength of that interplay. Therefore the choice of the mAb isotype and with that its constant domain determines the induced effector functions and the *in vivo* half-life [140-142]. For the design of therapeutic mAb the Ig isotypes IgG1, IgG2 and IgG4 are used exclusively, since these isotypes bind the FcRn. On the contrary to these isotypes IgG3 shows only low affinity for that receptor. These facts result in a half-life of IgG1, IgG2 and IgG4 of about 20 days in humans, whereas IgG3 is entirely degraded after 6–8 days [93, 97, 142]. For therapeutic mAb where effector functions such as ADCC and CDC are undesired IgG4 and IgG2 are the isotypes of choice, because they bind weakly to FcγR or components of the complement system. IgG2 has very poor complement fixation activity and IgG4 does not fix complement at all [73, 97, 142]. For the FcγR initiating ADCC as well as phagocytosis IgG4 possesses low affinity and IgG2 shows the lowest affinity [143-145]. Although IgG4 mAb seem to induce no effector function induced by the Fc fragment, they show an instability in the hinge region that results in the production of half-antibodies (10–30% of the total), which was already described for Natalizumab. The instability in the hinge region enables the exchange of their Fab arms with endogenous IgG4 *in vivo*, whereas the consequences of this phenomenon for the patient are not clear [146]. The subtype IgG1 is generally chosen when neutralization of the target cells by phagocytosis, ADCC or CDC is desired, for example in the case of cancer therapy. IgG3 also shows a high affinity for FcγR initiating these effector functions or the complement system, but due to its short half-life, this subtype is not suitable [143-145].

2.4. CD4 targeting therapy of autoimmune diseases

The human immune system is a highly complex system that furthermore needs to be flexible to protect the host against the sheer unlimited diversity of constantly evolving pathogens. Consequently, the cells of the immune system need mechanisms to identify various

pathogens, but in addition they need to discriminate between self-molecules of the host organism and foreign antigens. The immune system has evolved a large number of strategies to prevent the response to non-infectious antigens or self-molecules. But due to the complexity of the network of the immune system, responses may be induced that are directed against self and thereby cause autoimmunity.

2.4.1. Triggers for autoimmunity

A study in 1997 revealed that autoimmune diseases occur in 3–5 % of the general population [147]. The prevalence of autoimmune disease is remarkably high considering the fact that the immune systems evolved many mechanisms to prevent auto-reactivity. The development of autoimmune diseases is due to the host's genetics and environmental influences. The pathological auto-reactivity is generally initiated by the response to a single antigen. This self-peptide might be widely expressed throughout the host or only in one organ, and as the disease progresses, the response usually spreads to more antigens [148]. This effect is called epitope spreading and is defined as *de novo* activation of auto-reactive T cells by self-antigens that have been released after T cell or B cell mediated bystander tissue damage [68]. The genetic setup of the host can affect its susceptibility to autoimmunity by variable mechanisms. One option is that the genes affecting the overall reactivity of the immune system for example by polymorphisms in genes that plays key roles in the regulation of the immune system. Studies revealed that a polymorphism in CTLA-4 [149, 150], a protein which down-regulates T cell activation, or in IL-2 [151] which mediates growth, proliferation, and differentiation of T cells, is associated with the development of autoimmune diseases. Genes might also be involved in the onset of autoimmunity by affecting antigen presentation or recognition. It is known that different MHC class II alleles vary in their ability to present peptides to auto-reactive $CD4^+$ T cells. Therefore, it is possible that genes encoding for MHC class II are involved in autoimmune disease by preferring the presentation of peptides expressed by host tissue [148]. The expression products of genes could also be involved in protection of tissues from autoimmune damage. For example, the eye possesses mechanisms to block T cell infiltration by producing immunosuppressive cytokines like TGF-β [152]. Disrupting these mechanisms causes tissue damage by auto-reactive immune cells. In addition, autoimmunity can also be caused by environmental factors such as the adjuvantive effect of products from invading pathogens. Components of

microorganisms could stimulate the innate immune system like an adjuvant and thereby provide the second stimulus for auto-reactive T cells, which would normally not be activated due to the missing second trigger. Another environmental factor responsible for misarranged self-reactivity is molecular mimicry. This mechanism is based on the fact that an infiltrating pathogen codes for a peptide that is closely related to a peptide of the host. Studies also revealed that bacterial, viral or other parasitic infections indirectly induce auto-immune diseases by shifting the balance of Th1 and Th2 cells in the host. Infections with bacteria or viruses usually induce T cell differentiation into Th1 cells [153]. In contrast, helminthes infections direct the T cell differentiations towards Th2 cells [154]. The alteration in Th1/Th2 balance could abet autoimmunity. The previously described mechanisms demonstrate various possibilities for the onset of autoimmunity and auto-reactive T cells play a tremendous role in all of them by displaying a TCR specific for an endogenous peptide. They are responsible for the induction as well as the effector phase of the autoimmune response. In general auto-reactive T cells are eliminated within the thymus by a procedure called negative selection. However, some T cells escape this mechanism. These self-specific TCR expressing cells are usually not fully activated due to the weak affinity for endogenous peptides or missing co-stimulation. But for some reason, once these cells are activated they start to ”protect” the host against its own peptides. Interestingly, studies revealed that the presentation of some peptides are preferred compared to others. This might result from a favored antigen presentation of these peptides by specific MHC allelic variants. These polymorphic variants of the HLA gene are associated with the occurrence of autoimmune diseases. Specific haplotypes associated with autoimmune diseases such as type-I diabetes [155] and rheumatoid arthritis (RA) [156] were identified.

2.4.2. T cell directed therapeutic approaches for the treatment of autoimmune diseases

Different therapeutic approaches are employed that interfere with T cell function in order to inhibit or redirect self-reactive immune responses. The currently used agents appear to be more immune suppressive than tolerogenic. For example drugs like methotrexate and azathioprine, which interfere with DNA synthesis and thereby inhibit cell proliferation, are immunosuppressive and mediate an immune-suppressed status in the patient. Because

these conventional treatments bear high risks for the patients such as development of opportunistic infections or organ toxicity [157]. Therefore, the need for more specific and long lasting therapeutics arises. Therefore T cell-targeted therapy is a promising approach for the treatment of autoimmune diseases. The activation cascade of T cells shows that interfering with the development of auto-reactive T cells capable of damaging hosts´ tissue is a potential novel therapeutic approach. One mode of action is to disequilibrate the ternary complex consisting of the TCR-antigen-MHC-complex. This interaction relies not only on the affinity of the TCR to the antigen-MHC-complex, but also on the presence of various accessory molecules [158]. To disrupt the interaction of a TCR to its cognate MHC-peptide-complex, an antibody directed against CD3 -the signal transducing domain of the TCR- was designed. OKT3 (Muromab) is a murine IgG2a mAb that targets the epsilon subunit of CD3 and its primary application is in transplantation biology. The efficacy of OKT3 was originally accredited to a depletional mechanism of the mAb, but studies revealed that receptor modulation may play the bigger role in its immunosuppressive effect [159]. Besides CD3, CD4 also belongs to the class of co-receptors and is therefore a further suitable target. Because the co-receptor CD4 generally tightens the immunological synapse, CD4 specific mAb cause instability of the formed ternary complex, thereby disrupting T cell activation [158]. The mode of action and existing CD4 targeting mAb are discussed at a later point. The stimulation via co-stimulatory receptors expressed on T cells along with antigen recognition is crucial to induce T cell activation. The inhibition of T cell stimulation via targeting of co-stimulatory molecules is an alternative approach to promote anergic tolerance to self-peptides. The receptor CD28 is displayed on naive cells and triggered upon ligation with CD80 or CD86, which are expressed on APC. The direct targeting of CD28 via the mAb TGN1412 proved not successful due to strong side effects [160]. However the usage of blocking reagents to CD80 or CD86, like Belatacept or Abatacept, is sufficient to terminate the interaction of CD28 with the respective ligand. These drugs are fusion proteins combining the extracellular binding domain of CTLA-4 with the Fc portion of Ig. A further strategy to prevent tissue damage by auto-reactive T cells is the inhibition of leukocyte recruitment to sites of inflammation by targeting adhesion molecules such as CD2, LFA1 and LFA2. Within the past 20 years many drugs were designed to target either cytokines or their receptors. Cytokines, like IL-2, can trigger T-cell proliferation and differentiation, or induce the activation of other cells, thus enhancing the immune response. Especially blockage of

the effects of the proinflammatory cytokine TNF-α has been used by many mAb in the treatment of autoimmune diseases. Although the various TNF antagonists feature differences in molecular structure, it is suggested that their efficacy is caused by binding and subsequently neutralizing the soluble and transmembrane forms of TNF-α. The targeting of IL-1 and IL-6 has also been shown to be beneficial in the suppression of autoimmunity.

2.4.3. CD4 targeting mAb

As mentioned prior, many immune modulating mAb induce an overall suppression of the host. Therefore, a tolergenic state against self-peptides would be preferable in a therapeutic approach to minimize side effects. Since CD4 serves as co-stimulatory molecule on T cells, it significantly participates in T cell receptor-mediated stimulation. MAb against CD4 were the first to be found capable of inducing tolerance to protein antigens [161], although the mechanisms underlying tolerization by CD4 targeting mAb are not fully understood to date [162]. Furthermore Th cells, which are typically CD4 positive amongst CD3 positive T cells, coordinate immune responses. Consequently, CD4-targeted therapy holds the promise of being able to modulate the peripheral tolerance that is disturbed in autoimmune diseases. CD4-specific mAb could contribute to the recovery of peripheral tolerance by affecting multiple targets in self-directed T cell activation. The points of action by CD4-binding mAb are divided in three main checkpoints that influence T cell function: (1) The depletion of CD4 positive T cells, (2) the interaction with CD4 to reduce the function as a co-stimulatory molecule in T cell activation or (3) the triggering of an inhibitory signal to suppress self-reactive T cells. In therapy of autoimmune diseases non-depleting antibodies are preferred because they show a shorter period of non-specific immunosuppression compared with depleting antibodies [163] and it could also be demonstrated that cell depletion is not required for induction of tolerance [164]. The mode of action of non-depleting mAb has not been completely elucidated; however, it is known that several CD4-specific antibodies sterically inhibit CD4 interaction with MHC class II and thus reduce the overall avidity of the antigen-specific TCR with the corresponding MHC class II. Other CD4-targeting antibodies confer CD4 down modulation upon specific CD4 binding. In theory, the decrease of CD4 surface expression also reduces overall TCR MHC class II interaction. Nevertheless, recent data suggested that the CD4-specific antibodies conferring down modulation of CD4 induce

an agonistic signal that affects T cell function, causing Th cells to modulate instead of inducing immune responses. Thus Th cells stimulated with a CD4 down modulating mAb share properties of Treg cells. Since 1991, when the first CD4 specific murine IgG1 mAb BB14 was used to treat autoimmune diseases – [165], various different CD4 binding mAb have been developed that differ regarding the Ig subclass, the targeted CD4 epitope, the species they raised in (e.g. mouse and rat) or if it is chimeric, humanized or human [165]. Despite the first clinical trials being promising, to date no CD4 targeting mAb has been shown to be beneficial to the clinical outcome of autoimmune diseases like psoriasis or RA in further studies.

2.4.4. BT-061 – a new CD4-targeting mAb

In our studies we analyzed BT-061 (Tregalizumab), which is a new CD4-specific mAb. This mAb was designed for the treatment of autoimmune diseases such as psoriasis and RA. The molecule is derived from the CD4 targeting mAb hB-F5. BT-061 is a non-depleting IgG1 antibody, which was humanized by CDR grafting [166]. The drug is currently being tested in phase II trials and has been shown to confer beneficial effects on the symptoms of psoriasis and RA. Although upon BT-061 treatment T cells are not depleted, CD4 down-modulation of T cells was observed in the first clinical trials. It has been described that BT-061 is able to selectively activate Treg cells by binding to a specific epitope of CD4 [167]. BT‑061 is thought to be particularly safe, as the immune system remains functional against infection; however, the mode of action is not entirely clear [168].

Since it is assumed that the efficacy of BT-061 is dependent on CD4 down-modulation on T cells [167], initial work was focused on the establishment of an *in vitro* model that mirrors the circumstances observed in probants and enables the detection of CD4 down-modulation. This model was used to examine conditions that are critical for reduction of CD4 *in vitro*. Furthermore the results should help to elucidate the mechanism underlying CD4 down-modulation in patients. Since the mode of action of CD4-specific mAb is still unclear

3. Material and methods

3.1. Material

3.1.1. Human blood and human tonsils

The human whole blood was donated from colleagues of the Twincore, Hannover, Germany, and the buffy coats of healthy donors were provided by the blood donor center of the *Deutsches Rotes Kreuz* in Springe, Germany. The human tonsil specimens incurred in context of surgical intervention were used in compliance with regulations of the corresponding German responsible authorities. The sampling and the transport was organized by Sonja Kallendrusch and Prof. Ingo Bechmann from the institute of anatomy at the University of Leipzig, Germany.

3.1.2. Cell lines

The used fragment, crystallisable gamma receptor (FcγR) expressing cell lines were kindly provided by the group of Prof. Hengel. The cell lines are based on a T cell receptor (TCR) α, β and ζ negative BW5147 thymoma cell line. Upon transfection with pcDNA3.1 constructs encoding FcγR-ζ chimeras using Superfect (Qiagen GmbH, Germany) or AMAXA Nucleofection Kit V (Lonza Group, Germany) following manufacturer's instructions, the cells stably express a fusion receptor consisting of the extracellular domains of the different human FcγR linked to the transmembranic and intracellular domains of the murine TCR ζ-chain. The cloning of the complementary deoxyribonucleic acid (cDNA) encoding for the human FcγR cluster of differentiation (CD)16 (FcγRIIIA) (higher affinity variant with a valine in position 158), CD32A (FcγRIIA) (with histidine in position 131) and FcγRI have been described [169]. As a negative control the extracellular domain of human CD99 (mic domain) was fused to the intracellular domains of the murine TCR ζ-chain. The transfectants were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1 mM sodium pyruvate. To positively select the cells antibiotics were added to the culture. For CD99 expressing control cells 3 mg/ml Geneticin was used and for the selection of the FcγR expressing cells Zeocin was used in a

concentrations of 0.5 mg/ml [170]. The FcγR surface expression was determined by fluorescence activated cell sorting (FACS) analysis.

3.1.3. Antibodies, chemicals, cytokines, sera and hyperimmunglobulins

3.1.3.1 Antibodies

Table 3.1: List of antibodies used for FACS analysis or cell stimulation

Specificity	Isotype	Clone	conjugation	used amount	Manufacturer
BT-061	h IgG1	unspecified	unlabeled	indicated	Biotest AG
CD2	m IgG1, κ	RPA-2.10	FITC	1 µl	BioLegend
CD3	m IgG1, κ	UCHT1	APC	1 µl	BD Phamingen
CD3	m IgG1, κ	UCHT1	PerCP	1 µl	BioLegend
CD3	m IgG2b	OKT3	unlabeled	indicated	Janssen-Cilag
CD4	m IgG1, κ	RPA-T4	unlabeled	10 µg/ml	BD Phamingen
CD4	m IgG1, κ	SK3	FITC	1 µl	BioLegend
CD4	m IgG1, κ	SK3	PE	1 µl	BD Phamingen
CD8a	m IgG1, κ	RPA-T8	PE.Cy7	1 µl	BioLegend
CD14	m IgG2b, κ	MfP9.1	V450	1 µl	BD Phamingen
CD16	m IgG1, κ	3G8	PE	1 µl	BD Phamingen
CD19	m IgG1, κ	HIB19	PE	1 µl	BD Phamingen
CD25	m IgG1, κ	M-A251	V450	1 µl	BD Phamingen
CD28	m IgG1, κ	CD28.2	PE.Cy7	1 µl	BD Phamingen
CD32	m IgG2b, κ	FLI8.26 (2003)	FITC	1 µl	BD Phamingen
CD56	m IgG2a, κ	NCAM	APC	1 µl	ImmunoTools
CD56	m IgG2b, κ	NCAM16.2	FITC	1 µl	BD Phamingen
CD64	m IgG1, κ	10.1	FITC	3 µl	BD Phamingen
CD64 (LEAF)	m IgG1, κ	10.1	unlabeled	50 µg/ml	BioLegend
CD69	m IgG1, κ	FN50	PE.Cy7	1µl	BioLegend
CD86	m IgG2b, κ	IT2.2	PacificBlue	1 µl	BioLegend
CD99	m IgG1, κ	3B2/TA8	PE	1 µl	eBiosience
IFN-γ	m IgG1, κ	4S.B3	APC	10 µl (ICS)	BD Phamingen
IL-2	m IgG1, κ	5.344.111	PacificBlue	5 µl (ICS)	BD Phamingen
TNF-α	m IgG1, κ	MAb11	PE.Cy7	5 µl (ISC)	BD Phamingen
Iso IgG1	m IgG1, κ	MOPC-21	FITC	3 µl	BD Phamingen
Iso IgG1	m IgG1, κ	MOPC-21	PE	1 µl	BD Phamingen
Iso IgG2b	m IgG2b, κ	27-35	FITC	1 µl	BD Phamingen
CD15	m IgM, κ	HI98	FITC	1 µl	BD Phamingen
HLA-DR	m IgG2a, κ	L243	APC.Cy7	1 µl	BioLegend

APC=allophycocyanin; BD=Becton, Dickinson; Cy=Cyanine; h=human; FITC=fluorescein isothiocyanate; HLA=human leucocyte antigen; ICS=intracellular staining; IFN=interferon; IL=interleukin; m=murine; PE=phycoerythrin; PerCP=peridinin-chlorophyll-protein complex; TNF=tumor necrosis factor

3.1.3.2 Chemicals

Table 3.2: List of chemicals used for cell culture, buffers, cell stimulation or inhibition and RNA isolation

Reagent	Manufacturer
2-mercaptoethanol	Sigma
Ammoniumacetat	Merck
Brefeldin A (BFA)	BD Pharmingen
Cycloheximide (CHX)	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Merck
G418	Biochrom
Glutamax	Invitrogen
Ionomycin	Sigma
Lysophosphatidic acid (LPA)	Ambion
Natriumazid (NaN_3)	Merck
Paraformaldehyde (PFA)	Merck
Phosphoprotein phosphatase (PP1)	Biaffin
Phorbol-12-myristate-13-acetate (PMA)	Invitrogen
Zeocin	Invivogen

3.1.3.3 Cytokines

Table 3.3: List of cytokines used for cell differentiation and stimulation

Product	Manufacturer
Recombinant interferon (IFN)-γ	Peprotech
Recombinant interleukin (IL)-4	CellGenix
Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF)	CellGenix

3.1.3.4 Sera and hyperimmunglobulins

Table 3.4: List of sera and hyperimmunglobulines for cell handling and FACS analysis

Product	Manufacturer
AB serum, human	c.c.pro
FCS	Sigma
Polyglobin (Gamunex 10%)	Talecris Biotherapeutics

3.1.4. Buffers, media and solutions

3.1.4.1 Ready-to-use buffers, media and solutions

Table 3.5: List of buffers, media and solutions used for cell handling and FACS analysis

Product	**Manufacturer**
0.4% Trypan blue solution	Gibco
1 x Phosphate buffered saline (PBS)	Gibco
10 x Perm/Wash	BD Pharmingen
Auto MACS Pro Washing solution	Miltenyi Biotech
Biocoll	Biochrom
Cytofix/Cytoperm	BD Pharmingen
FACSclean	BD
FACSflow	BD
FACSrinse	BD
GMP Cell Grow DC	CellGenix
MACS Bovine serum albumin (BSA) Stock solution	Miltenyi Biotech
RPMI 1640 medium	Gibco
Sodium Pyruvate (100 mM)	Gibco
X-VIVO 15	BioWhittaker, Lonza

3.1.4.2 Selfmade buffers, media and solutions

All buffers, media and solutions were sterilized by autoclavation or filtration (0.45 µm) and stored at 4°C.

3.1.4.2.1 Buffers, media and solution used for cell culture

Culture medium for transfectants

FCS	10% (w/w)
Sodium pyruvat	1% (w/w)
Glutamax	1% (w/w)
RPMI	88% (w/w)

Freezing medium for transfectants

DMSO	10% (w/w)
RPMI	90% (w/w)

EDTA solution to detach cells

EDTA	1 mM
PBS	1 x
in H_2O	(pH 7.4)

3.1.4.2.2 Buffers, media and solutions for cell isolation

Dextran sedimentation buffer to isolate PML

Dextran (at least 100.000 MW)	6% (w/v)
NaCl	0.9% (w/v)
in H_2O	

NaCl solution to lyse RBC

NaCl	0.2 or 1.5% (v/w)
in H_2O	

MACS buffer for cell isolation using the AutoMACS

BSA	0.5% (w/v)
PBS	1 x
EDTA	2 mM (pH 8.0)
in H_2O	

3.1.4.2.3 Buffers, media and solutions used for FACS analysis

FACS buffer

BSA	2% (w/v)
PBS	1 x
EDTA	20 mM (pH 8.0)
NaN_3	0.03 % (w/v)
in H_2O	

PFA solution for cell fixation

PFA	1% (w/v)
PBS	1 x
in H_2O	

3.1.4.2.4 Buffers, media and solution for mAb coating

Coating buffer

Na_2HPO_4	0.1 M
in H_2O	pH 9.0

Blocking buffer

FCS	10% (v/v)
PBS	1 x
in H_2O	pH 9.0

3.1.5. Kits

Table 3.6: List of kits used cell enrichment by MACS, FACS analysis and micro array analysis

Product	Manufacturer
PKH-26 Red Fluorescent Cell Linker Kit	Sigma
Pan T cell Isolation Kit II, human	Miltenyi Biotech
CD14 MicroBeads, human	Miltenyi Biotech
CD19 MicroBeads, human	Miltenyi Biotech
NK cells Isolation Kit, human	Miltenyi Biotech
ELISA kit human IL-2	Biolegend
ELISA IL-2 murine	formerly BenderMed Systems
RNeasy Mini Kit	Qiagen
Human Genome whole 44k	Agilent
Low Input Quick Amp Labeling Kit	Agilent
RNA 6000 NanoChip Kit	Agilent
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit for 405 nm excitation	Invitrogen

3.1.6. Consumables (cell culture flasks, tubes etc.)

Table 3.7: List of consumables used for cell handling and cell preparation

Product	Manufacturer
10 ml pipettes	FALCON/BD
15 mL centrifugation tubes	FALCON/BD
24-well plates	FALCON/BD
25 ml pipettes	FALCON/BD
5 ml pipettes	FALCON/BD
50 mL centrifugation tubes	FALCON/BD
50 ml pipettes	FALCON/BD
96-well flat bottom plates	FALCON/BD
96-well flat bottom plates, Maxi Sorb	Nunc

Cryogenic storage tubes	Nunc
Disposable gloves	Manufix Sensitive, Braun
FACS tubes unsterile	FALCON/BD
Filter Cap Cell Culture Flask, T175	Nunc
Gentle MACS tubes	Miltenyi Biotech
Glas ware	Schott
Hemocytometer (Type Neubauer improved)	Roth
Pasteur pipettes, glas	VWR
Pipette tips	Eppendorf
Reagent Reservoirs	VWR
Sterile filter 0.45 µm	VWR
Sterile syringe filter	VWR
Syringes	Braun
Tubes (0.5 ml;1.5 ml and 2 ml)	Eppendorf

3.1.7. Software

Table 3.8: List of programs used to process data

Product	**Version**	**Manufacturer**
BD FACSDiva	6.1.2	BD
FCS Extractor	1.02	Earl F Glynn from the Stowers Institute for medical research
FlowJo	7.6.5	TreeStar
GeneSpring GX	11.0.2	Agilent
GraphPad Prism	5.02	GraphPad
Microsoft Office	2003	Microsoft
R	2.15.1	The R foundation for statistical computing

3.2. Methods

3.2.1. Cell biological methods

3.2.1.1 Cell culture handling

All procedures involving cells were performed in sterile conditions using a laminar flow cabinet (HeraSafe KS, Thermo Scientific). Cells were incubated in a CO_2-Incubator (CB210, Binder) at 37°C with 5% partial pressure of CO_2. To analyze cell morphology or to determine cell numbers microscopes were used (Axiostar plus, Axiovert 40C, Zeiss).

3.2.1.2 Isolation of PBMC from blood by density gradient centrifugation

To purify peripheral blood mononuclear cells (PBMC) from whole blood or buffy coats density gradient centrifugation using Ficoll was performed. The method is based on a protocol established by Ferrante and Thong, and Vissers *et al.* [171, 172]. Ficoll is a high-molecular-weight sucrose polymer (specific gravity 1.076 to 1.078 g/ml), that facilitates the required viscosity to the solution and allows rouleaux formation of erythrocytes. Due to the specific density of Ficoll the solution is denser than lymphocytes, monocytes, and platelets but less dense than granulocytes and erythrocytes at room temperature (RT). These differences concerning the density properties allow a separation of blood components in distinguishable phases following a centrifugation step. The upper phase contains platelets and plasma, whereas the lymphocytes and some platelets are located on the Ficoll layer as a white cloudy band. Granulocytes and erythrocytes form a cell pellet at the bottom of the tube [173].

3.2.1.2.1 Isolation of PBMC from whole blood

To obtain whole blood from healthy donors, blood was taken from the basilic vein and collected in heparinized tubes. The blood was diluted with an equal volume of room-temperatured 1x PBS and 25 ml of the blood/PBS-mixture was stratified in 50 ml tubes containing 15 ml of Ficoll. The samples were centrifuged at 900xg for 30 min at RT without brake to obtain the separation of blood components. To recover PBMC from the centrifuged

sample the white-cloudy fraction was transferred into a new 50 ml tube. To wash the cells the samples were filled up with magnetic-activated cell sorting (MACS) buffer at a final volume of 50 ml and centrifuged for 10 min at 600xg at 4°C. After the sedimentation the cell pellet was washed again with 50 ml MACS buffer. Upon washing steps cells of the same donor were pooled, resuspended in an appropriate amount of MACS buffer and the cell number was determined. From 50 ml of whole blood $8x10^7$ to $2x10^8$ PBMC were obtained.

3.2.1.2.2 Isolation of PBMC from buffy coats

The buffy coats of healthy donors were provided by the blood donor center of the *Deutsches Rotes Kreuz* in Springe, Germany. Buffy coats have a volume of 50 to 70 ml and contain the concentrated lymphocytes from a 500 ml whole blood donation. The whole content of a buffy coat was transferred to a T75 cell culture flask and filled up with 1xPBS to a total volume of 200 ml. The samples were further processed like samples obtained from whole blood (see above). From a buffy coat between $8x10^8$ and $2x10^9$ PBMC were obtained.

3.2.1.3 Isolation of neutrophils from whole blood

Neutrophils or polymorphonuclear leukocytes (PMN) can be isolated in large numbers and with high purity from human blood. Although the isolation protocol is easily feasible, this cell type is very sensible to temperature of other isolation conditions. To avoid activation of the PMS or cell death it is recommended to use fresh blood samples, to keep the samples at 4°C (when the protocols permit these conditions) and to work with plastic material to prevent cell adhesion. The used protocol is a combination from two protocols. The main part is based on the protocol published by Alfaro *et al.* 2011 [174] and the procedure to lysate cells was adopted from a protocol that was established by Boyum [175] and afterwards modified [176-179].

Peripheral blood was placed in a tube, mixed with the equal volume of cold 1xPBS and the equal volume of dextran sedimentation buffer. The samples were carefully inverted to ensure adequate mixing and incubated for 60 to 90 min until separation was completed. The yellowish supernatant was recovered and spun at 300xg for 12 min at 4°C with low brake.

The cell pellet was immediately resuspended in 1xPBS (at RT), whereas the amount of 1xPBS is equal to the starting volume of blood. Maximal 25 ml of the cell suspension was laid over 15 ml Biocoll (at RT) in a 50 ml conical tube. The samples were spun at 400xg for 40 min at RT. The top (saline) layer as well as the Biocoll layer was aspirated and the neutrophil- and red blood cell (RBC) containing pellet retained. To remove residual RBC each neutrophil/RBC pellet was mixed with 20 ml cold 0.2% $NaCl_2$ for exactly 30 sec. At the end of this period the isotonicity was restored by adding 20 ml ice-cold 1.6% $NaCl_2$. Afterwards the cells were centrifuged 6 min at 250xg and 4°C. The supernatant was discarded. The steps to lysate RBC were repeated once or twice until the cell pellets appeared free of RBC. After the final centrifugation the supernatant was discarded, the pellet was resuspended in 2 ml of 1xPBS per pellet and the samples were pooled. Finally the cell number was determined. $2–3x10^6$ neutrophils per ml blood can be expected.

3.2.1.4 TMC preparation

Located on the inside of the throat, the paired palatine tonsils form part of the first major barrier protecting the digestive and respiratory tracts from potentially invading microorganisms. Human tonsils are the most readily available lymphoid organs and are often used as a source of large numbers of cells characteristic for local lymphoid tissue.

The tonsils were stored in 1xPBS on ice after sampling. First the tonsils were cut into small pieces with scissors. Afterwards to prepare single cell suspensions the gentle MACS (Miltenyi Biotech) was used. The cell suspension named tonsillar mononuclear cell (TMC) were resuspended in X-VIVO 15 and the cell number was determined. Tonsil specimens typically weigh from 2 to 10 g. Total mononuclear cell yields will range from $5x10^8$ to $5x10^9$ cells per tonsil.

3.2.1.5 Determination of cell numbers and cell viability using Trypanblue

To count the cells and clarify the status of vitality a *Neubauer*-improved cell hemocytometer was used. Cells were diluted in Trypan blue which is a diazo dye that stains dead cells with incomplete membrane integrity. To receive a countable cell suspension the cells were

diluted in an appropriate amount of 0.4% Trypan blue and placed in the counting chamber of the hemocytometer. To determine the total cell number the cells within the four big squares of the *Neubauer* hemocytometer were counted. Each big square has an area of 0.04 mm^2 and a depth of 0.1 mm. From the described conditions arises a volume of 0.004 µl. To calculate the cell number the average value of the four big squares was multiplied by $1x10^4$ and the used dilution factor. The following formula was used:

$$C = N \times V \times 10^4$$

C = cell number per ml
N = average of counted cells in large squares
V = dilution factor
10^4 = chamber factor

The total cell number was calculated by multiplying the cell number per ml with the total volume of the cell suspension.

3.2.1.6 Collecting of cell supernatants

Sometimes it is insightful to analyze substances secreted by cells to study their kind of response to variable factors. For example released cytokines could help to clarify the immune status or mode of activation upon stimulation of the cell.

The cell culture supernatants were harvested and centrifuged at 300xg for 10 min at 4°C to remove cell debris. The supernatant was carefully collected and used immediately for analysis or stored at -20°C to prevent protein degradation.

3.2.1.7 Thawing, cryoconservation and passaging of cells

3.2.1.7.1 Thawing of cells

The medium of cryoconservated cells generally contains DMSO, which is on the one hand cytotoxic but on the other hand required to prevent cell damage by crystal formation during

the storage at low temperatures. To ensure that the cells do not start their metabolism before the DMSO is removed the cells were quickly thawed stored on dry ice for the transfer and immediately resuspended in 10 ml pre-warmed (37°C) culture medium. After centrifugation at 300xg for 7 min at RT the cell pellet is resuspended in an appropriate amount of fresh pre-warmed culture medium and seeded into cell culture flasks. The culturing was carried out in incubators with the conditions of 37°C, 5% CO_2 and 95% humidity.

3.2.1.7.2 Cryoconservation of cells

The method of cryoconservation is used to store primary or cell culture cells on the long term in liquid nitrogen or at -150°C. The freezing medium consists of the general culture medium supplemented with 10% DMSO. DMSO is used as a cryoprotectant that substitutes the water within the cells and reduces ice formation; thereby it prevents cell death during the freezing process [180].

First cell lines were grown to mid-log phase in a cell culture flask. When the appropriate cell density or confluence was achieved the cells were harvested. For harvesting suspension cells were pooled in a sterile container, whereas adherent cells were gently dislodged with an appropriate scraper or removed by trypsinization. Afterwards the cell number was determined and the cells were centrifuged for 7 min at 300xg at RT. The sediment was gently resuspended in freezing medium in a volume to achieve a final cell concentration of approximately $2x10^6$ cells/ml. 1 ml of resuspended cells were transferred to each labeled cryovial. The cryovials were immediately placed in a freezer storage box (Mr. Frosty) and frozen at −80°C freezer over night. Afterwards the samples were frozen at -150°C for long term storage. The freezer storage box ensures a decline of temperature by 1°C/min and therefore a mild freezing process.

3.2.1.7.3 Passaging of cells

Generally the protocols for cell passaging differ for adherent cells and for cells in suspension culture. Within the framework of this thesis exclusively murine suspension cells were used,

which does not need an enzymatically dispersing before passaging. The cells were passaged every 2 to 3 days.

When color changes from pink to yellow/orange in the medium, which indicated active cell metabolism passaging was performed. First the flask was removed from incubator and swirled to distribute cells evenly in the medium. The entire cell suspension was transferred to a sterile tube and the cell number was determined. The desired cell amount, which should be used for further cultivation, was placed in a fresh tube and centrifuged for 7 min at 300xg at RT. The sediment was resuspended in fresh pre-warmed (37°C) culture medium and transferred to a new culture flask. The flask was filled up with medium to a total volume of 30 ml and a final cell concentration of approximately $3x10^6$ cells/ml. The incubations were performed in a humidified 37°C and 5% CO_2 incubator.

3.2.1.8 Generation of myeloid imDC derived from monocytes

Dendritic cells (DC) are known as potent antigen-presenting cells (APC) that are important in the initiation and control of cellular immune responses. This type of cells is commonly used in T cell stimulation experiments. DC can be used either to stimulate T cell-mediated immunity or to induce immune tolerance, *in vitro* and *in vivo* [181]. Different methods to isolate DC from peripheral blood are used, but the drawback of these approaches is the small amount of DC, since DC represent about 0.1% of PBMC. The differentiation of monocytes to immature (im)DC is much more convenient since monocytes are plentiful in the peripheral blood (from 5 to 15% in the PBMC) and can be easily differentiated into large numbers of imDC by culturing in the presence of IL-4 and GM-CSF.

Monocytes obtained from PBMC were adjusted to $1x10^6$ per ml in CellGrow medium and differentiated by addition of IL-4 (1000 units (U)/ml) and GM-CSF (1000 U/ml). The cell suspension was seeded in a 24 well plate (1 ml per well) and incubated at 37°C. The cells were harvested 5 days post seeding. Generally 30 to 70% of monocytes developed to monocyte derived imDC.

3.2.1.9 Harvesting of primary adherent cells by EDTA

EDTA is widely used as a chelating agent. It binds metal bivalent ions such as calcium $(Ca)^{2+}$ and causes detaching of adherent cells.

For harvesting loosely adherent or nonadherent cells the suspension was resuspended by pipetting up and down. The cells were transferred and pooled in a sterile container. To collect the adherent cells EDTA solution (1 mM) was added to each well until the cells were well covered. To improve the detachment the plates were incubated at 4°C for 10 min. After the incubation the cells were harvested by pipetting up and down and added to the prior collected cells. The cell number was determined and following a centrifugation at 300xg for 7 min at RT the cells were resuspended in an appropriate volume of buffer or medium.

3.2.2. Immunological techniques

3.2.2.1 Immobilization of antibodies on 96-well plates

The properties of soluble or immobilized antibodies can differ in their effects on specific target bearing cells. For immobilization surface treated plates (MaxiSorb Nunc immune™ plate), which could optimize the adhesion of antibodies to the plate surface were used. Binding buffers additionally support the adsorptive immobilization of the antibodies to the plastic. Therefor for each antibody coating conditions were optimized. For OKT3 1xPBS and for BT-061 and RPT-A4 sodium phosphate buffer (for more details see chapter 3.1.4.2.4) were used.

The antibodies were diluted in binding buffer at the appropriate concentration. It is recommended to prepare the antibody dilution shortly before usage, because the molecule might unspecifically bind to the plastic surface of the tube. 200 µl of the solution was transferred per well and the coating was performed for 2 h at 37°C or over night at 4°C. After removal of the coating reagent, 100 µl blocking buffer was added to each well and incubated for 1 h at RT. Following the blocking procedure the wells were washed 3 times with 300 µl 1x PBS to remove unbound antibody residues. Afterwards cells were added to the antibody treated wells.

3.2.2.2 Enrichment of human cells from PBMC using MACS

To enrich specific cell types from PBMC (e.g. T cells) the MACS technology was used. MACS is a method for separation of various cell populations depending on their surface antigens. The MACS technology (Miltenyi Biotec GmbH) is based on the labeling of cell surface molecules with antibodies coupled to magnetic particles of approximately 50 nm in diameter (so called MicroBeads) and the subsequent separation using MACS columns. In order to induce a high-gradient magnetic field on the matrix of the columns the sample is loaded columns are placed in a strong permanent magnet of the AutoMACS pro (Miltenyi Biotec GmbH). The cell suspension contains labeled and unlabeled cells. Upon loading the samples on the column, unlabeled cells pass through the column and can be collected as negative fraction; on the other hand labeled cells are bound to the matrix and can be eluted as positive fraction after removal of the column from the magnetic field. With this system the cells can be separated positively or negatively with respect to the particular antigen(s) [182].

In the context of this thesis MACS kits for the isolation of B cells, monocytes, natural killer (NK) cells and T cells ($CD3^+$ and $CD4^+$) were used (for more details see Table 3.6). The cells were enriched according to the manufacturer's protocol. For the isolation of monocytes the recommended amounts of MicroBeads and buffer were reduced by 50%. For the positive isolation the *posseld*-program and for the negative selection the *depletes*-program was used, independent of the manufacturer's protocol recommendation.

3.2.2.3 T cell stimulation with antibodies

For stimulation with soluble BT-061 PBMC, MACS enriched T cells or TMC were incubated for 45 min at 4°C in X-VIVO 15 medium at a concentration of $1x10^6$/ml. BT-061 was added at the indicated concentrations. To remove unbound antibody the samples were centrifuged and the pellets were resuspened in fresh X-VIVO 15 medium. Afterwards the T cells were seeded at a concentration of $1x10^6$/ml and the PBMC or TMC at $4x10^6$/ml. In case the T cells were co-cultured with syngeneic immune cells or transfectants, these cells were added in a concentration of $4x10^5$/ml, except for the titration with the transfectants. To incubate T cells on coated BT-061 the cells were seeded at a concentration of $1x10^6$/ml. In a few experiments the syngenic immune cells were pretreated before the co-culturing with T cells.

In case for the monocytes $8x10^5$ cells were incubated with an anti-CD64 blocking antibody (50 µg/ml) or 0.1, 1, 10 or 50% human Ab serum for 45 min at 37°C. Afterwards T cells were added to the pretreated monocytes without removing the reagents. In case of the neutrophils the cells were treated with 500 U/ml IFN-γ for 22 h before the T cells were added. To measure the potency of secreted factors by monocytes co-cultured with BT-061 decorated T cells 100 µl of the supernatant was harvested and added to 100 µl T cells treated with BT-061 of the same donor. The T cells were seeded at a density of $1x10^6$/ml.

3.2.2.4 Blocking of Src kinases by using PP1

PP1 was identified as a high potency inhibitor of Src tyrosine kinase family members that acts as a competitive inhibitor of adenosine-5'-triphosphate (ATP) binding in 1996 [183].

To investigate the effect of Src (sarcoma) inhibition on BT-061 mediated CD4 down-modulation PBMC were pretreated with the Src kinases inhibitor PP1 at concentrations from 5 to 40 µM for 1 h at 37°C. Afterwards the cells were incubated for 18 h at 37°C in medium supplemented with BT-061 (10 µg/ml) and PP1 (from 5 to 40 µM). Subsequently to the incubation the CD4 surface expression was determined by FACS analysis.

3.2.2.5 Inhibition of protein biosynthesis by using CHX

CHX is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *streptomyces griseus*. CHX exerts its effect by interfering with the translocation step in protein synthesis (movement of two transfer ribonucleic acid (tRNA) molecules and messenger (m)RNA in relation to the ribosome) thus blocking translational elongation.

To analyze the effect of CHX treatment on CD4 down-modulation $2x10^5$ MACS enriched $CD4^+$ T cells were pretreated for one h with medium supplemented with CHX (10 µg/ml). Afterwards the cells were washed and subsequently incubated for three h in BT-061 (10 µg/ml) coated wells. Then CD4 expression was determined via FACS analysis.

To analyze the effect of CHX treatment on the renewal of CD4 after BT-061 induced down-modulation $2x10^5$ MACS enriched $CD4^+$ T cells were incubated for three h in wells coated with BT-061 (10 µg/ml), in medium supplemented with CHX (10 µg/ml) or in coating buffer treated wells. Afterwards the cells were further cultivated in untreated wells, medium supplemented with CHX (10 µg/ml) on the coated antibody or removed from the coated wells and cultured in medium or medium supplemented with CHX (10 µg/ml). Every 24 h CHX was added to the respective wells at a concentration of 10 µg/ml. The CD4 expression was determined after the 3, 24, 48 and 72 h by FACS analysis.

3.2.2.6 Proliferative assay for T cell function

A number of agents can specifically or nonspecifically induce or interact with T cell activation, which might result in cytokine production/inhibition, cytokine receptor expression or down-modulation, and ultimately proliferation or repression of proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes - in contrast to helper function for B lymphocytes or cytotoxicity - proliferation assays are reliable and have been widely used to assess the overall immunocompetence. In this thesis two ways to induce T cell proliferation were used. The first one is based on a protocol that describes the activation of unprimed T cells to cell-associated but unspecific antigens in the mixed leukocyte reaction (MLR). In the MLR suspensions of responder T cells are cultured with allogeneic stimulator immature DC. The activating stimulus is the foreign histocompatibility antigen (usually major histocompabitility complex (MHC) class I or class II molecules) expressed on the allogeneic stimulator cells. The other protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture by pharmacologic means. Here we used the therapeutic drug OKT3 [184].

3.2.2.6.1 T cell proliferation stimulated with OKT3

PBMC were isolated from buffy coats and stained with the fluorescent dye PKH-26. Stained PBMC were adjusted to a density of $2x10^6$ cells/ml in X-VIVO 15 medium. OKT3 was added to the medium in a final concentration of 10 µg/ml. Afterwards the cells were seeded in coating

buffer treated or BT-061 (10 µg/ml) coated wells and incubated for five days. Dilution of the fluorescence signal of labeled T cells was determined as a measure of T cell proliferation by FACS analysis.

3.2.2.6.2 T cell proliferation in MLR

Monocyte derived imDC were seeded in a serial log 2 dilution in 96-well plates starting with a concentration of $2.5x10^4$ cells/100 µl and ending at 0.625 cells/100 µl. T cells were MACS-enriched from buffy coats of another donor and stained with the fluorescent dye PKH-26. Stained T cells were adjusted to a density of $1x10^6$ cells/ml in X-VIVO 15 medium and 100 µl of that suspension was added to all wells containing imDC. The cells were seeded on untreated or BT-061 (10 µg/ml) coated wells and incubated for seven or nine days. Dilution of the fluorescence signal of the labeled T cells was determined as a measure of T cell proliferation by FACS analysis after 9 d of culturing.

3.2.2.7 Stimulation of $CD4^+$ T cells for transcriptome analysis

$2x10^5$ MACS enriched $CD4^+$ T cells were incubates with medium or soluble BT-061 (10 µg/ml) for 45 min at 37°C, whereas the cell density was adjusted to $1x10^6$/ml. Afterwards the cells were washed and $2x10^5$ cells seeded in coating buffer treated wells or wells coated with BT-061 (10 µg/ml) or RPT-A4 (10 µg/ml) for 3 h. 3 equally treated samples were pooled and centrifuged at 300xg for 7 min at RT. The pellets were resuspended in 350 µl RLT buffer (buffer contains 1% β-mercaptoethanol and is sufficient for $<5x10^6$ cells, for $5x10^6$ – $1x10^7$ 600 µl RLT was used) and stored at -20 °C until RNA isolation was performed.

3.2.2.8 Immunological analysis using flow cytometry

The flow cytometry technique was first published in 1965 in *Science* [185]. It allows the discrimination of cells based on their size and granularity. The specific technique of fluorescent activated cell sorting (FACS) furthermore enables the detection of cells stained with fluorochrome labeled antibodies to perform phenotypic cell analysis, determination of cytokine production, proliferation or apoptosis. The optical system of a FACS machine is composed of lasers and optical filters that permit the detection of the variable

fluorochromes and the physical cell properties parameters simultaneously. The fluid system ensures that the cell suspension is analyzed on a single cell basis by transporting the cells separately and in a defined manner through the optical system. This is possible by providing different pressures between the sheath fluid and the sample fluid. This pressure system allows that the sample core remains separate but coaxial within the sheath fluid and the cells separately pass through the laser beam. The cell size is detected via the forward scatter (FSC), which is located at the opposite site of the exciting laser, whereas the side scatter (SSC), which detects cell granularity, is 90° with respect to the exciting light. The emitted fluorescence is determined at the same location as the SSC. The display of the FSC/SSC allows the separation between cell debris and viable cells. The principle of FACS consists in the excitation of the electrons of the fluorochrome at a definite wavelength by a monochromatic laser beam. The power of the laser elevates the electrons to a higher energy level. The electrons drop subsequently back to their original energy level, but meanwhile they emit energy in form of photons. The released photon concentration is detected via a photomultiplier, whereas the measured value is proportional to the amount of cell bound antibody. The simultaneous measurement of variable fluorochromes is possible in spite of similar excitation wavelength, because the fluorochromes could differ concerning their emission wavelength. The electronic system of the FACS machine - here LSRII sorb (BD) - converts the fluorescence signals into electronic signals that can be analyzed by a computer. Acquisition and analysis software help to record and analyze the data provided by the cytometer.

3.2.2.8.1 Analysis of surficial expressed molecules by FACS analysis

To analyze cells by flow cytometry $2x10^5$ to $1x10^6$ cells per sample were stained with flourochrome labeled antibodies. The cells were placed in a conical 5 ml tube and resuspended in 50 µl FACS buffer. To block unspecific binding of the antibodies to FcγR expressed on the cell surface 5 µl human polyglobine (Gamunex 10%) was added to the cell suspension. Afterwards fluorochrome-labeled cell surface specific antibodies were added in the appropriate concentration (final dilution between 1:20 and 1:55). The cell-antibody suspensions were mixed and incubated for 30 min at 4°C protected from light. To prevent further binding 1 ml FACS buffer was added to the sample followed by centrifugation at

300xg and 4°C for 7 min. After centrifugation the supernatant was removed and the cell pellet was resuspended in 200 µl FACS buffer and fixed with 50 µl 1% PFA. The samples were stored at 4°C in the dark until the measurement was performed. Analysis was carried out by FACS LSRII sorb flow cytometer (BD). The obtained data were analyzed using FlowJo software (TreeStar).

3.2.2.8.2 Detection of intracellular proteins by FACS

The procedure of intracellular cytokine staining in conjunction with flow cytometry allows to determine the number or phenotype of cells that produce a specific cytokine. To prevent the release of cytokines from the cell secretion inhibitors such as BFA were used. BFA inhibits the intracellular transport of proteins by the induction of Golgi apparatus degradation. This blockage causes an accumulation of proteins in the endoplasmic reticulum [186]. Because of the high loss of cells due to the protocol it is recommended to use more cells then in the surface staining protocol specified. Generally $6x10^5$ cells per sample were used for intracellular FACS staining. Cells were resuspended in 50 µl 1x PBS and 5 µl Gamunex 10% and stained with the indicated antibodies for surface staining (for more details see Table 3.1). To fix the antibodies that are specifically bound to the surface a fixation buffer with PFA was used, which additionally contains permeabilization reagents (Cytofix/Cytoperm).

The cells were incubated in 100 µl Cytofix/Cytoperm solution for 20 min at RT in the dark. Afterwards 100 µl 1x Perm/Wash was added to the samples. The cells were centrifuged at 350xg for 5 min at 10°C. 50 µl 1x Perm/Wash and the antibodies in the indicated amounts for the intracellular staining were pipetted to the cell pellet. Antibody binding was performed for 15 min at RT protected from light. Afterwards the samples were washed twice with 300 µl 1x Perm/Wash. Finally the cells were resuspended in 200 µl FACS buffer. The samples were stored at 4°C and protected from light until the measurement was performed. Analysis was carried out by FACS LSRII sorb flow cytometer (BD). The obtained data were analyzed using FlowJo software (TreeStar).

3.2.2.8.3 Flow cytometric analysis of cell division by dye dilution

As cells of the immune system, i.e. T cells, might undergo considerable expansion as response to a variety of stimuli. Therefore the ability to determine the division of cell populations undergoing proliferation is particularly useful in the study of immunological events. There are a number of techniques available to investigate cell proliferation, such as incorporation of BrdU or tritiated thymidine or intracellular fluorescent labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE) or PKH to tag proliferating cells directly. In this thesis the labeling with PKH-26 was the technique of choice, whereas the "26" indicates the emitting wave length upon laser excitation. This method to label and to track cell proliferation was first published in 1993 by Ashley *et al.* [187]. PKH-26 is a lipophilic dye incorporating in the membrane bilayer without leaking or toxic side effects. The used PKH-26 fluorescent cell linker kit allows proprietary membrane labeling to stably incorporate a yellow-orange fluorescent dye with long aliphatic tails (PKH-26) into lipid regions of the cell membrane. Incorporated PKH-26 is divided equally between daughter cells and allows discrimination between resting and dividing cells via flow cytometry [188].

At the beginning the desired amount of cells (maximum $2x10^7$ cells) were placed in a tube. After centrifugation at 300xg at RT for 7 min the cell pellet was gently resuspended by pipetting in 1 ml of Diluent C (provided with the kit). To prepare the staining solution 4 µl of PKH-26 dye were diluted in 996 µl Diluent C. It is recommended to prepare the staining solution shortly prior usage. After the staining solution was added to the cell suspension and the liquids were properly but gently mixed an incubation of 2 to 5 min was performed. The staining was stopped by adding an equal volume (2 ml) of 1% BSA and a further incubation for 1 min. Following the addition of 4 ml X-VIVO 15 medium the cells were centrifuged for 10 min at 300xg at RT. The cell sediment was resuspended in 10 ml X-VIVO 15 and centrifuged again. The washing steps were repeated three times to remove fluorescence dye residues. For more information concerning the protocol see manufacturers' protocol.

3.2.2.9 Quantitative protein detection using ELISA method

The principle of the enzyme-linked immunosorbent assay (ELISA) method was first introduced in 1971 by Engvall and Perlmann [189]. ELISA is a method to detect the presence

of soluble antigens secreted by cells, i.e. cytokines. In the framework of this thesis double antibody–sandwich ELISA kits were used to detect murine and human IL-2 (for more details see Table 3.6). The plates are pre-coated with capture antibody specific for the protein, which should be detected – in this case murine and human IL-2. The supernatant from cells, which should be analyzed for the release of the protein, are added to the wells coated with the capture antibodies and are incubated. In this step the specific antigens are recognized and immobilized by the capture antibody. Afterwards the plates are washed and subsequently incubated with a biotinylated detection antibody which is specific for a different epitope of the antigen. After incubation, unbound conjugate was removed. Finally the amount of antigens is detected by adding an enzyme-linked streptavidin followed by a fluorogenic substrate that produces a visible signal in proportion to the quantity of antigen in the sample. Fluorogenic substrates are detected with a spectrophotometer. In this protocol tetramethylbenzidine (TMB) was used as a substrate for streptavidin-linked horseradish peroxidase (HRP). HRP turns TMB to a blue-colored product, which changes to yellow upon addition of an acid sulphur or phosphoric acid containing stop solution.

The ELISA kits were used according to the manufacturers' protocol and with the buffers and solutions provided with the kit.

3.2.3. Genetic engineering methods

3.2.3.1 mRNA expression analysis of human T cells using Agilent microarrays

The microarray is a method that allows the analysis of several thousands of genes through detecting the corresponding mRNA levels in cells simultaneously. On a Microarray-Chip, mRNA is hybridized to specific oligonucleotides thus allowing the detection and quantification of thousands of known sequences in parallel. A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Each DNA spot contains a specific DNA sequence, also known as probes. The probes can be a short section of a gene or other DNA elements that are used to hybridize a cDNA under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. After the

hybridisation procedure the array was washed and analyzed with the Agilent DNA Microarray Scanner (Agilent; Santa Clara; USA).

3.2.3.1.1 RNA-Isolation via RNeasy kit

The RNeasy procedure represents technology for RNA purification. The technology is based on the selective binding properties of a silica-based membrane. A high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy column, where the total RNA binds to the membrane and contaminants are removed. RNA is then eluted in 30 - 100 µl water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified (RNeasy Mini Handbook 09/2010).

The RNeasy kit was used according to the manufacturers' protocol and with the buffers and solutions provided with the kit. Afterwards the RNA was precipitated using ethanol precipitation.

3.2.3.1.2 RNA precipitation with ethanol

The isolated nucleic acid was additionally precipitated with ethanol to concentrate the product and remove contaminations. First 1 µl linear Acrylamide (5 µg/ml), 0.5 volume 7.5 M ammonium acetate and 2.5 volume ice-cold 95% ethanol were added and mixed. The samples were kept for at least 1 h at -80°C. After centrifugation at maximum speed for 30 min at 4°C, the precipitate was washed twice with 400 µl ice-cold 80% ethanol. Afterwards the nucleic acid was dried using a vacuum centrifuge and resuspended in 13 µl Diethylpyrocarbonate (DEPC) treated H_2O. The sample was stored at -80°C.

3.2.3.1.3 Quality control of the isolated RNA and Gene Array

The subsequent preparations including the Gene Array analysis was performed by the facility "Gene Analytics" at the Helmholtz Centre for Infection Research in Braunschweig. The sample reactions were performed using the Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturers' protocol.

3.2.3.1.4 Quality control by NanoChip

To analyze the quality and integrity of the total RNA the NanoChip Kit (Agilent) was used combined with the 2100 Bioanalyzer (Agilent). With the NanoChip RNA molecules are separated by size. Charged biomolecules like RNA are electrophoretically driven by a voltage gradient, similar to gel electrophoresis. Because of a constant mass-to-charge ratio and the presence of a sieving polymer matrix, the molecules are separated by size. Smaller fragments are migrating faster than larger ones. Dye molecules intercalate into RNA strands and these complexes are detected by laser-induced fluorescence. An RNA ladder standard is run on every chip used as a reference for data analysis. The software automatically compares the unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify the ribosomal RNA peaks.

3.2.3.1.5 Microarray hybridization and analysis

500 ng of total RNA were applied for Cy3-labelling reaction using the one color Quick Amp Labeling protocol (Agilent Technologies; Waldbronn, Germany). Afterwards labeled cRNA was hybridized to Agilent´s human 4x44k microarrays for 16 h at 68°C and scanned using the Agilent DNA Microarray Scanner. Expression values were calculated by the software package Feature Extraction 10.5.1.1 (Agilent Technologies; Waldbronn, Germany). Statistical analysis of the expression data was performed using the Gene Spring Software package (Agilent Technologies; Waldbronn, Germany).

3.2.4. Computer-based and statistical analysis

3.2.4.1 FACS data processing to determine CD4 down-modulation and cell proliferation

To quantify the percentage of CD4 down-modulated T cells in a standardized manner a new protocol to process FACS data was established together with Prof. Frank Klawonn (Department of Computer Science Ostfalia University of Applied Sciences). For this approach an algorithm was developed setting a gate for analysis of CD4 down-modulation independent from the varying percentage of the population of $CD3^+CD4^-CD8^-$. The establishment of a computer based method for standardized gate definition was necessary in order to be able to summarize data sets obtained from many different experiments and donors.

First the results which were obtained via FACS analysis were processed using FlowJo. Therefore lymphocyte population was gated based on physical parameters such as cell size and granularity (forward scatter (FSC)/sideward scatter (SSC)). To determine CD4 down-modulation in a next step $CD3^+CD8^-$ T cells were gated, which by definition represent $CD3^+CD4^+$ T cells. Within the gate of $CD3^+CD8^-$ T cells CD4 down-modulation was determined. To quantify the percentage of CD4-down-modulated T cells the fluorescence intensity of CD4 staining was plotted in a histogram. These histograms were exported from FlowJo in an FCS2 format. To transcribe the containing information into a format that is legible by the analyzing program, a comma-separated values (CSV) converting program was used (free download: http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/index.htm). These CSV files were processed with a procedure named compute.and.apply.gate programmed in “R”. The algorithm defines a gate independent from the varying percentage of the population of $CD3^+CD4^-CD8^-$- using principles from robust statistics. It is assumed that the raw data of BT-061 untreated samples follow a normal distribution, which is corrupted by an additional noise distribution originating from the varying percentage of the population of $CD3^+CD4^-CD8^-$-. The estimates for the expected value and the standard deviation of the normal distribution are based on the median and the interquartile range. The interquartile range is the length of the interval containing the middle 50% of the data in the sample. For a normal distribution, the interquartile range must be multiplied by a factor of about 0.741 to obtain the standard deviation. Based on this

normal distribution a gate is set as 1% quantile of the corresponding normal CD4 expression. This gate was then applied on histograms showing CD4 expression in BT-061 treated samples. Next, the percentage of $CD3^+CD4^-CD8^-$- T cells was determined in untreated cells and this value was subtracted from the percentage of $CD3^+CD4^-CD8^-$- cells determined within the gate upon BT-061 treatment of cells. The described procedure was also used to calculate T cell proliferation, whereas instead of calculation of CD4 staining of $CD3^+CD8^-$ lymphocytes the PKH-26 staining of $CD3^+$ lymphocytes was determined.

3.2.4.2 Visualization of results obtained by micro array using hierarchical cluster analysis (HCA)

The obtained data sets from micro array were processed via the software Gene Spring GX (Agilent.SingleColor.26652). To analyze such a data set the results were initially filtered on the basis of specific criteria. The fluorescence intensity was normalized among all tested arrays and subsequently compared with each other. mRNA with a fluorescence intensity in a range between 20 and 100% were used in the following process. To provide a global comparison of the response of different donors to the variable treatments the data were analyzed using HCA. HCA is an unsupervised agglomerative clustering algorithm that is widely used in the analysis of gene expression data [190] and is beginning to be used in the analysis of immunological data [191, 192]. The HCA is based on the principle that similar data sets were summarized in clusters whereas the similarity is decided following a specific algorithm. These clusters could further be separated in sub-clusters to visualize relations based on similarity. Furthermore the statistical significance (p-Value less than 0.05) of the gene regulation of all samples was calculated with Oneway analysis of variance (ANOVA) test. Genes, that were 2-fold up- or down-regulated compared to the unstimulated control, were incorporated in the subsequent heat map. Hierarchical clustering was used to identify responder gene profiles with respect to treatment. Similarity measures used as a metric for clusterization was “uncentred Pearson correlation” combined with the “complete” linkage rule creating the dendrogram structure. Heat maps are used to visualize a variety of information for a couple of data sets simultaneously. The type of gene regulation (up or down compared to unstimulated controls) and the magnitude is encoded with color values.

Red indicates the up-regulation and blue the down-regulation of a gene compared with average of all samples.

3.2.4.3 Statistical analysis

Statistical analyses were done using GraphPad Prism 5. The two-tailed non parametric Mann-Whitney test was used for analysis of differences between groups with unmatched pair values and two-tailed non parametric Wilcoxon signed rank test was used for analysis of differences between groups with matched pair values. Statistical significance was indicated when p-values <0.05. Error bars in graphs indicate standard error of mean (SEM).

4. Results

Autoimmune diseases affect 3–5% of the general population [148]. During the past decade efforts have focused on the development of novel drugs for the therapy of autoimmunity. Conventional treatments include the long-term administration of immunosupressants such as steroids, azathioprine and cyclosporine [193], all of which, however, can cause severe adverse effects such as development of opportunistic infections or organ toxicity [157]. Therefore, alternative approaches that allow more specific and long-term treatment are needed. Monoclonal antibodies (mAb) promise to fulfill these requirements due to their unique target specificity. CD4-targeted therapy has the potential to modulate peripheral T cell tolerance, which often is dysregulated in autoimmune diseases. Theoretically, CD4-specific mAb can contribute to the reestablishment of peripheral tolerance by triggering different mechanisms. On the one hand self-reactive T cells represent a possible target for therapy by CD4-specific mAb. The interaction of the mAb with CD4 could disrupt the interaction of the T cell receptor (TCR) with major histocompatibility complex (MHC)-self-peptide-complex or trigger the inhibition of the TCR mediated activating signal induced by self-peptides. On the other hand the activation of regulatory T cells (Treg cells), which usually suppress auto-reactive T cells, might be a possible mode of action to prevent autoimmunity by CD4-specific mAb [162]. Tregalizumab (BT-061) is a new CD4-specific mAb, which is currently in phase II trials for the treatment of psoriasis and rheumatoid arthritis (RA) [168]. BT-061 is the humanized variant of a murine mAb specifically binding the human CD4 receptor. BT-061 is a non-depleting immunoglobulin (Ig)G1 antibody, which was humanized by engraftment of the antigen-specificity conferring complementarity determining regions (CDR) [166]. Although BT-061 did not confer depletion of T cells, in first clinical trials, BT-061 treatment induced CD4 down-modulation of T cells. Furthermore it is described that BT-061 is able to selectively activate Treg cells by binding to a specific epitope of CD4 [167]. Preclinical studies imply that BT‑061 is particularly safe. Although the administration of BT-061 to healthy volunteers induced transient sequestering of CD4, CD8 and natural killer cells (NK cells), no cellular depletion was observed. Slightly elevated cytokine levels were detected in the serum of BT-061 treated individuals, which had no detectable clinical relevance. Furthermore, under BT-061 treatment no indications for

generalized immunosuppressive effects were detected. Thus, collectively the data indicated that BT-061 was well tolerated [167]. Of note, so far the exact mode of action of BT-061 is not resolved [168].

4.1. Cellular conditions of BT-061 mediated CD4 down-modulation of T cells

First clinical studies indicated that CD4 expression of T cells is down-modulated upon administration of BT-061 [167]. Similar phenomena, i.e. CD4 down-modulation upon mAb binding, have been observed with other CD4-specifc mAb before [194]. Nevertheless, still the cellular and molecular conditions playing a role in BT-061 mediated induction of CD4 down-modulation are not fully understood. Furthermore, dose dependence and kinetics of the reaction has not been extensively studied. Therefore, a fluorescence activated cell sorting (FACS) based method was established that allowed monitoring of CD4 expression of T cells upon binding of the CD4-specific therapeutic mAb BT-061. To characterize the mechanism of CD4 down-modulation, human peripheral blood mononuclear cell (PBMC) of healthy donors were isolated, incubated with different concentrations of BT-061, and CD4 surface expression was analyzed at multiple time points. Furthermore, the contribution of different immune cell subsets comprised within PBMC to confer BT-061 mediated CD4 down-modulation was examined.

4.1.1. Analysis of the impact of mAb concentration and incubation time on BT-061 induced CD4 down-modulation of T cells in PBMC

In the first set of experiments PBMC were treated with BT-061 and CD4 expression of T cells was examined cytofluometrically. For determination of CD4 surface expression cells were stained with anti-CD3-APC, anti-CD8a-PE.Cy7 and the anti-CD4-PE clone SK3, which does not compete with BT-061 binding to CD4. For data analysis in a first step based on physical parameters, such as cell size and granularity (forward scatter (FSC)/sideward scatter (SSC)), the lymphocyte population was gated. In a next step $CD3^+CD8^-$ T cells were gated, which by definition represent $CD3^+CD4^+$ T cells (Figure 4.1A). Within the gate of $CD3^+CD8^-$ T cells CD4 down-modulation was determined. Analysis of primary FACS data was based on

quantification of the degree of CD4 down-modulation of T cells upon BT-061 treatment as indicated by the mean fluorescence intensity (MFI) of CD4 staining relative to controls. This analysis strategy has been used in many previously published studies addressing the reduction of surface receptor expression [194-196]. The degree of CD4 down-modulation of BT-061 treated cells was calculated as percentage down-modulation relative to the MFI of the anti-CD4 staining of untreated cells. To eliminate the influence of variable laser settings and auto-fluorescence of cells the MFI of the anti-CD4 staining of $CD3^+CD8^+$ was defined as background (BG) and was subtracted from the MFI of BT-061 treated and untreated samples. The following formula was used to calculate the degree of CD4 down-modulation:

$$100 - \ 100 \times \left(\frac{MFI_{experimental}[MFI_{SK3}\ (CD3^+CD8^-\ sample + BT061)] - \ MFI_{BG\ experimental}\ [MFI_{SK3}(CD3^+CD8^+ sample + BT061)]}{MFI_{100\%control}[MFI_{SK3}\ (CD3^+CD8^-\ sample - BT061)] - \ MFI_{BG\ 0\%control}[MFI_{SK3}(CD3^+CD8^+ sample - BT061)]} \right)$$

Values for the degree of CD4 down-modulation as determined by MFI also allowed combination of results from PBMC of different donors and different experiments. Of note, MFI were measured in a logarithmic scale. Therefore, this method was very sensitive when it came to the quantification of very minor effects, which allowed the exact determination of the degree of CD4 down-modulation. However, also minor inherent experimental variations might cause deviating results. Furthermore the calculated value of the MFI allowed no conclusion, whether this value corresponded to a uni- or multimodal distribution of the fluorescence intensity. The detailed data analysis revealed that in many PBMC samples already before application of BT-061 a subset of $CD3^+CD4^-CD8^-$ was detected. Of note, the prevalence of this subset varied from 6.2 to 11.8% in the population of $CD3^+CD8^-$ T cells between different donors (Figure 4.1B, black striped area). This subset of $CD3^+CD4^-CD8^-$ typically constitutes 0.5-2% of human T cells in peripheral blood and comprises cells such NKT cells, Treg cells and others [197-199]. Because of the already mentioned mathematical sensitivity of the MFI, the subset of $CD3^+CD4^-CD8^-$ influenced the determination of the degree of CD4 down-modulation, which was calculated on the basis of the MFI, to a varying extent between the donors. Thus, for exact quantification of CD4 down-modulation of T cells upon BT-061 treatment, the percentage of the population of $CD3^+CD4^-CD8^-$ cells that was already present in untreated controls, had to be subtracted from the percentage of the population of $CD3^+CD4^-CD8^-$ in BT-061 treated samples. To take the varying subset of $CD3^+CD4^-CD8^-$ into consideration, another approach was followed to quantify CD4 down-

modulation. For this approach an algorithm was developed that allowed a standardized procedure to set a gate for analysis of CD4 down-modulation independent of the percentage of the population of $CD3^+CD4^-CD8^-$ and allowed the quantification of the percentage of CD4 down-modulated T cells. This gate was defined by using a control sample that was not treated with BT-061. The establishment of a computer based method for standardized gate definition was necessary in order to be able to summarize data sets obtained from many different experiments with PBMC of different donors. Therefore, using the values obtained from the raw FACS data including the varying population of $CD3^+CD4^-CD8^-$ cells (Figure 4.1B, black striped area) the mean was calculated and based on this value a normal was estimated. Since the population of $CD3^+CD4^-CD8^-$ cells considerably influenced the mean, the estimate of the normal distribution did not mirror the distribution of the values obtained from the raw data (Figure 4.1B, red curve). Therefore as a robust alternative the median was used to estimate the expected value for a normal distribution (Figure 4.1B, green curve). The median is not as sensitive to outliners, compared to the mean. For the same reasons, the estimation of the standard deviation was based on the interquartile range instead of the sample standard deviation. In a next step the analysis gate was placed that exactly 1% of the counts for CD4 expression were included within the gate (Figure 4.1B, blue line). This procedure assured proper placement of the gate independent of the size of the $CD3^+CD4^-CD8^-$ subset. Next, the percentage of $CD3^+CD4^+CD8^-$ T cells was determined in untreated cells and this value was subtracted from the percentage of $CD3^+CD4^+CD8^-$ cells determined within the gate upon BT-061 treatment of cells. Values for CD4 down-modulation determined by standardized gate placement allowed combination of results from PBMC of different donors and different experiments independent from the subset of $CD3^+CD4^-CD8^-$ cells. In conclusion, by applying two different data analysis procedures, BT-061 induced CD4 down-modulation was described (i) by the degree of CD4 surface receptor down-modulation using the MFI and (ii) the percentage of T cells that showed CD4 down-modulation using a standardized gate.

To study the modulation of CD4 expression upon BT-061 binding of T cells in an *in vitro* assay, the aim was to incubate PBMC from healthy donors with mAb concentrations that corresponded to mAb concentrations used in clinical trials. Considering the fact that a person with an averaged body weight of 70 kg, typically has a blood volume of 5 – 7 l, whereas 50% of that volume is contributed by cells and the other 50% by serum, upon intravenously (i.v.) administration a mAb is diluted in an average in a volume of 2.5 – 4 l

serum [200]. In a phase IIa trial BT-061 was i.v. injected at concentrations between 0.5 to 25 mg per patient [201], resulting in serum mAb concentrations between approximately 0.125 and 10 µg/ml. To also study these relevant concentrations in an *in vitro* model, PBMC were incubated with BT-061 at concentrations ranging from 0.1 to 30 µg/ml. After the indicated time intervals cells were harvested and CD4 surface expression was determined cytofluometrically.

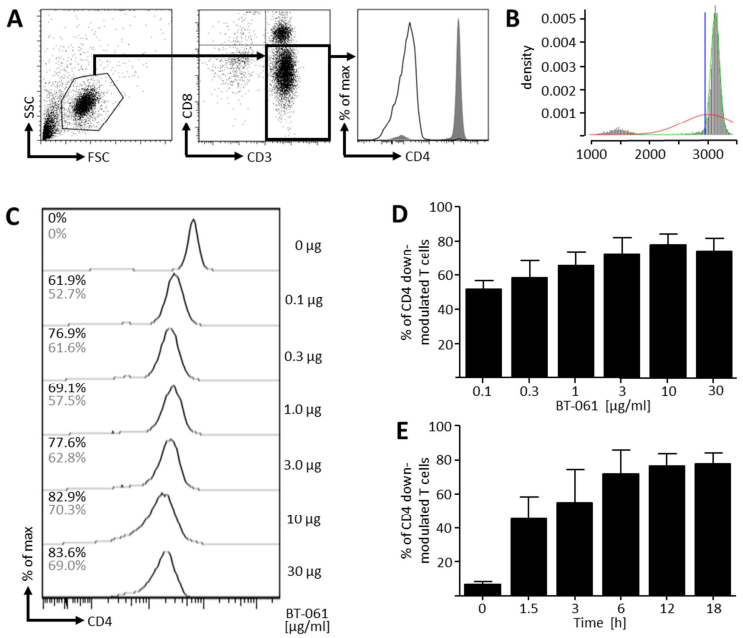

Figure 4.1: Upon BT-061 treatment of PBMC CD4 down-modulation of T cells turned out to be time and dose dependent. (A) For quantification of CD4 down-modulation, $2x10^5$ $CD3^+$ T cells isolated from PBMC by MACS purification were stained with anti-CD3-APC, anti-CD8a-PE.Cy7 and anti-CD4-PE clone SK3 that binds a different epitope than BT-061 (filled grey), or with the anti-CD4 isotype control IgG1κ (black line). CD4 expression was quantified by gating on lymphocytes using physical parameters (FSC/SSC) and then on $CD3^+CD8^-$T cells, which are by definition $CD3^+CD4^+$. (B) Depiction of representative data used to determine the percentage of gated T cells that showed CD4 down-modulation by defining a gate independent from the $CD3^+CD4^-CD8^-$ population. Therefore, using the values obtained from the raw FACS data including the varying population of $CD3^+CD4^-CD8^-$ cells (black striped area) the mean was calculated and based on this value a normal distribution was estimated (red curve). As a robust alternative the median was used to estimate the expected value for a normal distribution (green curve). In a next step, the analysis gate was placed that exactly 1% of the counts for CD4 expression were included within the gate independent from the $CD3^+CD4^-CD8^-$ population (blue line). Next, the percentage of $CD3^+CD4^+CD8^-$ T cells including the $CD3^+CD4^-CD8^-$ population was determined in untreated cells and this value was subtracted from the percentage of $CD3^+CD4^-CD8^-$ cells determined within the gate upon BT-061 treatment of cells. (More details concerning processing and the used software see chapter 3.2.4.1). The presented data are representative for the results obtained with at least 100 samples. (C) $8x10^5$ PBMC were treated for 45 min at 4°C with BT-061 at concentrations

ranging from 0.1 to 30 µg/ml. Then cells were washed and incubated for additional 18 h at 37°C. Values printed in black indicate the percentage of gated T cells that showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the $CD8^-$ T cell population. One representative experiment out of 2 similar ones with cells derived from 3-5 donors is shown. (D) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation (E) $8x10^5$ PBMC were pre-treated with 10 µg/ml BT-061 for 45 min at 4°C. After washing, the cells were cultivated for 1.5, 3, 6, 12 and 18 h at 37°C and then CD4 expression was determined cytofluometrically. Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation with data of 2 similar experiments with cells derived from 3-5 donors. Error bars indicate SEM.

The analysis of all performed experiments revealed that upon incubation of PBMC with BT-061 the percentage of significantly CD4 down-modulated T cells increased starting at concentrations of 0.1 µg/ml and reached maximal effects upon incubation with 10 µg/ml (Figure 4.1C and D). Higher dosages of 30 µg/ml did not enhance CD4 down-modulation (Figure 4.1D). To investigate the kinetics of CD4 down-modulation in PBMC, the CD4 surface expression was measured at different time points post seeding. Upon incubation of PBMC with the most effective concentration in CD4 down-modulation of 10 µg/ml BT-061 at 37°C, a maximal percentage of CD4 down-modulated T cells was observed as early as 6 h after incubation and reached a plateau at 12 h after incubation (Figure 4.1E), which declined after approximately 24 h (data not shown). In summary, the results shown in Figure 4.1 demonstrated a dependency of CD4 down-modulation of T cells in PBMC on the incubation time until 12 h and the dosage of used BT-061 between concentrations of 0.1 and 10 µg/ml.

4.1.2. Quantitative analysis of CD4 down-modulation of T cells conferred by soluble or plate bound BT-061

After investigating the impact of BT-061 treatment on CD4 surface expression of T cells in PBMC, this phenomenon was next studied using purified T cells. To this end, MACS enriched $CD3^+$ T cells were incubated with soluble BT-061. To examine, whether the immobilization of BT-061 influenced CD4 surface expression on enriched T cells, in another set of experiments the mAb was coated to a 96-well plate and then T cells were added.

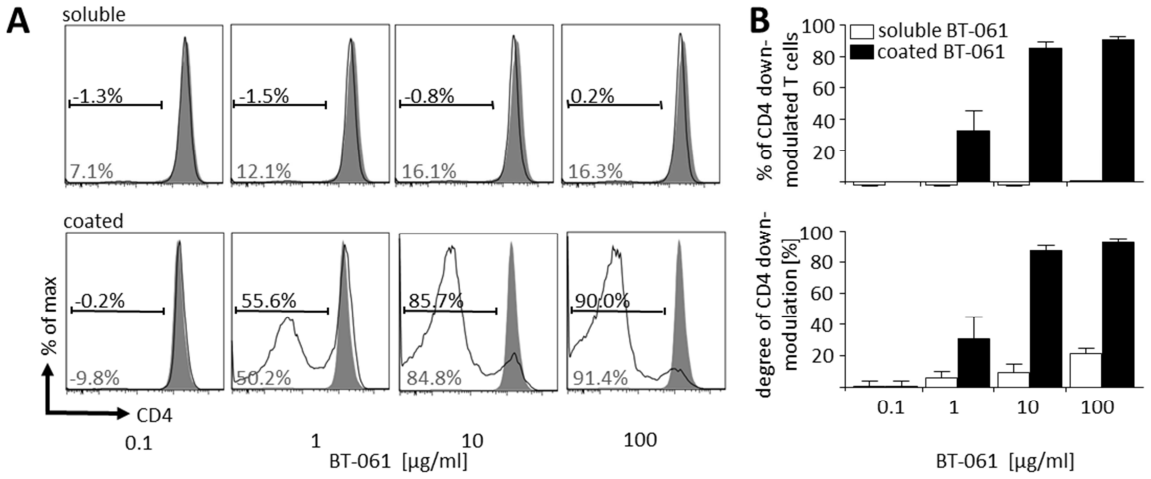

Figure 4.2: Immobilized BT-061 conferred strong CD4 down-modulation, whereas soluble mAb only marginally affected the degree of CD4 down-modulation of T cells and not affected at all the percentage of CD4 down-modulated T cells. $2x10^5$ $CD3^+$ T cells isolated from PBMC by MACS purification were treated either with soluble or with coated BT-061 and cultivated for 18 h. Then CD4 expression of T cells was determined cytofluometrically as detailed in the legend of Figure 4.1. (A, upper row) $CD3^+$ T cells were cultured with medium (filled grey) or medium supplemented with soluble BT-061 at concentrations of 0.1, 1, 10 and 100 µg/ml (black line) for 45 min at 4°C. Then cells were washed, cultivated for 18 h at 37°C and CD4 expression was determined cytofluometrically. (A, lower row) $CD3^+$ T cells were cultured in either untreated wells (filled grey) or in wells coated with BT-061 at concentrations of 0.1, 1, 10 and 100 µg/ml (black line). Afterwards cells were cultivated for 18 h at 37°C and CD4 expression was determined cytofluometrically. Values printed in black indicate the percentage of gated T cells that showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the $CD8^-$ T cell population. Representative data of one experiment from cells derived from 3 donors (upper row) or from 2 similar experiments with cells derived from 5 donors (lower row) are shown. (B) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation (upper graph) and the degree of CD4 down-modulation of the $CD8^-$ T cell population (lower graph). Error bars indicate SEM.

The performed experiments revealed that soluble BT-061 compared to untreated controls did not affect the percentage of CD4 down-modulated T cells at concentrations ranging from 0.1 to 100 µg/ml (Figure 4.2A, values printed in black in upper panel, and B, upper graph). However, the same concentrations of soluble mAb induced a slight decrease of the degree of CD4 surface expression of T cells (Figure 4.2A, values printed in grey in upper panel, and B, lower graph). The incubation of purified T cells in wells coated with BT-061 at concentrations ranging from 1 to 100 µg/ml induced a clear CD4 down-modulation, whereas the administration of 0.1 µg/ml BT-061 showed no effect. The addition of purified T cells to wells coated with BT-061 at a concentration of 1 µg/ml induced a significant CD4 down-modulation in 30% of the T cells. The incubation with coated BT-061 at a concentration of

10 µg/ml mediated that 85% of T cells showed a significant CD4 down-modulation. BT-061 at a concentration of 100 µg/ml induced in 90% of T cells a significant CD4 down-modulation (Figure 4.2A, lower panels, and B). Therefore the data revealed that CD4 down-modulation increased depending on the dosage of the coated mAb in concentration ranging from 0.1 to 100 µg/ml, whereas soluble BT-061 in equal concentrations did marginally affected the degree of CD4 down-modulation, but not the percentage of significantly CD4 down-modulated T cells.

4.1.3. Analysis of cell subsets that confer CD4 down-modulation of BT-061 decorated T cells

Based on the results described above, i.e. soluble BT-061 conferred CD4 down-modulation on T cells within PBMC, whereas the same mAb only marginally affected CD4 expression when added to purified T cells, it was hypothesized that PBMC contain one or more immune cell subsets that played a role in conferring CD4 down-modulation of BT-061 decorated T cells. To identify which cell type crucially conferred this effect, BT-061 treated T cells were either incubated with PBMC, various different immune cell subsets MACS enriched from PBMC, or the corresponding fraction of PBMC depleted from the respective cell subsets.

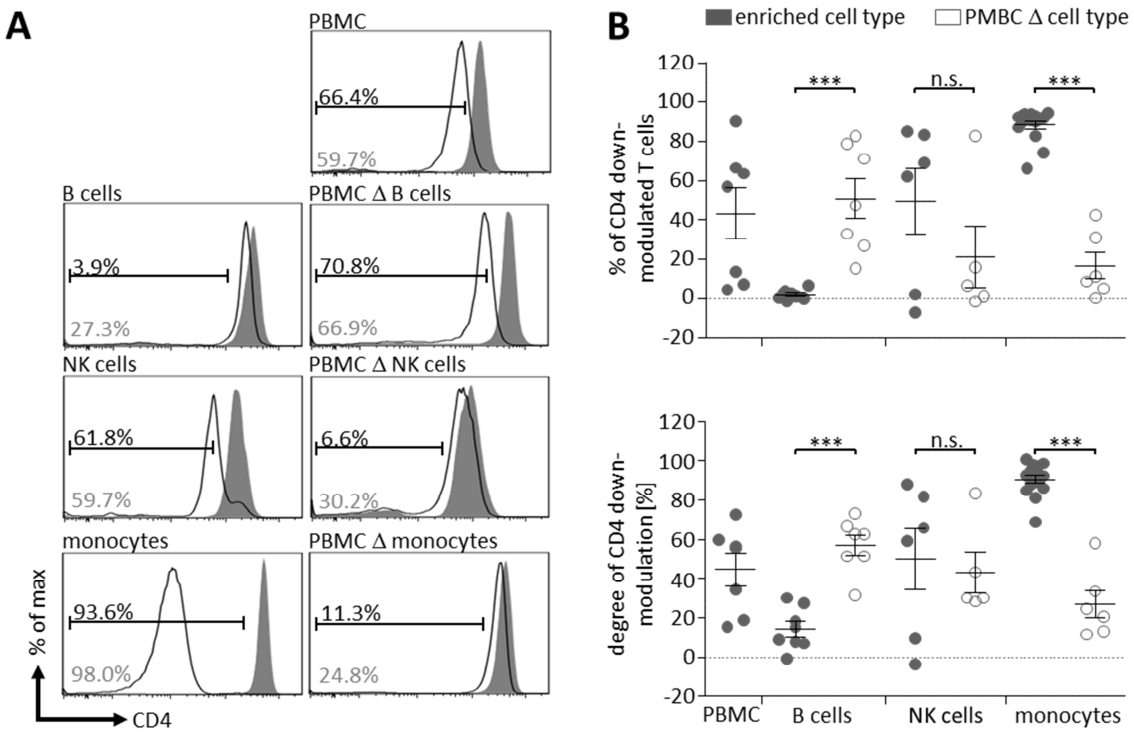

Figure 4.3: Monocytes conferred CD4 down-modulation of BT-061-decorated T cells. (A) 2x10^5 purified T cells were treated for 45 min at 4°C with medium (filled grey) or with 10 µg/ml BT-061 (black line). Upon washing, T cells were co-cultured for 18 h at 37°C with either 8x10^5 PBMC, or enriched immune cell subsets isolated from PBMC, as indicated, or PBMC depleted of the respective immune cell subset. Afterwards CD4 expression was determined cytofluometrically. Values printed in black indicate the percentage of gated T cells that showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the CD8$^-$ T cell population. Representative data are shown of 4 similar experiments performed with cells derived from 7 donors (co-stimulation with PMBC), 4-5 experiments with cells derived from 7-8 donors (co-stimulation with B cells or B cell depleted PBMC), 3-4 experiments with cells derived from 5-6 donors (co-stimulation with NK cells or NK cell depleted PBMC), and 4-8 experiments with cells derived from 6-15 donors (co-stimulation with monocytes or monocyte depleted PBMC). (B) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation (upper graph) and the degree of CD4 down-modulation of the CD8$^-$ T cell population (lower graph). Error bars indicate SEM.

BT-061 decorated T cells incubated with PBMC showed significant CD4 down-modulation in 43% of the analyzed T cells (Figure 4.3B, upper graph), whereas the calculation of the overall degree of CD4 down-modulation amounted at 45% (Figure 4.3B, lower graph). PBMC depleted of B cells induced a similar CD4 down-modulation as observed with complete PBMC, while isolated B cells conferred only a very minor effect (Figure 4.3A, second panel, and B). These results indicated that B cells did not play a crucial role in conferring CD4 down-modulation. Purified NK cells conferred a similar CD4 down-modulation as observed with

PBMC (Figure 4.3A, third panel, and B), whereas NK cells depleted PBMC induced a reduced effect (Figure 4.3A, third panel, and B). Although the results showed some variations between cells tested from different donors, they clearly indicated that enriched NK cells were able to confer CD4 down-modulation, whereas they are not the only and most important immune cell subset, because PBMC depleted of NK cells still were able to induce CD4 down-modulation. In a next experiment, BT-061 decorated T cells were incubated with enriched monocytes or PBMC depleted of monocytes. Interestingly, isolated monocytes induced a stronger CD4 down-modulation than NK cells, whereas PBMC depleted of monocytes caused only minor CD4 down-modulation (Figure 4.3A, fourth panel, and B). Importantly, monocytes are the most abundant antigen presenting subset in PBMC representing approximately 15-20% of all nucleated cells [202]. Thus, the experiments indicated that monocytes represented the immune cell subset that critically contributed to CD4 down-modulation of BT-061-decorated T cells.

4.1.4. Analysis concerning the contribution of secreted factors to the induction of CD4 down-modulation on BT-061 decorated T cells

To determine whether cell-cell interaction alone or in addition some soluble factors secreted by monocytes or T cells played a critical role in antibody-mediated CD4 down-modulation of BT-061 decorated T cells, cell-free supernatants of BT-061 decorated T cells co-incubated for 18 h with monocytes were isolated and added to BT-061 treated T cells.

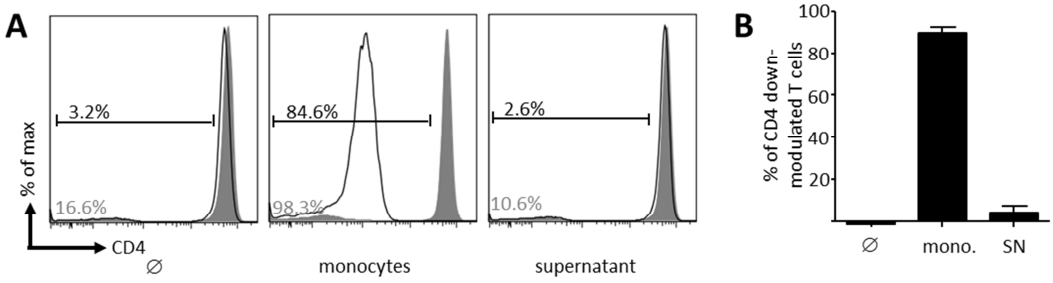

Figure 4.4: Soluble factors secreted by cells that were involved in CD4 down-modulation were not sufficient to confer CD4 down-modulation of BT-061 decorated T cells. (A) T cells were incubated in medium (filled grey) or medium supplemented with BT-061 at a concentration of 10 µg/ml (black line). After washing either medium was added (left panel), or the cells were co-incubated with $8x10^5$ monocytes (middle panel), or they were incubated in cell-free supernatant taken from BT-061 decorated T cells co-incubated with monocytes for 18 h (right panel). After 18 h of incubation at 37°C the CD4 expression was determined

cytofluometrically. Values printed in black indicate the percentage of gated T cells that showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the $CD8^-$ T cell population. One representative result obtained from 2 similar experiments with cells derived of a total of 3 donors is shown. (B) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation. Error bars indicate SEM.

Upon incubation of BT-061 decorated T cells with conditioned supernatant only very minor CD4 down-modulation was observed (Figure 4.4A, right panel, and B), which was comparable with the CD4 down-modulation induced by the addition of soluble antibody to T cells alone (Figure 4.4A, left panel, and B). Thus, the data indicated that soluble factors secreted by cells that were involved in CD4 down-modulation were not sufficient to confer CD4 down-modulation of BT-061-decorated T cells.

4.2. Characterization of FcγR interactions in BT-061 induced CD4 down-modulation of T cells

The above experiments indicated that BT-061 decorated T cells needed interaction with monocytes and maybe some other less dominant cell subsets in order to confer CD4 down-modulation. Fragment, crystallizable γ receptor (FcγR) are known to be differentially expressed on immune cells and to interact with the Fc moiety of IgG. The FcγR in the human system are classified in three main types, the high affinity CD64 and the medium or low affinity receptors CD32 and CD16 [73]. BT-061 is designed as an IgG1 antibody. Although to date many licensed antibodies are IgG1 molecules, their interaction with FcγR expressing immune cell subsets is still not fully understood.

To study the role of FcγR in BT-061 mediated CD4 down-modulation, and in particular, to dissect the contribution of CD64 vs. CD32 and CD16, transfected cell lines stably expressing the extracellular portion of the human FcγR CD16, CD32A, CD32B or CD64 were used (Figure 4.5). The cells have been generated by transfection of a murine thymoma cell line with constructs encoding chimeric FcγR-ζ chain proteins.

4.2.1. Analysis of the of the interaction of FcγR expressing transfectants with plate bound BT-061

To control whether the different transfected cell lines expressed the desired FcγR on the surface the expression levels of the respective receptors were determined cytofluometrically

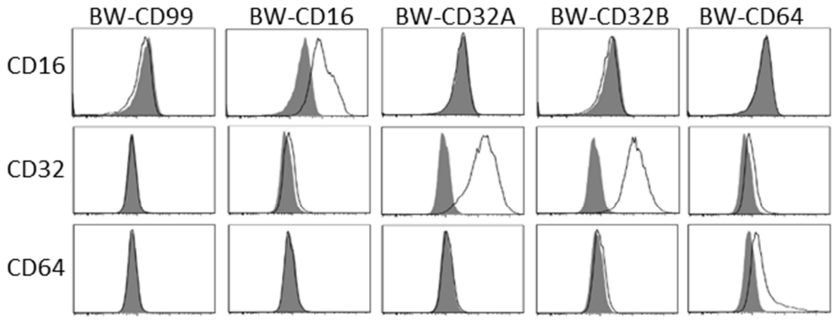

Figure 4.5: FcγR expression level of differently transfected cell lines. $2x10^5$ cells of the indicated transfectants were stained with antibodies specifically binding the FcγR CD16, CD32 or CD64 and the respective isotype controls. The expression of CD16, CD32 and CD64 (black lines) or the appropriate isotype controls (filled grey) were monitored cytofluometrically. Representative data from 3 similar experiments are shown.

The data obtained by FACS analysis, revealed that cells, which were transfected with a construct containing the sequence for the extracellular portion of the human FcγR CD16, displayed CD16 on the surface and showed no expression of the other FcγR CD32 or CD64. The examination of cells, which were transfected with constructs containing the sequence for the extracellular portion of the human FcγR CD32A, B and CD64, revealed that the different cell lines expressed exclusively the expected receptors on the cell surface (Figure 4.5).

As mentioned, the transfectants were engineered to express the extracellular domain of each human FcγR. This extracellular domain is linked to an intracellular ζ-chain of the murine T cell receptor (TCR). In addition, these cells carry an intracellular response element that leads to the production of murine interleukin (IL)-2 once a signal via the ζ-chain of the murine confers a signal into the cell. This signaling can only occur when the FcγR is cross-linked at the cell surface. Thus, the amount of IL-2 production is directly related to the cross-linking of the FcγR and indicates the quantity of the interaction with the tested antibody. To determine the interaction of FcγR expressing transfectants with plate bound BT-061, the mAb was coated to plastic surface and the transgenic cell lines (one cell line for each FcγR)

were added. After incubation for 18 h at 37°C the supernatant was harvested and the amount of produced IL-2 was measured using an enzyme-linked immunosorbent assay (ELISA) method.

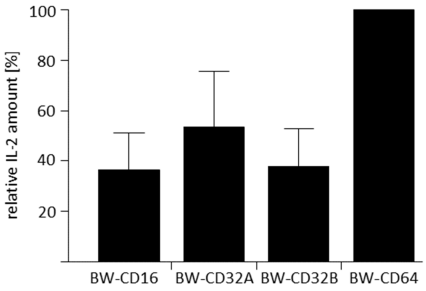

Figure 4.6: CD64 expressed by a murine transfected cell line revealed the strongest interaction with BT-061. $2x10^5$ FcγR expressing cells were added to untreated wells or wells coated with 12.5 µg/ml BT-061. The cells were incubated for 18 h at 37°C. Afterwards cell-free supernatant was collected and the amount of IL-2 was determined by an ELISA method. The total IL-2 amount released by CD64 expressing transfectants was set 100% and the IL-2 amount released by the other transfectants is calculated relative to that value. Statistical analysis of 2 similar experiments, whereas one is performed in doublets and the other one in triplicates, are shown. Error bars indicate SEM.

Due to the fact that the total amount of measured IL-2 varied considerably between different experiments, in Figure 4.6 the relative amount of produced IL-2 is indicated. The results of all tested FcγR expressing transfectants showed that the variable FcγR expressing transfectants differed in their interaction intensities with coated BT-061. The transfectant expressing the high affinity receptor CD64 released the highest amount of IL-2 upon incubation with coated BT-061 in comparison to the other FcγR expressing transfectants. The amount of secreted IL-2 using the CD32A expressing transfectant amounted 54% of the CD64 expressing transfectant. The receptors CD16 and CD32B interacted weakly with the coated mAb. The CD16 expressing transfectant reached 36% of the relative IL-2 amount compared to the CD64 expressing transfectant, and the CD32B expressing transfectant 38% (Figure 4.6). These data indicated that CD64 expressed on a transfected murine cell line showed the strongest interaction with coated BT-061 compared with CD16, CD32A and CD32B measured by the secreted amount of IL-2.

4.2.2. Investigation of the potential of different FcγR expressing transfectants to mediate CD4 down-modulation

In the next set of experiments it was examined whether the potential of different FcγR expressing transfectants to interact with coated BT-061 correlated with the potential to mediate CD4 down-modulation on BT-061 decorated T cells. Therefore, BT-061 treated T cells were incubated with FcγR expressing transfectatants and CD4 expression was examined cytofluometrically after 18 h co-culturing.

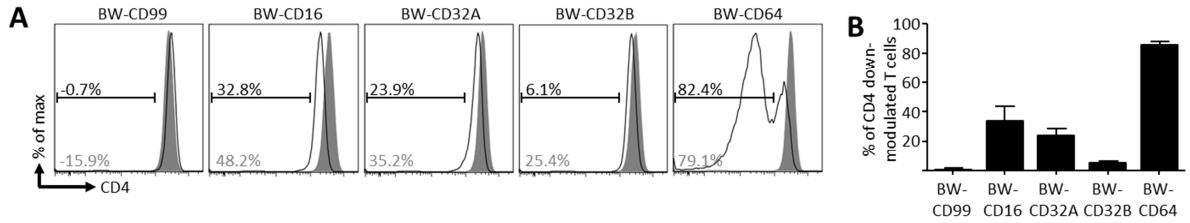

Figure 4.7: The high affinity FcγR CD64 expressed by a murine transfected cell line conferred the highest BT-061 mediated CD4 down-modulation on T cells. (A) BT-061 decorated T cells prepared as described in the legend of Figure 4.3 were co-incubated with $8x10^5$ transfectants expressing the human extracellular domain of CD99 (MIC domain as negative control), or the human extracellular domain of CD32A, CD32B or CD64. After 18 h of incubation at 37°C CD4 expression was determined cytofluometrically. Values printed in black indicate the percentage of gated T cells that showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the $CD8^-$ T cell population. Representative data are shown of 2 similar experiments performed with cells derived from 4 donors (co-stimulation with BW-CD99 expressing transfectants), 3 experiments with cells derived from 7 donors (co-stimulation with BW-CD16 expressing transfectants), 3 experiments with cells derived from 7 donors (co-stimulation with BW-CD32A expressing transfectants), 2 experiments with cells derived from 4 donors (co-stimulation with BW-CD32B expressing transfectants), and 3 experiments with cells derived from 7 donors (co-stimulation with BW-CD64 expressing transfectants). (B) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation. Error bars indicate SEM.

The data demonstrated that the transfectant expressing the high affinity receptor CD64 was most potent in conferring CD4 down-modulation on BT-061 decorated T cells (Figure 4.7A, fifth panel, and B). The co-culturing of BT-061 decorated T cells with CD64 expressing transfectants induced in 86% of T cells a significant CD4 down-modulation (Figure 4.7B). Even at reduced cell numbers of $2x10^5$ cells, instead of the shown $8x10^5$ cells, the CD64 expressing transfectant maintained its ability to robustly down-modulate CD4 (data not shown). In contrast, the co-incubation of mAb treated T cells with CD32B expressing

transfectants only marginally affected CD4 surface expression (Figure 4.7A, fourth panel, and B) compared to a CD99 (mic domain, irrelevant molecule) expressing control transfectant (Figure 4.7A, first panel, and B). CD16 (Figure 4.7A, second panel, and B) and CD32A expressing transfectants (Figure 4.7A, third panel, and B) influenced CD4 down-modulation to a higher extent than CD32B expressing transfectants but lesser intense than CD64 expressing transfectants. The incubation with CD16 expressing transfectants induced in 35% of T cells a significant down-modulation of CD4, whereas CD32A expressing transfectants affected 35% of the analyzed T cells (Figure 4.7B). The data revealed that interaction of different FcγR expressing transfectants with coated BT-061, quantified by measuring secreted IL-2, correlated partially with the potential of the transfected cells to mediate CD4 down-modulation on BT-061 decorated T cells. However, both experimental approaches indicated that CD64 is most potent compared to the other FcγR to interact with BT-061 and thereby inducing CD4-down-modulation in BT-061 decorated T cells.

4.2.3. Influence of FcγR expressed by immune cells on CD4 down-modulation of BT-061 decorated T cells

In the following set of experiments the role of FcγR expressed on human immune cells in BT-061 mediated CD4 down-modulation was examined cytofluometrically. These investigations help to investigate immune cells potentially interacting with the mAb after administration.To examine, which immune cell subsets possesses the potential to interact via its FcγR with BT-061, the surface expression of FcγR on MACS enriched immune cell subsets were analyzed cytofluometrically.

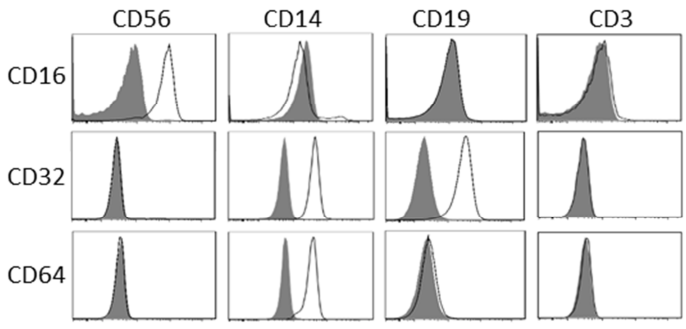

Figure 4.8: FcγR expression on human immune cells. NK cells, monocytes, B cells and T cells were enriched using the MACS method and stained with subset specific markers using anti-CD56 for NK cells, anti-CD14 for monocytes, anti-CD19 for B cells and anti-CD3 for T cells.

They were co-stained with antibodies for human CD16, CD32 and CD64 (black line) or with the respective isotype controls (filled grey). Representative data from 3 similar experiments are shown.

The obtained data revealed that $CD56^+$ NK cells exclusively expressed CD16 (Figure 4.8, first row). A minor population of $CD14^+$ monocytes displayed CD16 on the cell surface, whereas the predominant part expressed the high affinity receptor CD64 and the low affinity receptor CD32 (Figure 4.8, second row). $CD19^+$ B cells showed exclusively expression of CD32 (Figure 4.8, third row), which is known to be the inhibitory receptor CD32B [203]. A rare population of T cells displayed CD16 on the surface (Figure 4.8, fourth row). The results shown in Figure 4.8 were in line with former reports concerning the expression of FcγR on the analyzed immune cells.

4.2.4. Blockade of CD64 on monocytes reduces CD4 down-modulation on BT-061 decorated T cells

Combining the observation that monocytes are the only identified immune cell subtype expressing CD64 (Figure 4.8) and that they were the most potent analyzed immune cell subset in conferring CD4 down-modulation on BT-061 treated T cells (Figure 4.3A and B), the question arose, whether CD64 expressed by monocytes was crucial for CD4 down-modulation. To address this hypothesis the receptor CD64 on monocytes was blocked when monocytes were incubated with BT-061 decorated T cells. Afterwards CD4 down-modulation was analyzed cytofluometrically.

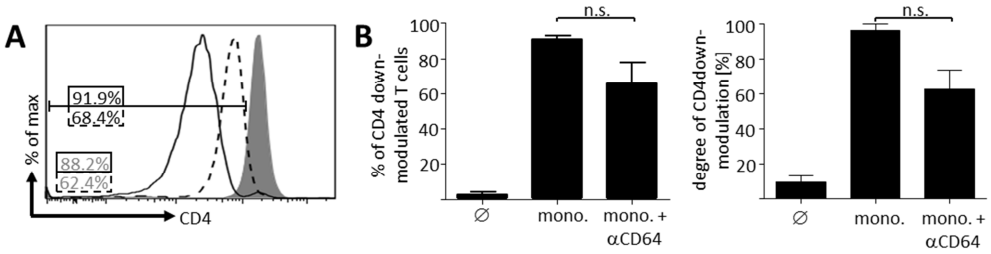

Figure 4.9: The blockade of CD64 reduced monocyte induced BT-061 mediated CD4 down-modulation of T cells. (A) $8x10^5$ monocytes were incubated with an anti-CD64 blocking antibody (50 µg/ml) (black dotted line) or medium (continuous black line) for 45 min at 37°C. Afterwards the differently treated monocytes or medium (filled grey) were added to BT-061 (10 µg/ml) decorated T cells. After 18 h incubation at 37°C CD4 expression was determined cytofluometrically. Values printed in black indicate the percentage of gated T cells that

showed CD4 down-modulation, whereas values printed in grey indicate the degree of CD4 down-modulation of the $CD8^-$ T cell population. In the continuously framed boxes values of CD4 down-modulation of T cells are indicated for BT-061 decorated T cells co-incubated with monocytes, whereas in the dotted framed boxes values for CD4 down-modulation of BT-061 decorated T cells co-incubated with and anti-CD64-pretreated monocytes are shown. One representative experiment out of 2 similar ones with cells derived from 3 donors is shown. (B) Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation (left graph) and the degree of CD4 down-modulation of the $CD8^-$ T cell population (right graph). Error bars indicate SEM.

The results of all performed experiments showed that monocytes induced a significant CD4 down-modulation on BT-061 treated T cells in 91% of the T cells population. However, the blockade of CD64 on monocytes reduced the effect to 66% (Figure 4.9B, left graph). This difference mediated by anti-CD64-pre-treatment of monocytes was even more pronounced considering the degree of CD4 down-modulation (Figure 4.9A and B, right graph). Although the statistical analysis revealed that blockade of CD64 did not significantly reduced CD4 down-modulation mediated by monocytes on BT-061-treated T cells, a decrease of monocytes-mediated CD4 down-modulation was measured in every performed experiment when anti-CD64 was added. Experiments to identify concentrations of the anti-CD64 blocking antibody, which would totally block CD64 on the CD64 expressing cell line, revealed that even at high concentrations the reagent was not able to completely inhibit CD64 mediated CD4 down-modulation on BT-061 decorated T cells (data not shown). Therefore, the remaining CD4 down-modulation might be due to an incomplete inhibition of CD64 by the blocking reagent. Altogether, the results demonstrate that CD64 on monocytes considerably participated in monocyte mediated CD4 down-modulation on BT-061 decorated T cells.

4.2.5. Analysis of CD4 down-modulation mediated by serum treated monocytes and IFN-γ stimulated neutrophils

Previous studies suggested that CD64 displayed on monocytes was saturated under steady state conditions with naturally produced IgG in the serum [204]. Therefore, to examine whether serum treatment of monocytes influenced monocyte mediated CD4 down-modulation on BT-061 decorated T cells, monocytes were pre-incubated with concentrations of human serum ranging from 0.1 to 10% before co-culturing with BT-061 decorated T cells.

Furthermore, previous reports indicated that during a proinflammatory state, like during the active state of autoimmune diseases, the expression patterns of FcγR may vary on immune cells, like neutrophils. To mimic proinflammatory conditions it has been suggested to treat neutrophils with IFN (Interferon)-γ [205]. To investigate whether expression of FcγR on neutrophils changed under proinflammatory conditions, neutrophils were incubated with IFN-γ and afterwards the expression of CD16, CD32 and CD64 was analyzed cytofluometrically. Furthermore, the capacity of neutrophils to down-modulate CD4 on BT-061 decorated T cells should be determined.

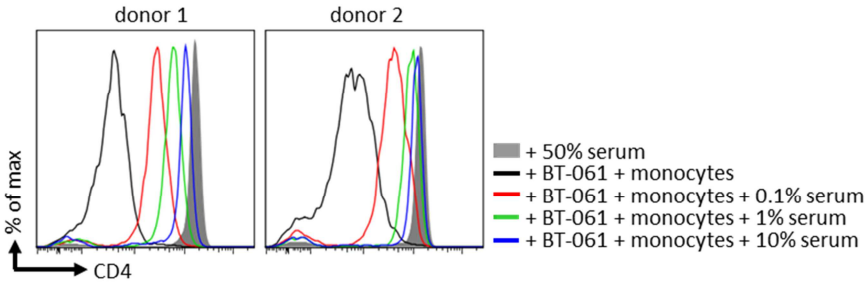

Figure 4.10: CD4 down-modulation on BT-061 decorated T cells induced by monocytes was diminished by serum treatment. $8x10^5$ monocytes were incubated in medium enriched with AB serum at concentrations ranging from 0.1 to 10% of serum (0.1% red line, 1% green line and 10% blue line) or medium (black line) for 45 min at 37°C. Afterwards the differently treated monocytes or medium supplemented with 50% serum (filled grey) were added to untreated or BT-061 (10 µg/ml) decorated T cells. After 18 h of incubation at 37°C the CD4 expression was determined cytofluometrically. Data from one experiment with cells derived from two individual donors are shown.

Pre-treatment of monocytes with 0.1% serum massively reduced BT-061 mediated CD4 down-modulation induced by monocytes (Figure 4.10, red line) compared to CD4 down-modulation induced by untreated monocytes (Figure 4.10, black line). The increase of serum concentrations to 10% enhanced the inhibitory effect on CD4 down-modulation conferred by monocytes on mAb treated T cells (Figure 4.10, blue line). Higher concentrations of up to 50% serum showed no enhanced inhibitory effect on monocyte induced BT-061 mediated CD4 down-modulation on T cells (data not shown). Pre-treatment of monocytes from two donors with concentrations of serum ranging from 0.1 to 10% revealed that Ig containing serum inhibited monocyte conferred CD4 down-modulation on BT-061 decorated T cells.

In the next set of experiments the potential of neutrophils to mediated CD4 down-modulation on BT-061 decorated T cells should be investigated. It is known that on neutrophils the degree of FcγR surface expression differs between steady state and

proinflammatory conditions [205, 206]. Therefore FcγR expression on untreated and IFN-γ treated neutrophils was determined cytofluometrically.

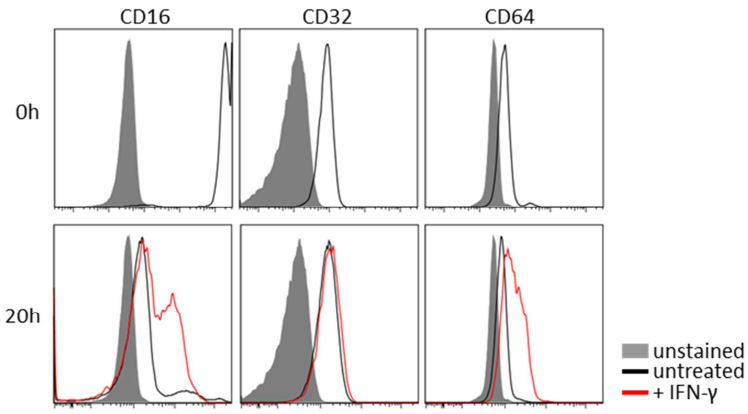

Figure 4.11: CD64 on neutrophils was up-regulated upon IFN-γ treatment. $8x10^5$ neutrophils were seeded and treated with 500 U/mL IFN-γ for 20 h. The FcγR expression was measured immediately after the isolation (0 h) and following the incubation with medium or IFN-γ (22 h). The cells were stained with anti-CD16-PE, anti-CD32A-FITC and anti-CD64-FITC. The surface expression was determined cytofluometrically. The exemplary data from 2 experiments with a total of 2 donors are shown.

The examination of FcγR expression on neutrophils revealed that these cells expressed high levels of CD16 and displayed moderate levels of CD32 on the cell surface after isolation from buffy coats (Figure 4.11, 0 h). The high affinity receptor CD64 was expressed on a low level on cells surface. The incubation for 20 h without the addition of IFN-γ clearly increased FcγR expression of CD16 on the cells. The additional treatment with IFN-γ enhanced clearly CD64 and CD16 surface expression in comparison to untreated controls (Figure 4.11, 20 h). The determination of the expression levels of the activation markers CD63 and CD18, revealed that these surface molecules were up-regulated after isolation from buffy coats compared to unstained controls (data not shown). Because, neutrophils mediated CD4 down-modulation on T cells after co-culturing, which was independent from BT-061 treatment of T cells (data not shown), it could not be clarified whether neutrophils participated in BT-061 mediated CD4 down-modulation on T cells. This might be due to a pre-activated status of the neutrophils after isolation, indicated by the up-regulation of the activation markers. In summary, it was not fully reproducible *in vitro* whether neutrophils conferred BT-061 mediated CD4 down-modulation under steady state or proinflammatory conditions. However, a clear up-regulation of CD16 and CD64, which are shown to mediate CD4 down-modulation on BT-061 decorated T cells, upon IFN-γ treatment was detected.

4.2.6. Investigations concerning the capacity to down-modulate CD4 on BT-061 treated T cells by monocyte derived imDC and cells prepared from human tonsils

Although therapeutic mAb initially encounter immune cells represented in the peripheral blood upon i.v. application, they gradually accumulate in other organs and tissues. Furthermore BT-061 is also applied via the *sub cutaneous* (s.c.) route. In the following experiments the role of immune cells, which are typically found in the skin or lymphoid tissues, which also might interact with BT-061, was investigated.

Because dendritic cells (DC) are located e.g. in the skin (known as Langerhans cells [207, 208]), their capacity to interact with BT-061 decorated T cells needed to be clarified. In order to identify the role of immature (im)DC in the interaction with BT-061 it was necessary to determine the expression pattern of FcγR. Furthermore, the potential of imDC to down-modulate CD4 on BT-061 treated T cells was tested cytofluometrically. ImDC are represented in small amounts within PBMC isolated from buffy coats, therefore imDC derived from monocytes were used to obtain satisfactory amounts of cells.

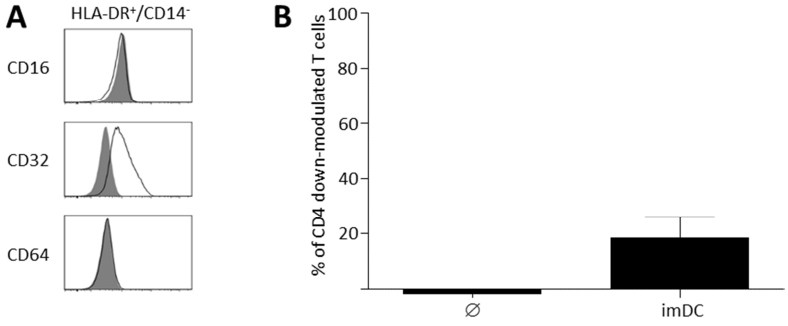

Figure 4.12: imDC only marginally conferred CD4 down-modulation of BT-061 decorated T cells. (A) $2x10^5$ HLA-DR$^+$ and CD14$^-$ monocyte derived DC were stained with FcγR specific antibodies for CD16, CD32 or CD64 (black line) and the respective isotype controls (filled grey). The exemplary data from 2 experiments with a total of 5 donors are shown (B) $2x10^5$ purified T cells were treated for 45 min at 4°C with medium or with 10 µg/ml BT-061. Upon washing, T cells were co-cultured for 18 h at 37°C with medium or $8x10^5$ syngenic imDC. Afterwards CD4 expression was determined cytofluometrically. Statistical analysis of the percentage of CD4 down-modulated T cells of all experiments is shown. The results were obtained from one experiment with cells derived from a total of 4 donors. Error bars indicate SEM.

The measurement of FcγR on monocyte-derived imDC revealed that these cells exclusively display CD32 on the surface, whereas the used flourochrome-labeled mAb is not able to distinguish between CD32A and CD32B (Figure 4.12A). Previous data showed that CD32A as well as CD32B is expressed by monocyte-derived imDC [209]. After the co-incubation of BT-061 decorated T cells with imDC, CD4 was down-modulated in 19% of analyzed T cells (Figure 4.12B). In comparison to CD4 down-modulation observed with enriched immune cells like monocytes that induced a CD4 down-modulation in 88% of T cells (Figure 4.3B), the effect of imDC on CD4 down-modulation upon BT-061 treatment appeared marginal.

Because it is likely that therapeutic mAb accumulate in the draining lymph node upon s.c. administration, it was examined whether CD4 is down-modulated upon application of BT-061 in cells prepared from tonsils. Therefore, single cell suspensions were prepared from tonsils, which derive so called tonsillar mononuclear cells (TMC). TMC were treated with BT-061 at concentrations ranging from 0.1 to 30 µg/ml and CD4 expression was determined cytofluometrically. Furthermore, FcγR expression pattern and composition of immune cell subsets of TMC were investigated.

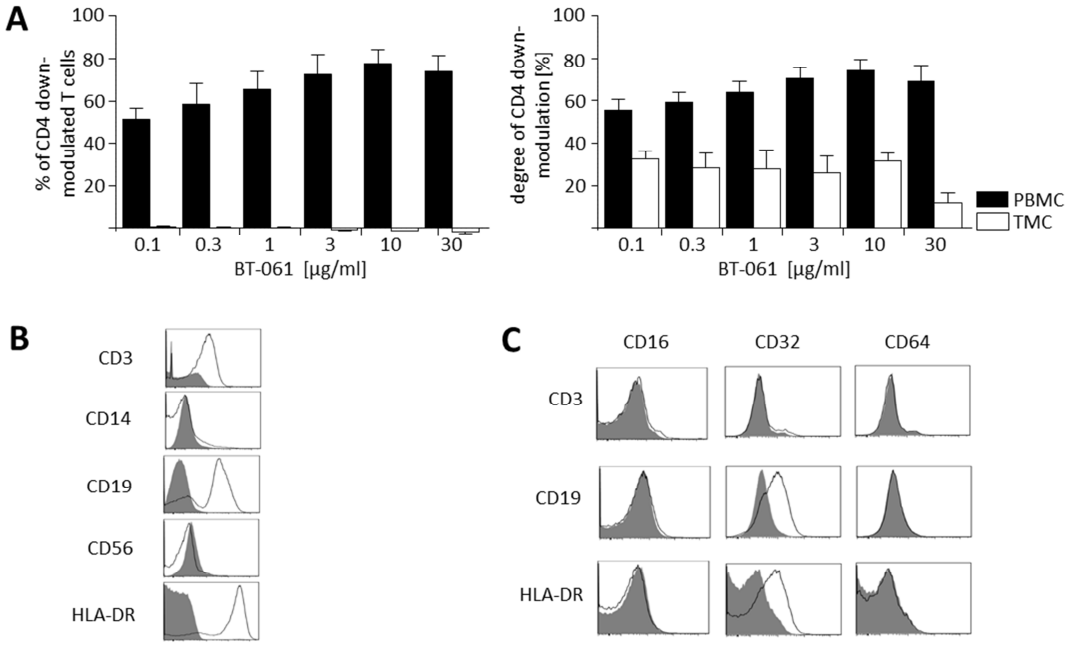

Figure 4.13: Upon BT-061 treatment, T cells in TMC show reduced CD4 down-modulation when compared with T cells in PBMC. (A) 8x10^5 PBMC (black filled bars) or TMC (white filled bars) were treated for 45 min at 4°C with BT-061 at concentrations ranging from 0.1 to 30 µg/ml. Then cells were washed and incubated for additional 18 h at 37°C. Afterwards CD4 expression was determined cytofluometrically. Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation (left graph) and of the degree of CD4 down-

modulation of the $CD8^-$ T cells population (right graph) is shown for data of 2 similar experiments with cells derived from a total of 3 donors (TMC) and 2 similar experiments with cells derived from a total of 3 to 5 donors (PBMC). (B) TMC were stained with subset specific markers for CD3, CD14, CD19, CD56 and HLA-DR (black line), whereas unstained cells were used as negative control (filled grey). Data from one experiment with cells derived from one donor are shown. (C) TMC were stained with subset specific markers for CD3, CD19 and HLA-DR and additionally with antibodies for CD16, CD32 and CD64 (black line) or with the respective isotype controls (filled grey). Data from one experiment with cells derived from one donor are shown.

The experiments with TMC revealed that BT-061 treatment at concentrations ranging from 0.1 to 30 µg/ml did not affect the percentage of CD4 down-modulated T cells within TMC compared to untreated controls. Interestingly, the same concentrations of BT-061 induced in T cells within PBMC, a significant CD4 down-modulation. Even at a concentration of 10 µg/ml of BT-061 that was most effective in PBMC, no significant CD4 down-modulation of T cells within TMC was induced (Figure 4.13A). The examination of TMC incubated with soluble BT-061 revealed that the degree of CD4-down-modulation was affected, but less pronounced than in PBMC treated with the same mAb concentrations. Furthermore no enhanced effect on the degree of CD4 down-modulation was detectable with increasing mAb concentrations, which was observed in PBMC (Figure 4.13). Analysis of the composition of immune cells being represented in TMC indicated that T cells, which are $CD3^+$, were present in TMC. Furthermore, $CD19^+$ B cells and $CD14^-HLA\text{-}DR^+$ DC were detected within single cell suspensions prepared from tonsils. The investigations demonstrated that no $CD56^+$ NK cells were present in the prepared TMC (Figure 4.13B). The analysis of FcγR expression of $CD3^+$ T cells from TMC showed, if at all a minor population of T cells expressed CD16. $CD19^+$ B cells and $CD14^-HLA\text{-}DR^+$ were shown no be exclusively positive for the expression of CD32, whereas CD64 was detected on no of the tested cell populations (Figure 4.13C). From this data it was concluded that cells within TMC were able to affect the degree of CD4 down-modulation of T cells, but to a lesser extent than cells within PBMC. Interestingly, the percentage of CD4 down-modulated T cells was not influenced upon BT-061 treatment, whereas the treatment of PBMC with the mAb showed a clear effect on that parameter.

The data suggested that BT-061 needed to be cross-linked to induce a decrease of CD4 on T cells upon BT-061 treatment. This cross-linking was mediated within PBMC by monocytes expressing the FcγR CD64. The high affinity receptor was sufficient to induce a CD4 down-modulation on BT-061 decorated T cells, whereas secreted factors were not crucial to

minimize CD4 expression. Additional *in vitro* experiments gave reasons to believe that *in vivo* CD64 on monocytes is partially saturated by serum Ig. This was hypothesized, because BT-061 mediated CD4 down-modulation on mAb treated T cells induced by monocytes was clearly abrogated upon pre-incubation of monocytes with human serum. The obtained data also revealed that imDC hardly mediated CD4 down-modulation on BT-061 decorated T cells. Interestingly, no effect on the percentage of CD4 down-modulated T cells was detectable within TMC upon BT-061 treatment at all applied concentrations.

4.3. Characterization of CD4 down-modulation, its implication on signaling cascade induction and activation status of T cells

In the previous chapter the cellular requirements, kinetics and dose dependency, of CD4 down-modulation on T cells upon BT-061 treatment were analyzed. To gain insight into the mode of action of BT-061 and its impact on targeted T cells, it was important to identify e.g. involved signaling pathways or activation status of T cells upon treatment with BT-061. The antibody was developed for the therapy of the autoimmune diseases psoriasis and RA. The question arose how binding of the CD4 molecule – expressed on T helper cells (Th cells) and regulatory T cells (Treg cells) – by BT-061 could stimulate T cells to improve the disordered conditions during an autoimmune disease. The following sets of experiments were performed to investigate induced signaling cascades, involved signaling molecules or released cytokines upon BT-061 treatment.

4.3.1. Influence of protein biosynthesis and activation of LcK on BT-061 mediated CD4 down- modulation on T cells

In order to evaluate the contribution of protein biosynthesis in the renewal of CD4 after BT-061 mediated down-modulation cycloheximide (CHX) was used. CHX is primarily used as an inhibitor of protein biosynthesis in eukaryotic organisms [210]. Thereby CHX enables the analysis of the participation of short-lived molecule(s) on variable effects, because these structures are sensitive to the exposure of CHX. Furthermore CHX is used to examine whether synthesis of new proteins is needed for an analyzed effect or whether the involved proteins are permanently present in the cell. To investigate the dependency on protein

biosynthesis of BT-061 induced CD4 down-modulation, $CD4^+$ T cells were pre-treated with CHX at a concentration of 10 µg/ml and afterwards incubated on coated BT-061. After 3 h the percentage of CD4-down-modulated T cells was analyzed cytofluometrically. In another experiment the involvement of a T cell specific pathway was investigated. In general triggering via the TCR includes amongst other events the phosphorylation of lymphocyte protein-tyrosine kinase (Lck) and ζ-chain-associated protein kinase 70 (Zap-70) (for more details see chapter 2.1.3). To investigate the role of Lck activation in BT-061 mediated CD4 down-modulation the sarcoma (Src)-kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]- Pyrimidin 1 (PP1) was used. PP1 is known to inhibit Src kinases [183], but it is most specific for Lck in T cells [211-213]. To elucidate the dependency of BT-061 mediated CD4 down-modulation on Lck activation PBMC were pre-treated with PP1 at concentrations ranging from 5 to 40 µM. Afterwards the cells were incubated in wells coated with BT-061 at a concentration of 10 µg/ml. PBMC were cultured for 6 and 18 h and the percentage of CD4 down-modulated T cells was measured cytofluometrically.

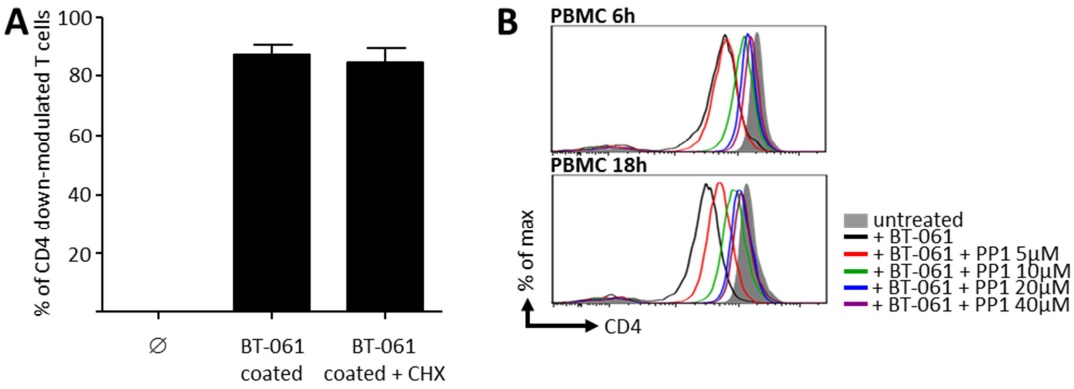

Figure 4.14: BT-061 mediated CD4 down-modulation did not depend on protein biosynthesis, but partially on activation of Lck. (A) $2x10^5$ MACS enriched $CD4^+$ T cells were pre-treated for one h at 37°C with medium or medium supplemented with CHX at a concentration of 10 µg/ml. Afterwards the cells were seeded in untreated or BT-061 (10µg/ml) coated wells and incubated for 3 h. Then cells were stained and CD4 expression was determined cytofluometrically. Statistical analysis of the percentage of gated T cells that showed CD4 down-modulation of one experiment with cells derived from 3 donors is shown. Error bars indicate SEM. (B) $8x10^5$ PBMC were pre-treated 45 min with medium or medium supplemented with PP1 at a concentration of 5, 10, 20 or 40 µM at 37°C. Afterwards the cells were washed and incubated for 6 and 18 h at 37°C in medium, medium supplemented with BT-061 at a concentration of 10µg/ml or medium supplemented with PP1 (at concentrations ranging from 5 to 40 µM) and BT-061 (10µg/ml). Subsequently the cells were stained and CD4 surface expression was determined cytofluometrically. One experiment with cells derived from a total of 3 donors is shown.

3 h after seeding 87% of T cells cultured on immobilized BT-061 at a concentration of 10 µg/ml showed significant CD4 down-modulation compared to T cells incubated with medium. Interestingly, when T cells were pre-treated with CHX and incubated on coated BT-061, CD4 was significantly down-modulated in 85% of the analyzed T cells (Figure 4.14A). These results revealed that the pre-treatment of T cells with the protein biosynthesis inhibitor CHX did not abolish CD4 down-modulation induced by coated BT-061. Furthermore, the data indicated that molecules needed to induce the reduction of CD4 on the surface of T cells did not belong to the group of short-lived molecules or needed to be synthesized upon BT-061 treatment.

The experiments performed with the Src inhibitor PP1 revealed that 6 h after addition of soluble BT-061 at a concentration of 10 µg/ml to PP1 (5 µM) pre-treated PBMC no influence of Lck blockade on BT-061 mediated CD4 down-modulation compared to PBMC, which were not incubated with PP1, was detected (Figure 4.14, upper panel, black and red line). However, after 18 h a clear reduction of BT-061 mediated CD4 down-modulation on T cells upon pre-treatment with PP1 at a concentration of 5 µM was observed (Figure 4.14, lower panel, black and red line). This effect was even more pronounced with higher dosages of PP1 ranging from 10 to 40 µM after 6 and 18 h (Figure 4.14, upper and lower panel, green, blue and purple line). Interestingly, even the pre-treatment with 40 µM PP1 did not inhibit the antibody induced CD4 down-modulation completely, although according to manufacturers' instructions the half maximal inhibitory concentration (IC50) is at 5 µM to specifically inhibit Lck [183]. These data revealed that BT-061 mediated CD4 down-modulation in T cells was partially dependent on activation of Lck.

4.3.2. Impact of protein biosynthesis on recovery of CD4 surface expression upon BT-061 mediated CD4 down-modulation

Previously performed experiments indicated that upon BT-061 mediated CD4 down-modulation the receptor reappeared on the surface of T cells after approximately 18 or 24 h (data not shown). The data shown in Figure 4.14A demonstrated that CD4 down-modulation on T cells induced by immobilized BT-061 was not affected by the blockade of protein biosynthesis, but the influence on the reappearance still needed to be investigated. In order

to evaluate the contribution of protein biosynthesis in the recovery of CD4 after BT-061 mediated down-modulation, $CD4^+$ T cells were incubated on coated BT-061 at a concentration of 10 µg/ml to down-modulate CD4. After 3 h cells were harvested from mAb coated wells and were cultured in medium and medium supplemented with CHX at a concentration of 10 µg/ml. The percentage of CD4 down-modulation was examined after 0, 24, 48 and 72 h cytofluometrically.

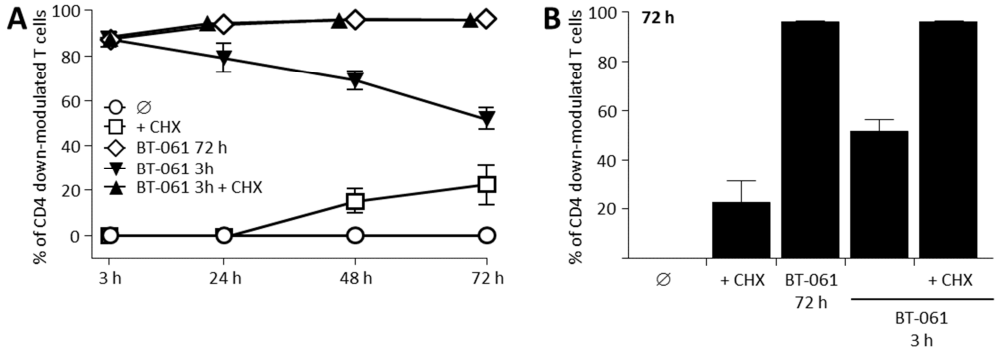

Figure 4.15: The recovery of CD4 on the surface of T cells after down-modulation induced by BT-061 depended on CD4 *de novo* synthesis. (A) $2x10^5$ MACS enriched $CD4^+$ T cells were incubated for 3 h in untreated wells, in medium supplemented with CHX at a concentration of 10µg/ml or in wells coated with BT-061 at a concentration of 10µg/ml, . Afterwards untreated cells were harvested and further cultivated in untreated wells (○), whereas CHX pre-treated cells were harvested and further cultivated in medium supplemented with CHX (10 µg/ml) (□). BT-061 treated T cells were harvested and further cultured on coated BT-061 (10 µg/ml)(◇), in medium (▼) or in medium supplemented with CHX (10 µg/ml) (▲).Every 24 h CHX was added to the respective wells at a concentration of 10 µg/ml. The CD4 expression was determined cytofluometrically after 3, 24, 48 and 72 h. The statistical analysis of the percentage of gated T cells that showed CD4 down-modulation of one experiments with cells derived from a total of 3 different donors is shown. (B) Depiction of the percentage of gated T cells that showed CD4 down-modulation after 72 h in a bar diagram. The data are based on the same experiments as shown in (A). Error bars indicate SEM.

When T cells were incubated for 3 h in wells treated with coated BT-061 CD4 was, as expected from previous experiments, significantly down-modulated in 87% of the analyzed T cells (Figure 4.15A). Interestingly, when down-modulated T cells were after 3 h harvested and further incubated in medium the percentage of significant CD4 down-modulated T cells decreased to 52% after 72 h (Figure 4.15A and B). In contrast, when T cells, which were incubated for 3 h on coated BT-061, were harvested and incubated in medium supplemented with CHX, the percentage of significant CD4 down-modulated T cells did not decrease (Figure 4.15A and B). However, the percentage of CD4-down-modulated T cells

upon culturing on immobilized BT-061 for 3 h and after cultivation in untreated wells did not reach the level of CD4 expression of untreated T cells, although a clear tendency of recovery of CD4 surface expression was observed. An elongation of the experiment was not possible, due to the fact that culturing of human primary T cells is no longer feasible. The results obtained from T cells cultured with CHX for 3 days also demonstrated that even without the addition of BT-061 CD4 expression decreased. Therefore it was hypothesized that CHX treatment affected natural turnover of CD4 and thereby induced a down-modulation of CD4 independent from BT-061. Additionally the results demonstrated that recovery of CD4 surface expression upon BT-061 mediated down-modulation required CD4 *de novo* synthesis, whereas CD4 recycling from internal pools did not contribute to that recovery.

4.3.3. Effect of various cross-linking procedures of BT-061 on different T cells surface markers

In a next set of experiments the impact of BT-061 on the expression of important T cell surface markers such as CD2, CD3, CD25, CD28, CD69 and CD86 was determined. In addition to the influence of BT-061 treatment on the expression of surface molecules on T cells the effects of different cross-linking procedures of the mAb including coating of BT-061, co-culturing with monocytes, and co-culturing with CD64 expressing transfectants were studied. Therefore T cells were incubated for 18 h under the described conditions and surface expression of CD2, CD3, CD25, CD28, CD69 and CD86 was determined cytofluometrically.

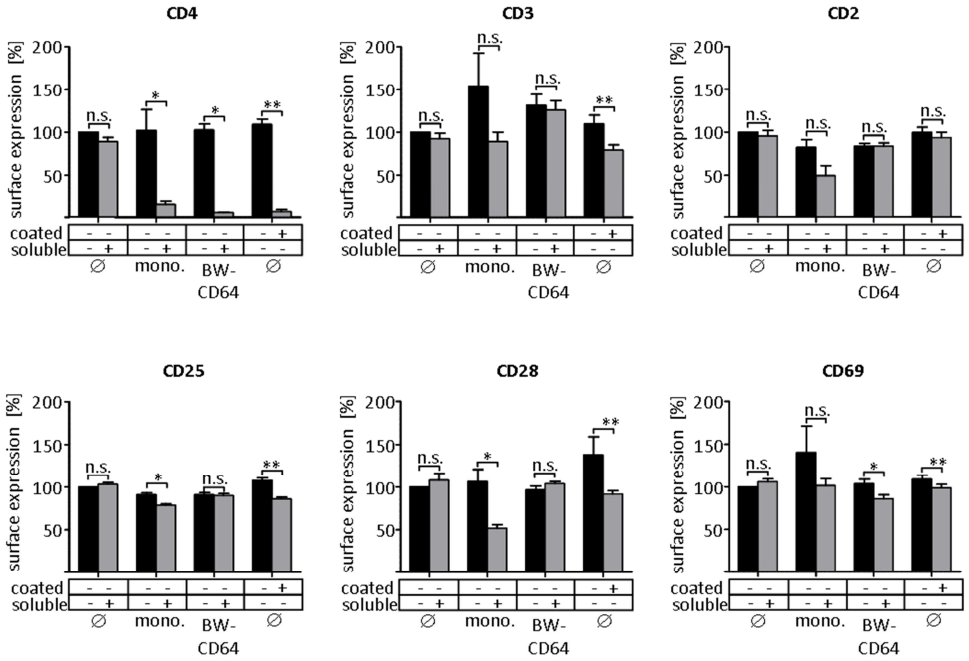

Figure 4.16: Different cross-linking procedures regulated down-modulation of selected cell surface markers of BT-061 decorated T cell diversely. $2x10^5$ BT-061 decorated $CD4^+$ T cells were co-incubated with $8x10^5$ monocytes, $8x10^5$ CD64 expressing transfectants or T cells were incubated in wells coated with BT-061 (10 µg/ml). After 18 h of incubation at 37°C the expression of CD2, CD3, CD4, CD25, CD28, CD69 and CD86 of all cells was analyzed cytofluometrically. The percentage of surface expression is calculated as MFI of treated T cells relative to untreated controls. Statistical analysis of 3 similar experiments with cells derived from a total of 6-8 donors. Error bars indicate SEM. These data are also shown in the master thesis of Daniela Buschjäger, 2012. The experiments were planned and mainly performed by Stephanie Vogel.

Except for CD86 (data not shown) and CD2, which were not affected by the indicated treatments, all other T cell markers were differentially co-regulated (Figure 4.16). In the case of CD4 the degree of down-modulation depended on the used cross-linking procedure. The co-incubation of BT-061 decorated T cells with monocytes or CD64 expressing transfectants induced a less pronounced CD4 down-modulation than the incubation of T cells on coated BT-061. Coated BT-061 significantly decreased CD3, CD25, CD28 and CD69 expression levels when compared with controls, which were not treated with BT-061. Of note, the degree of down-modulation of those markers was not as pronounced as that of CD4 with the same treatment. Upon co-incubation of BT-061 decorated T cells with monocytes the expression of the markers CD25 and CD28 was significantly decreased. Although CD64 expressing transfectants induced strong CD4 down-modulation of BT-061 decorated T cells, exclusively CD69 expression also was decreased under such conditions. The data revealed that addition of soluble BT-061 did not influence the surface expression of any tested surface molecule

compared to untreated controls. Although all used cross-linking procedures served to immobilize BT-061, i.e. by coating the mAb to a well or interaction with its Fc fragment via a FcγR, they affected the regulation of important T cells markers differentially. The data additionally demonstrated that under biologically relevant settings, i.e. co-incubation of BT-061 decorated T cells with monocytes, not only CD4 but also CD25 and CD28 expression levels were reduced. Interestingly none of the tested activation markers were up-regulated upon treatment with immobilized BT-061.

4.3.4. Influence of BT-061 treatment on protein expression of IL-2, TNF-α and IFN-γ

BT-061 was described to possess agonistic effectiveness [167]. An agonistic drug is capable of binding to a cell surface receptor and causing a response similar to the endogenous receptor binding partner. However, a superagonistic pharmaceutical substance is a compound that is capable of producing a greater maximal response than the endogenous agonist for the target receptor, and thus has an efficacy of more than 100%. TGN1412 is a first-in-class superagonist mAb and is specific for the T cell co-stimulatory molecule CD28 [214, 215]. This drug induced upon application in six healthy young men a life-threatening systemic release of pro-inflammatory cytokines, termed a cytokine-release syndrome (CRS) [160]. The agonistic mAb OKT3, which is specific for the TCR component CD3 and has been in clinical use for decades to treat transplant rejection, is a CRS inducer as well [216]. To examine whether BT-061 was able to induce CRS specific cytokines like TGN1412 and OKT3, the release of tumor necrosis factor (TNF)-α, interleukin (IL)-2 and interferon (IFN)-γ after BT-061 stimulation was tested. These cytokines were chosen, because they are described as cytokines typically released during CRS [93, 217, 218]. Furthermore the analysis of released cytokines upon BT-061 treatment might help to elucidate the effect of the drug on various T cell subsets. It is known that IL-2 is generally generated by activated $CD4^+$ T cells [219, 220] and IFN-γ is a prototypical Th1 cytokine [221]. To investigate the release of IL-2, IFN-γ and TNF-α $CD3^+$ T cells were stimulated with coated BT-061. After 6 h intracellular cytokine staining (ICS) was performed and the percentage of cells, which were positive for the specific cytokines, was determined cytofluometrically. 6-hour incubation was chosen, because this

time period was shown to be comparable for the kinetics of the CRS caused in humans with TGN1412.

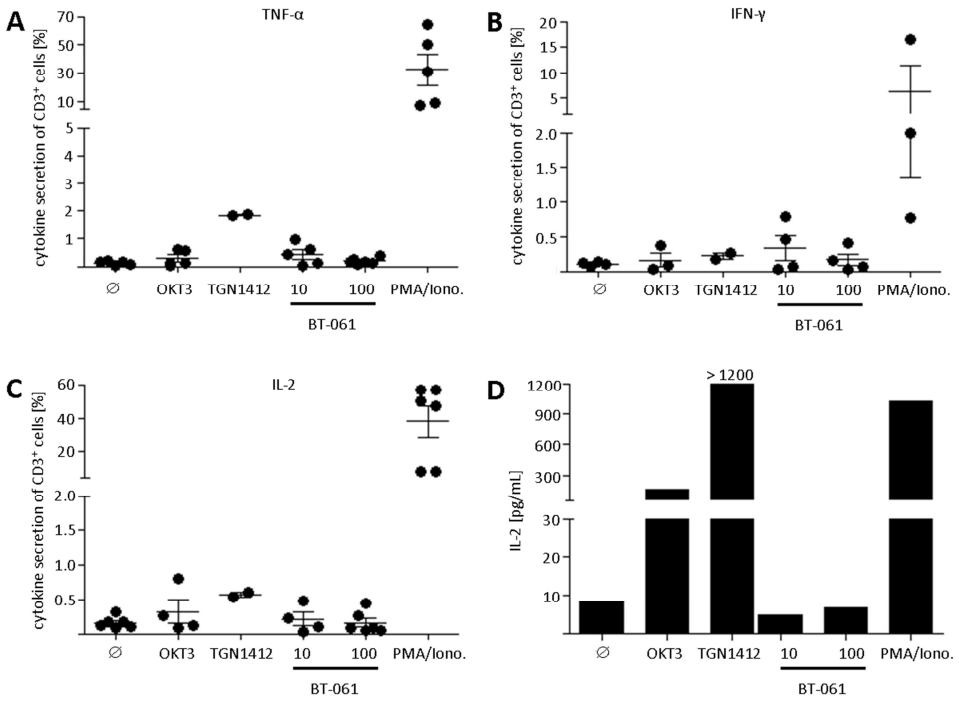

Figure 4.17: Upon treatment with coated BT-061 IFN-γ, TNF-α or IL-2 production was not detected intracellular in T cells. $CD3^+$ MACS enriched T cells were treated for 6 h with coated BT-061 at a concentration of 10 or 100 µg/ml, coated TGN1412 at a concentration of 1 µg/ml, coated OKT3 at a concentration of 10 µg/ml or phorbol-12-myristate-13-acetate (PMA) (0.05 µg/ml)/Ionomycin (0.75 µg/ml). To prevent cytokine secretion Brefeldin A (BFA) was added at a concentration of 1 µg/ml. Afterwards the cells were stained with anti-CD3, anti-CD4 and anti-CD8a. Then the cells were permeabilized and stained for (A) IFN-γ-, (B) TNF-α- and (C) IL-2-specific antibodies. The percentage of $CD3^+$ cells for the respective cytokine was measured cytofluometrically. The statistical analysis of all similar performed experiments is shown. The results obtained with TNF-α were from 1 - 5 similar experiments with cells derived from a total of 2 to 6 donors. The results obtained with IFN-γ were from 1 - 5 similar experiments with cells derived from a total of 2 to 4 donors. The results obtained with IL-2 were from 2 - 5 similar experiments with cells derived from a total of 2 to 6 donors. Error bars indicate SEM. (D) $CD3^+$ MACS enriched T cells were treated for 18 h with coated BT-061 at a concentration of 10 or 100 µg/ml, TGN1412 at a concentration of 1 µg/ml, OKT3 at a concentration of 10 µg/ml or PMA (0.05 µg/ml)/Ionomycin (0.75 µg/ml). Cell-free supernatant was collected and the IL-2 amount released by the cells was determined by ELISA method. The red line indicates the limit of detection of the used ELISA. The graph presents data from one experiment with supernatant derived from cells from a total of one donor. These data are also shown in the master thesis of Daniela Buschjäger, 2012. The experiments were planned by and performed with the support of Stephanie Vogel.

After 6 h exclusively in the positive control of T cells treated with PMA/Ionomycin 7% of $CD3^+$ cells were induced to produce IFN-γ. Even the control antibodies TGN1412 and OKT3 did not increase the frequency of IFN-γ producing cells above background staining level. Although the summary of the experiments indicated variances between the different samples, the results demonstrated that the treatment of $CD3^+$ T cells with coated BT-061 at concentrations of 10 and 100 µg/ml did induce if at all marginally IFN-γ production (Figure 4.17B). The measurement of the frequency of TNF-α producing $CD3^+$ T cells obtained with immobilized mAb revealed comparable results as the ones obtained with the measurement of IFN-γ producing $CD3^+$ T cells, except for TGN1412 treated cells. BT-061 at a concentration of 10 and 100 µg/ml as well as OKT3 did not cause TNF-α production in $CD3^+$ T cells. However, coated TGN1412 was less stimulatory than the addition of PMA/Ionomycin, but it induced a clear TNF-α production in 2% of $CD3^+$ cells (Figure 4.17B). The treatment of $CD3^+$ T cells with immobilized BT-061 at a concentration of 10 and 100 µg/ml did not induce IL-2 secretion above background level. However, the stimulation with OKT3 and TGN1412 induced in a minor population of T cells IL-2 production in comparison to the PMA/Ionomycin treated cells (Figure 4.17C). Even the detection of totally released IL-2 in cell-free supernatant via ELISA method confirmed the results obtained with ICS. Under these conditions TGN1412 stimulated cells released more than 1200 pg/ml IL-2, which was more than the amount of IL-2 secreted by cells treated with PMA/Ionomycin. The incubation of $CD3^+$ T cells with OKT3 induced the production of IL-2, resulting in 156 pg/ml (Figure 4.17D). The cytokine production of IL-2, IFN-γ and TNF-α was additionally analyzed after the treatment of PBMC with soluble BT-061 and after co-incubation of BT-061 decorated T cells with monocytes. Also in this setting no production IL-2, IFN-γ and TNF-α was detected (data not shown). The mAb TGN1412 and OKT3, which induced a CRS in clinical trials, mediated to some extend the release of TNF-α and IL-2, but much less than expected from experiments reported by others. In contrast, T cells incubated on coated BT-061 did not stimulate any IL-2-, IFN-γ- and TNF-α production. Even the measurement of mRNA for IL-2, IFN-γ or TNF-α showed no up-regulation in $CD3^+$ T cells upon stimulation with coated BT-061 (data not shown).

4.3.5. Impact of BT-061 stimulation on the transcriptome of CD4$^+$ T cells

To further investigate the mode of action of BT-061 and its effect on T cell signaling and eventually on various T cell subsets an analysis of mRNA expression via microarray analysis was performed. The analysis of mRNA expression via microarray is a method that allows the analysis of several thousands of genes through the simultaneous detection of corresponding mRNA levels in cells. To investigate whether BT-061 induced a specific set of genes compared to other CD4-targeting mAb another CD4 binding mAb was also tested. BT-061 was compared with the mAb RPA-T4. This clone is often used to specifically stain CD4 T cells in FACS analysis. RPT-A4 binds another epitope of CD4 than BT-061 [222]. To determine the impact of soluble vs. coated BT-061 and RPA-T4 stimulation on T cells, CD4$^+$ T cells were incubated in untreated wells or with soluble or coated mAb (10 µg/ml) for 3 h. Subsequently the mRNA was isolated from the cells and mRNA expression was measured using microarray analysis.

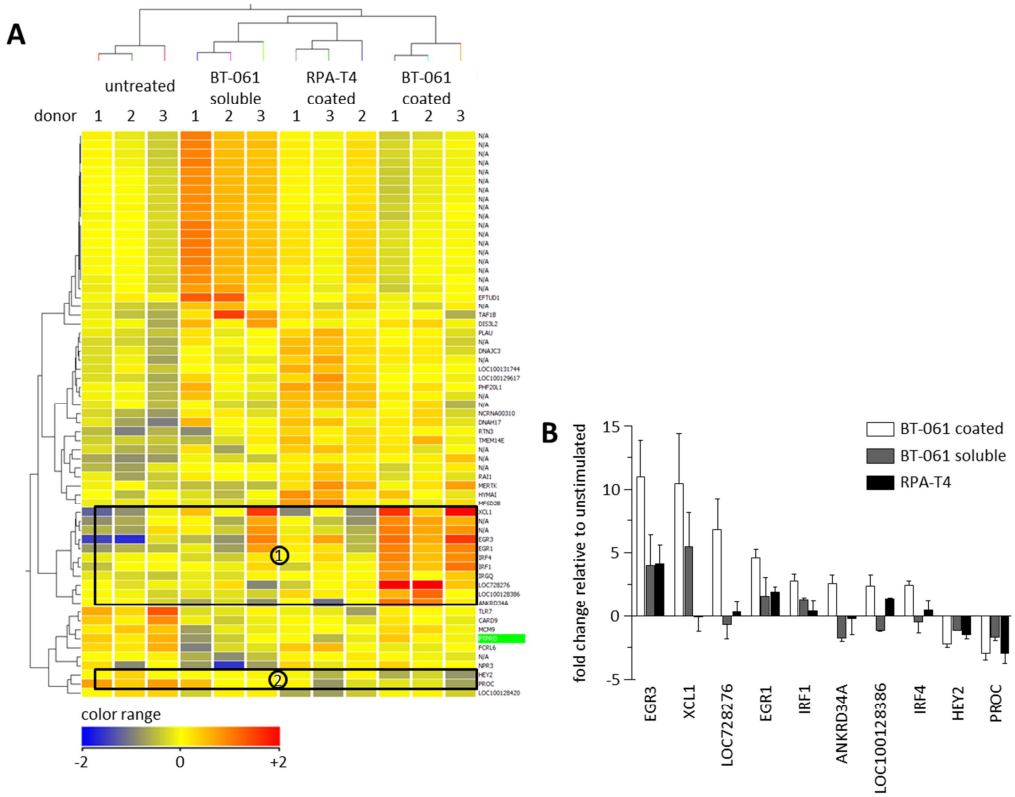

Figure 4.18: Treatment of T cells with coated BT-061 induced regulation of specific genes compared with soluble BT-061 or another coated CD4 targeting mAb. CD4$^+$ MACS enriched T cells were treated for 3 h with medium, coated RPA-T4 at a concentration of 10 µg/ml, soluble BT-061 at a concentration of 10 µg/ml or coated BT-061 at a concentration of 10 µg/ml. 500 ng of total RNA were applied for Cy3-labeling reaction and afterwards scanned using the Agilent DNA Microarray Scanner. (A) The data shown in the heat map are

processed as described in the following: raw intensities were log2 transformed, globally normalized using “scale to mean of all samples” and subsequently expressed as mean centralized relative gene intensity. For further data stratification samples were clustered according to their treatment classes. ANOVA statistics were applied to determine differential gene expression (p-value less than 0.05 with a minimal fold change of two fold for at least one possible comparison). Hierarchical clustering was used to identify responder gene profiles with respect to treatment. Similarity measures used as a metric for clusterization was “uncentred Pearson correlation” combined with the “complete” linkage rule creating the dendrogram structure. The fold change induction relative to the mean of all measured values is encoded by colors ranging from blue (for minimal mRNA expression) to red (for maximal mRNA expression). The framed sections indicate gene patterns specifically regulated upon treatment with coated BT-061. ① shows a set of genes, which is up-regulated after incubation with coated BT-061. ② indicates genes, which are down-regulated upon treatment with coated BT-061. (B) Statistical analysis of the fold change induction of genes relative to unstimulated controls, which are specifically regulated when the T cells were incubated with immobilized BT-061. The results obtained with samples of the same donors are also shown in the master thesis of Daniela Buschjäger, 2012, but the data were processed differently. The experiments were planned by and performed with the support of Stephanie Vogel.

To analyze such a data set the results were initially filtered. The fluorescence intensity was normalized among all tested arrays and subsequently compared with each other. mRNA with a fluorescence intensity in a range between 20 and 100% were considered for the hierarchical cluster analysis (HCA), whereas the other values were deleted. To provide a global comparison of the response of different donors to the variable treatments the data were analyzed by HCA. The HCA is based on the principle that similar data sets were summarized in clusters, whereas the similarity is decided following a specific algorithm. These clusters could further be separated in sub-clusters to visualize relations based on similarity. Furthermore the statistical significance (p-Value less than 0.05) of the gene regulation of all samples was calculated with Oneway ANOVA test. Genes that were 2-fold up- or down-regulated compared to the unstimulated controls, were considered in the subsequent heat map. Hierarchical clustering was used to identify responder gene profiles with respect to treatment. Similarity measures used as a metric for clusterization was “uncentred Pearson correlation” combined with the “complete” linkage rule creating the dendrogram structure. The type of gene regulation (up or down compared to unstimulated controls) and the magnitude is encoded with color values. Red indicates the up-regulation and blue the down-regulation of a gene compared with average of all samples (Figure 4.18A). The horizontal clustering of the regulated genes led to the arrangement of similar treated samples of different donors in one group. This fact indicated that the patterns of

regulated genes upon equal treatments were more similar to each other than patterns of the same donors differently treated. These results confirmed the consistence of data obtained from variable donors but similarly treated samples. Furthermore the results depicted in the heat map demonstrated that the immobilization of BT-061 induced the regulation of a specific set of genes. These genes were neither regulated upon the treatment with soluble BT-061 nor with RPA-T4 (Figure 4.18A). The vertical HCA showed that the pattern of genes regulated upon treatment with soluble BT-061 were more similar to the pattern of regulated genes upon incubation with coated RPT-A4 than with coated BT-061. This observation indicated that soluble BT-061 influenced T cells upon binding to the target although CD4 down-modulation was not detected (Figure 4.18A), whereas further statistical analysis revealed that the impact of soluble BT-061 on gene regulation of $CD4^+$ T cells is not significant in relation to unstimulated controls (data not shown). The specifically regulated genes were summarized in patterns (indicated by black framed boxes in Figure 4.18A), which were regulated similarly. On the one hand coated BT-061 resulted in the up-regulation of a set of genes (Figure 4.18A in ①), but on the other hand the immobilized mAb induced the down-regulation of another set of genes as well (Figure 4.18A in ②). The statistical analysis of the fold change of genes relative to the unstimulated control revealed that coated BT-061 specifically induced the up-regulation of the mRNA encoding for early growth response protein (EGR)3, chemokine (C motif) ligand (XCL)1, LOC728276, EGR1, interferon regulatory factor (IRF)1, Ankyrin repeat domain-containing protein 34A (ANKRD34A), LOC100128386 and IRF4. On the contrary, the treatment of $CD4^+$ T cells with immobilized mAb mediated the down-modulation of the mRNA encoding for for hairy/enhancer-of-split related with YRPW motif protein 2 (HEY2) and protein C (PROC).

The analysis of mRNA expression to identify regulated genes revealed that coated BT-061 affected a specific set of genes in $CD4^+$ T cells, which are neither regulated by soluble BT-061 nor by another CD4 binding mAb – RPT-A4. These data additionally indicated that BT-061 need to be cross-linked to develop its function.

4.4. Analysis of differently induced T cell proliferation upon BT-061 treatment

The previously described results partially helped to clarify the mechanism underlying BT-061 mediated CD4 down-modulation on T cells. Additionally, the data revealed information about the influence of the mAb on targeted T cells, like their activation status or differentiation status, but still the mode of action of BT-061 remained partially elusive. From different agents it is known that they specifically or nonspecifically induce or interact with T cell activation. These effects upon interaction can result in cytokine production or inhibition, receptor expression or down-modulation, and ultimately proliferation or repression of proliferation of the stimulated T cells. Therefore, to elucidate the influence of cross-linked BT-061 on targeted T cell proliferation induced by various stimuli was examined. Furthermore the proliferation assays could reveal data on the proliferative potential of coated BT-061 itself.

4.4.1. Investigation of anti-CD3 induced proliferation with simultaneous BT-061 treatment in PBMC

In the first set of experiments the therapeutic mAb OKT3 was applied to induce proliferation in PBMC. Because OKT3 needs to be cross-linked to establish its proliferative function like BT-061, [223] the experiments were carried out with PBMC. Monocytes could provide the cross-linking by the interaction of their FcγR and the Fc portion of OKT3 [224]. The impact of cell density was not clear, because on the one hand OKT3 needs to be cross-linked by nearby cultured cells, but on the other hand T cells have to interact with BT-061 coated to the bottom of the plate. Therefore the experiment was performed with $2x10^5$ and $8x10^5$ cells per well. OKT3 recognizes the ε component of the CD3 signal-transduction complex. It possesses mitogenic abilities and thereby induces T cell proliferation and large-scale release of cytokines such as TNF-α. Therefore this drug, which was long time used in clinics, was used to investigate the influence of coated BT-061 on TCR triggered proliferation.

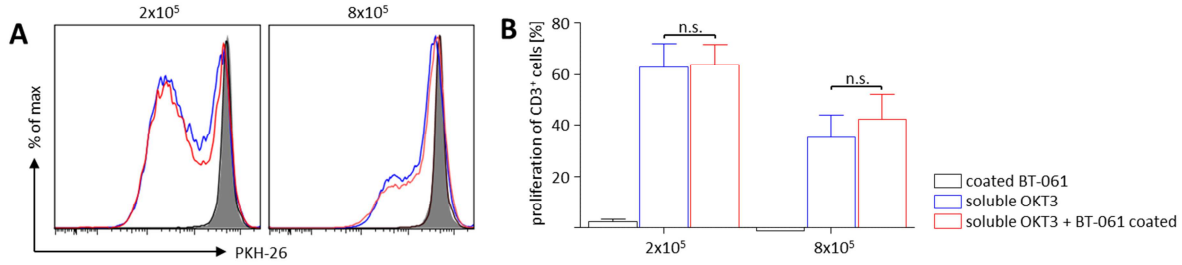

Figure 4.19: Treatment with coated BT-061 did not influence OKT3 induced proliferation in PBMC (A) PBMC were isolated from buffy coats and labeled with PKH-26. $2x10^5$ or $8x10^5$ labeled cells were seeded in untreated or BT-061 (10 µg/ml) coated wells and incubated in medium or medium supplemented with OKT3 at a concentration of 10 µg/ml. The cells were incubated for 5 days at 37°C. Afterwards the cells were harvested and stained with flourochrome labeled antibodies for CD3, CD4 (clone SK3) and CD8a. To measure proliferation of T cells PKH-26 dilution of living $CD3^+$ singlets was determined cytofluometrically. Because of CD3 down-regulating properties of OKT3, the CD3 gate was aligned on $CD3^{dim}$ for OKT3 treated samples. One representative experiment of 3 similar experiments with cells derived from a total of 8 donors (with $2x10^5$ cells) and 2 similar experiments with cells derived from a total of 6 donors (with $8x10^5$cells) is shown. (B) Statistical analysis of the percentage of $CD3^+$ T cells that showed proliferation Error bars indicate SEM.

The data obtained with PBMC incubated with soluble OKT3 at a concentration of 10 µg/ml revealed that culturing of $8x10^5$ cells per well less efficiently induced OKT3 driven proliferation of $CD3^+$ T cells in PBMC than culturing of $2x10^5$ per well. Interestingly, the simultaneously administration of coated BT-061 to OKT3 treated PBMC had no significant impact on the proliferation rate of $CD3^+$ T cells in both tested cell concentrations. The incubation of $2x10^5$ cells per well in the presence of OKT3 and coated BT-061 at a concentration of 10 µg/ml induced 64% $CD3^+$ T cells to proliferate compared to 63% proliferation induced only by OKT3. When $8x10^5$ cells were cultured with both mAb 42% of $CD3^+$ T cells showed proliferation, whereas OKT3 on its own induced 35% of $CD3^+$ T cells to proliferate (Figure 4.19). Furthermore the data revealed that addition of soluble BT-061 at a concentration of 10 µg/ml to PBMC did not induce proliferation of $CD3^+$ T cells. The data demonstrated that proliferation induced by the TCR triggering reagent OKT3 was not affected by the simultaneous application of coated BT-061 and that BT-061 had no proliferative effect on PBMC.

4.4.2. Examination of a MLR with BT-061 treated T cells

Additionally the effect of BT-061 on T cell proliferation induced by allo-reactivity was analyzed. Therefore $CD3^+$ T cells isolated from buffy coats were co-cultured with monocyte derived imDC. In this kind of mixed lymphocyte reaction (MLR) T cells are activated by the stimulus given from the foreign histocompatibility antigen (MHC class I or class II molecules) expressed on the allogenic imDC. To analyze the impact of BT-061 on proliferation in MLR $CD3^+$ T cells were isolated and stained with PKH-26. The T cells were cultured with different concentrations of allogenic monocyte derived imDC. Furthermore the cells were cultured in untreated or wells coated with BT-061 at a concentration of 10 µg/ml. 7 and 9 days post seeding proliferation and CD4 expression was determined cytofluometrically.

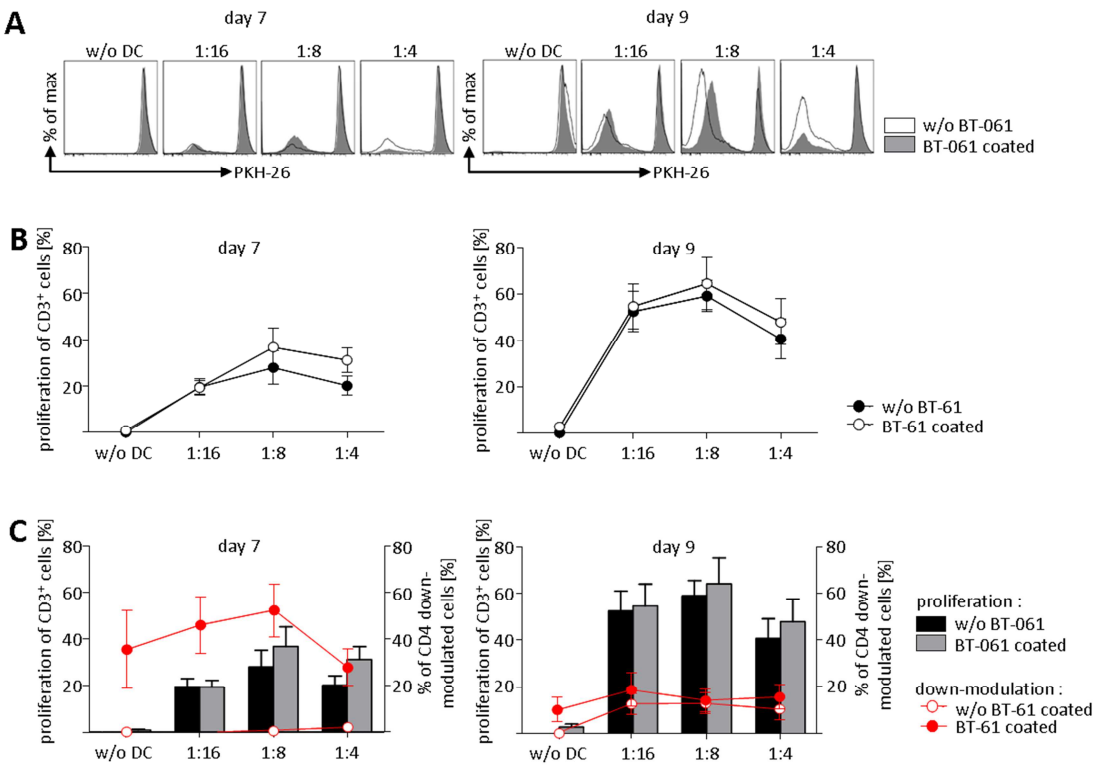

Figure 4.20: Treatment with coated BT-061 did not influence T cell proliferation in a MLR. (A) $CD3^+$ T cells were isolated from buffy coats and labeled with PKH-26. $1x10^5$ stained T cells were seeded in untreated or BT-061 (10 µg/ml) coated wells. 5 days before allogenic monocytes were isolated from buffy coats and differentiated to monocyte-derived imDC in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF). These imDC were titrated as indicated to the T cells, whereas $1x10^5$ T cells are constantly seeded. The cells were incubated for 7 and 9 days at 37 °C. Afterwards the cells were harvested and stained with flourochrome labeled antibodies for CD3, CD4 (clone SK3) and CD8a. To measure the proliferation of T cells the PKH-26 dilution of living $CD3^+$ singlets was

determined cytofluometrically. The CD4 down-modulation was measured as described in the Figure 4.1. One experiment out of 2 similar ones with cells derived from 6 donors is shown. (B) Statistical analysis of the percentage of $CD3^+$ T cells that showed proliferation (C) Statistical analysis of the percentage of $CD3^+$ T cells that showed proliferation compared with the percentage of gated T cells that showed CD4 down-modulation. Error bars indicate SEM.

The data revealed that T cell proliferation triggered by foreign histocompatibility antigen expressed by allogenic imDC, as expected, increased after 9 days compared to 7 days at all tested ratios between T cells and imDC. Although, the proliferation intensity varied between the experiments with cells derived from different donors, in general the culturing of T cells with imDC in an 1:8 ratio mediated the strongest proliferation in T cells (Figure 4.20A, B and C). Further analysis concerning the proliferating T cell subset revealed that the proliferating cells were mainly $CD4^+$ T cells (data not shown). Additionally the experiments indicated that culturing on wells treated with coated BT-061 at a concentration of 10 µg/ml did not influence significantly the proliferation of T cells in contrast to culturing without immobilized mAb (Figure 4.20B, right and left graph). The co-culturing of $1.25x10^4$ imDC with $1x10^5$ T cells (1:8 ratio) for 7 days induced a proliferation in 28% of $CD3^+$ T cells and the additional incubation on coated BT-061 triggered 37% of $CD3^+$ T cells to proliferate (Figure 4.20B, right graph). This marginal higher percentage of proliferating cells upon culturing on immobilized BT-061 in contrast to the incubation on untreated wells even decreased after 9 days of treatment (Figure 4.20B, left graph). The data obtained after 7 days revealed a profound difference in the percentage of CD4 down-modulation on T cells in an MLR between the culturing without BT-061 or carried out on BT-061 coated wells at a concentration of 10 µg/ml (Figure 4.20C, left graph). The co-culturing of imDC with T cells in an 1:8 ratio for 7 days induced a CD4 down-modulation of 0.7% of $CD3^+$ T cells. In contrast, the additional incubation on coated BT-061 mediated a CD4 down-modulation in 52% of $CD3^+$ T cells (Figure 4.20C, 7 days). This clear difference in CD4 expression after 7 days between T cells in a MLR performed without the addition of BT-061 and with T cells in a MLR performed on coated BT-061 is no longer detectable after 9 days of culturing (Figure 4.20C, right graph). In summary these data demonstrated that CD4 was clearly down-modulated after 7 days upon culturing on coated BT-061 compared to untreated controls, whereas this effect was not detectable after 9 days post seeding. On the contrary, the proliferation after 7 and after 9 days did not differ significantly between the culturing on untreated or BT-061 coated wells.

The data gave reasons to hypothesize that BT-061 CD4 down-modulation on T cells did not affect T cell proliferation induced by allogenic imDC. Furthermore the experiments demonstrated that coated BT-061 at a concentration of 10 µg/ml possessed to proliferative potential on purified $CD3^+$ T cells under the tested conditions.

5. Discussion

Currently approximately 3-5% of the general population is affected by autoimmune diseases worldwide [147]. Because of inflammatory symptoms and the resulting health impairment of the patients a medicamentous therapy is generally indispensable. Currently therapies approved by the US Food and Drug Administration (FDA) for autoimmune diseases are predominantly focusing on systemic inhibition of immune inflammatory activity [68]. This therapeutic approach induces a systemic immune suppression of the host that is unfortunately often coupled with numerous side effects. Monoclonal antibodies (mAb) promise to improve the therapy of autoimmune diseases due to their unique target specificity. Previous studies demonstrated that cluster of differentiation (CD)4-specific mAb could contribute to the recovery of peripheral tolerance, which often is deregulated in autoimmune diseases, by affecting multiple targets in self-directed T cell activation. However, the mechanisms underlying tolerization by CD4-targeting mAb are not yet fully understood [162]. In contrast to the encouraging results of *in vitro* studies, in animal models or even in initial clinical trials, until now no CD4-targeting mAb was shown to be beneficial to clinical symptoms of autoimmune diseases like psoriasis or rheumatoide arthritis (RA) on the long run [225-227]. The aim of this study was to characterize the new CD4-specific mAb BT-061 (Tregalizumab). Especially the capacity of BT-061 for CD4 down-modulation was studied in detail. These investigations helped to clarify the mode of action of BT-061 exemplary for CD4-targeting mAb. The data additionally facilitates the evaluation of potential interactions with immune cells that are able to induce side effects posed by therapeutic mAb.

Initial analysis were performed to establish an *in vitro* setting, which enables the measurement of CD4 down-modulation comparable to that, which was observed in patients treated with BT-061 [167]. Therefore a cytometrically based method to detect the fluorescence of the CD4-specific mAb SK3, which does not compete with the binding of BT-061, was established (Figure 4.1A and B). Two different data analysis procedures were applied to analyze BT-061 induced CD4 down-modulation. To quantify the degree of CD4 surface receptor down-modulation the mean fluorescence intensity (MFI) of CD4 staining was used and to calculate the percentage of T cells that showed CD4 down-modulation a standardized gate was applied. To investigate effective dosages of BT-061 to down-modulate CD4 and the kinetics of CD4 down-modulation upon BT-061 treatment, human peripheral

blood mononuclear cell (PBMC) isolated from buffy coats of healthy donors were used. PBMC were chosen, because these cells are generally used to examine effects e.g. cytokine release syndrome (CRS) mediated by immunmodulatory mAb [228-231]. The investigations with BT-061 treated PBMC revealed that the reduction of CD4 on the surface of T cells followed a bell-shaped curve in relation to the applied mAb dosage. The minimal dosage of 0.1 µg/ml was already effective while the CD4 down-modulation peaked using 10 µg/ml (Figure 4.1C and D). The data achieved by the treatment of human PBMC with BT-061 are comparable with results obtained with other CD4-specific mAb. The saturating amount for the CD4-targeting mAb BB14, BF8, BL4, 13B8.2 and F101-69 were calculated at 5 – 10 µg/ml, whereas the maximal CD4-down-modulation induced by BB14 was detected at lower concentrations [196]. The investigations of Bartholomew *et al.* using the CD4-binding mAb Campath-9h also revealed the highest reduction of CD4 surface expression in PBMC with 10 µg/ml [194]. Besides the determination of optimal values to detect the decrease of CD4 the experiments were performed to establish an *in vitro* system to mimic the patients´ *in vivo* system. Therefore, the question arose whether results obtained in the former described *in vitro* model mirror effective dosages for patients´ treatment with BT-061. We assume that the level of CD4 down-modulation is a parameter of the mAb efficacy and reflects the most effective dosage concerning the clinical outcome. Data obtained within the clinical trial 967 monitoring the psoriasis area and severity index (PASI) of patients treated with 0.5, 2.5, and 20 mg BT-061 intravenously (i.v.) revealed that approximately 50% of the probands respond with a 50% reduction of PASI [201]. These effective dosages nearly correspond to the maximal effective dosage identified via the previously described *in vitro* system. Supposing that a person with an averaged body weight of 70 kg, has typically a blood volume of 5 – 7 l, whereas 50% of that volume is contributed by cells and the other 50% by serum, upon i.v. administration a mAb is diluted in an average in a volume of 2.5 – 4 l serum [200]. Under these circumstances the most effective dosage of 10 µg/ml in PBMC corresponds to the application of 25 to 40 mg i.v. The comparison of results obtained with other CD4-specific mAb and the efficacy of BT-061 in clinical trials reveal that the usage of PBMC is suitable to draw initial conclusions about the potential effects mediated by BT-061.

BT-061 was designed as an immunmodulatory mAb, which is effective by inducing a signal into the cell upon binding. In contrast blocking mAb, which inhibit the interaction between a receptor and its´ ligand, function upon the mere adhesion to their target. On the contrary to

the data obtained with PBMC experiments with purified T cells revealed that the sole binding of soluble BT-061 to its´ target structure was not effective in triggering a signal that mediated the down-modulation of CD4 on the cell-surface (Figure 4.2A and B). Interestingly, a clear CD4 down-modulation was detected upon the incubation of purified T cells with immobilized BT-061 (Figure 4.2A and B). The results demonstrate that a bi-specific cross-linking provided by the treatment of purified T cells with soluble BT-061 was insufficient to mediate CD4 down-modulation by BT-061. Due to the fact that BT-061 is an immunglobulin (Ig)G1 isotype it possesses bivalent monomeric properties *in vivo* [232], which offer the cross-linking of two CD4 receptors simultaneously at the most. Therefore it was concluded that CD4 needs to be „hyper"-cross-linked, which is not accomplished by soluble BT-061, to transmit the requested signal into the cell to mediate CD4 down-modulation. The observation that the non-T cells within PBMC (Figure 4.3A and B), in contrast to purified T cells alone (Figure 4.2A and B), were sufficient to induce CD4 down-modulation was strong evidence that fragment, crystallizable γ receptor (FcγR) are crucial to provide the formerly mentioned „hyper"-cross-linking of BT-061. However, the treatment of T cells with plate-bound antibody also induced a decrease of CD4 (Figure 4.2A and B). Thus, the CD4 down-modulation induced by direct interaction of FcγR with the mAb constant region is obviously not depending on any additional secreted factors by other cells within the PBMC. Furthermore, monocytes which are capable to induce BT-061-mediated CD4 down-modulation, still mediated that effect after the blockade of cytokine secretion by Brefeldin A (BFA) (data not shown). It was also shown, that the supernatant of monocytes co-cultured with BT-061 decorated T cells was not able to cause a reduction of CD4 on BT-061-treated T cells (Figure 4.4A and B). Nearly all immune cells express FcγR on their surface, except from T cells, which display minor amounts of CD16 on a subset [83]. Considering that fact it was interesting that not all tested immune cell subsets within the PBMC were effective in inducing a CD4 down-modulation on BT-061-decorated T cells (Figure 4.3A and B). This observation demonstrated that not all classes of FcγR possess equal potential to decrease CD4 on mAb-treated T cells. Investigations concerning the participation of variable FcγR using a murine cell line expressing the extracellular domain of the different human FcγR revealed that CD64 is mainly responsible for the observed CD4 down-modulation on T cells. These data are in line with the finding that monocytes displaying high levels of CD64 (Figure 4.5) induced the strongest down-modulation of CD4 on T cells within PBMC (Figure 4.3A and

B). By contrast an involvement of B cells and thereby CD32B, which is the only FcγR expressed on B cells [203], was not detectable (Figure 4.3A and B). Furthermore, the potential of natural killer cells (NK cells) to mediate a decrease of CD4 on T cells treated with BT-061 was examined. Concerning this immune cell subtype, which is described to exclusively display CD16 [80, 85, 95], the participation in the measured CD4 down-modulation in PBMC was not as clear compared to monocytes and B cells. The data obtained with CD16 expressing transfectants demonstrated a lower potential to mediate a CD4 down-modulation in comparison to CD64 but higher than the other analyzed FcγR CD32B and CD32A (Figure 4.6 and Figure 4.7A and B). However, the data obtained with NK cells were ambiguous (Figure 4.3A and B). To enrich NK cells a kit was used, which depletes all non-NK cells, i.e. T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells. This mode of isolation ensures the depletion of NKT cells and the enrichment of the $CD56^{dim}$ as well as the $CD56^{bright}$ population of NK cells. $CD56^{dim}$ $CD16^{bright}$ NK cells represent at least 90% of all peripheral blood NK cells [233] and contain much more perforin, granzymes and cytolytic granules than their $CD56^{bright}$ $CD16^{-}$ counterpart [234]. Because of the high CD16 expression of this population these cells possess the potential to interact with the Fc portion of BT-061. One reason for the observed divergent levels of CD4 down-modulation seen with NK cells from different donors might be the variable percentage of $CD56^{bright}$ $CD16^{-}$ and $CD56^{dim}$ $CD16^{+}$ cells isolated from peripheral blood. Poli *et al.* discussed that different groups made the observation that an expansion of $CD56^{bright}$ NK cells occurred during the course of several diseases [235]. This cell expansion directly influenced the ratio between the formerly described NK cell populations and the total number of $CD16^{+}$ cells, which potentially interacted with the Fc fragment of BT-061. Another explanation for the detected variability between the tested donors might be the expression of different allelic variants of CD16 on the analyzed NK cells. The most prominent single nucleotide polymorphism (SNP) described for CD16, which affects the binding affinity for IgG subclasses, is at position 158 of the amino acid sequence. Wu *et al.* and Koene *et al.* at described that a substitution from a valine (V) into a phenylalanine (F) resulted in a stronger affinity of the V158 allotype for IgG1 compared to F158 [91]. The likelihood that the NK cells used in the experiments obtained from different donors expressed variable allelic variants of CD16 that influence the interaction potential with the Fc fragment of BT-061 was supported by a report of Lernbecher *et al.*. In this study the genotypcial variants of the low-affinity FcγR

at position 158 were investigated in 223 Caucasian blood donors. The results demonstrated that approximately half of all tested probands expressed F homozygously. 39% of the analyzed donors revealed a heterozygous phenotype and 11% owned two V alleles [236]. Therefore the variable results concerning CD4 down-modulation of BT-061 treated T cells obtained with NK cells from different donors might be due to the allelic variants of CD16 of the tested donors. Finally the data obtained with NK cells did not definitely clarify the participation of CD16 in the PBMC mediated CD4 down-modulation on BT-061 decorated T cells. The investigations using transfectants expressing CD16 revealed a clear potential of CD16 to interact with BT-061, even though to a much lesser extent than CD64. However, it should be considered that the transfected extracellular domain of the human FcγR CD16 represents the high-affinity allelic variant and therefore possesses maximal potential to interact with BT-061. Nevertheless, the potential of BT-061 to induce an undesired depletion of $CD4^+$ T cells was estimated at a very low level. Neither life-dead-staining nor macroscopic analysis in our experiments showed an indication for a cytotoxic effect by BT-061 triggered NK cells (data not shown) nor a reduction of T cell counts was observed within preclinical trials [167]. Although the data obtained with monocytes and the transfectans were clear concerning the involvement of CD64 in BT-061 mediated CD4 modulation on T cells, the participation of CD32A, which is also expressed on monocytes, was not as obvious. The blockade of CD64 on monocytes, which additionally express CD32A and B, demonstrated a reduction of CD4 down-modulation on BT-061 decorated T cells, albeit no total inhibition (Figure 4.9A and B). Although variable blocking reagents were used to suppress the influence of CD64, no of the used antibodies was able to block the down-modulation of CD4 by the CD64 expressing transfectants completely (data not shown). Therefore it is not conclusively clarified whether the detected remaining CD4 down-modulation by CD64-blocked monocytes is induced by residues of unblocked CD64 or by CD32A. The data using the CD32A expressing transfectants (Figure 4.7A and B) and monocyte derived immature (im) dendritic cells (DC) (Figure 4.12B), which exclusively express CD32A and B (Figure 4.12A and [209]), revealed the potential of CD32A to mediate a BT-061 induced CD4 down-modulation. Even though the reduction was observed to a lesser extent compared with CD64 and CD16. Collectively these observations indicated that CD64 on monocytes was necessary and sufficient to confer BT-061 mediated CD4 down-modulation and that other receptors or soluble factors expressed by monocytes did not play a critical role. The importance of

monocytes concerning BT-061 mediated CD4-down-modulation was supported considering the fact that monocytes comprise approximately 15-30 % of PBMC. The role of CD16 expressed by NK cells is rather subordinated, with respect to the fact that neither cytotoxicity in *in vitro* studies nor T cell depletion in clinical trials was observed. Furthermore NK cells represent only 5 – 10 % of PBMC. The examination of imDC, which were generated by the differentiation of monocytes, demonstrated a minor potential to interact with BT-061 and mediate a down-modulation of CD4 (Figure 4.12B). However, these data did not entirely exclude the opportunity of DC to interact with BT-061, because Bayry *et al.* demonstrated that blood derived myeloid DC express CD64 as well but to a lower extend than monocytes [237]. In contrast to that cell type plasmacytoid DC do not display the high affinity receptor on their surface [238]. Postulating the hypothesis that CD64 is predominantly responsible for a CD4 down-modulation on BT-061 decorated T cells, the question what kind of cell type plays the critical role *in vivo* still needs to be answered. On the one hand the data obtained from the previously described *in vitro* experiments demonstrated that monocytes and to a lesser extend DC could be crucial for the mAb efficacy. On the other hand previously studies query about the high affinity nature of CD64 under serum conditions [94]. The investigations of van der Poel *et al.* support the hypothesis that CD64 is saturated under serum conditions, because of the high IgG content in serum [204]. Our studies using monocytes pre-incubated with different concentrations of human serum support this hypothesis. The capacity of serum-pre-treated monocytes to down-modulate CD4 on BT-061-decorated T cells was severely hampered (Figure 4.10). BT-061 was developed for the treatment of autoimmune diseases like psoriasis und RA. Therefore, the question arose, whether CD64 expression changed under pro-inflammatory conditions and thereby enables an interaction of BT-061 with CD64 in spite of serum contained in the blood. The data described in the literature concerning that question were ambivalent. Laurent *et al.* demonstrated that monocytes from RA patients showed a small decrease in the proportion of $CD64^+$ cells, although the MFI of CD64 revealed a slight increase, compared to healthy controls [230]. In contrast two other studies did not support the hypothesis that monocytes increase CD64 surface expression under proinflammatory conditions within the course of autoimmune diseases [239, 240]. Because of the ambiguous reports in the literature it is still not clear, whether CD64 on monocytes is up-regulated in the course of autoimmune diseases. Therefore it is not known, whether the saturation by serum IgG under steady-state conditions could be overcome

under pro-inflammatory conditions and the receptor could interact with BT-061. However, the permanent expression of a constantly blocked receptor seems unlikely.

Focusing on cells that also express CD64 neutrophils should be considered as well. On the one hand it is generally accepted that neutrophils do not or at a low level display CD64 receptors on their surface under steady-state conditions. On the other hand the receptor expression is up-regulated as a physiological response upon the exposure to microbial wall components such as lipopolysaccharide (LPS) [206], complement split products and some cytokines such as interferon-γ (IFN-γ) [205] and granulocyte colony stimulating factor (G-CSF) [241]. Neutrophils constitute approximately 40-60% of the white blood cell population [242] and are therefore the most abundant type of white blood cells in mammals. During active phases of RA, inflamed joints are massively infiltrated with neutrophils comprising 80-90% of the total cell population [243]. Due to that fact an up-regulation of CD64 in the course of autoimmune diseases could offer a new pool of CD64 molecules potentially interacting with BT-061. In previous studies the up-regulation of CD64 on neutrophils isolated from synovial fluid (SF) from RA patients was shown [244-246]. In contrast many studies investigating this aspect for peripheral blood neutrophils reported no decrease of CD64 expression on neutrophils of RA patients compared to healthy controls [246, 247]. It was not possible to clarify whether neutrophils have the potential to interact with BT-061 and induce CD4 down-modulation via FcγR under steady-state or under proinflammatory conditions induced by IFN-γ-treatment. However, an up-regulation of CD16 and CD64 was detected upon IFN-γ-treatment (Figure 4.11). The observation that neutrophils mediate a reduction of CD4 expression on the surface of T cells after co-culturing independent from BT-061 treatment (data not shown), made it impossible to investigate the influence of neutrophils on BT-061 mediated CD4-down-modulation. This observation might be due to the often described pre-activated status of isolated neutrophils and was shown in our experiments by the enhanced expression of CD63 and CD18 (data not shown). Besides the formerly discussed immune cell types other immune cells might interact with BT-061 via their FcγR. Macrophages are also described as CD64 expressing cells [80, 85, 95], which thereby offer a high potential to mediate a BT-061 mediated CD4 down-modulation on T cells. Although this cell type is strategically located throughout the body tissues like the bone, lung, gut or in the brain as microglia [248] it is not clear, whether macrophages potentially encounter BT-061 decorated T cells. This interaction might take place in the spleen, where macrophages are resident in

the marginal zone or in other secondary lymphoid organs like lymph nodes (LN) [248]. LN are furthermore interesting, when searching for potential interaction partners for BT-061-decorated T cells or the clarification of the impact upon application in the patient. The palatine tonsils belong to the peripheral lymphoid tissue like LN and are therefore a suitable model to represent this immunological structure. Tonsils feature a different compartimentation, distribution of cell subtypes and activation status of the cells compared to the blood. For example central memory T cells, being predominantly $CD4^+$, are enriched in LN and tonsils [249]. The subset of follicular T helper cells (Tfh cell) is also present in tonsils, where they display CD40 ligand(L) and inducible T cell co-stimulator (ICOS) on their surface and provide spontaneous help to B cells [42, 250, 251]. It is likely that therapeutic mAb will accumulate in the draining LN upon administration. Therefore the question arose, whether the diverse conditions, e.g. cell composition and activation status of cells, within a tonsil compared to blood, were reflected in a different level of CD4 down-modulation on T cells within tonsillar mononuclear cells (TMC) compared to PBMC. Although we expected macrophages to be present in the isolated TMC [252], no $CD14^+$ expressing cells were detected (Figure 4.13B). The predominant cell populations represented in the isolated TMC were $CD3^+$ T cells, $CD19^+$ B cells and HLA-DR^+ DC (Figure 4.13B). The examination of FcγR expression in TMC revealed that CD32A and B were displayed on the cells within TMC (Figure 4.13B). The analysis of CD4 expression in TMC upon BT-061 administration demonstrated that the addition of the mAb to TMC at different concentration did not affect the percentage of CD4 down-modulated T cells. This result indicated that the circumstances in TMC that differ from that in PBMC, concerning the activation status of cells and the cell distribution, did not influence the percentage of CD4 down-modulated T cells on the resident cells. In contrast to our data obtained with potentially activated T cells in TMC, Bartholomew *et al.* demonstrated by using the CD4-targeting mAb Campath9-h that pre-activated T cells obtained from a T cell line showed a significant CD4 down-modulation. Interestingly, this decrease was not dependent on FcγR interactions [194]. However, the preparation of a fragment antigen-binding $(Fab)_2$ fragment of BT-061 could clearly show the dependence on FcγR mediated interaction for CD4-down-modulation on T cells. In summary, results obtained with the humanized IgG1 mAb BT-061 mirror the observation described in literature concerning the binding pattern on IgG1 isotypes to human FcγR. CD64 features the highest affinity for IgG [93, 97, 142]. In contrast to the indicated potential of CD32A and

CD16 to interact with BT-061, Bruhns *et al.* reported that monomeric polyclonal IgG exclusively bind to CD64. However, it was not analyzed whether preparations of BT-061 only contain monomeric mAb. The data from Jeffris *et al.*, demonstrated an equal interaction level of IgG1 with CD32 and CD16, which was not seen in our studies. The data reviewed in the publication of Brennan *et al.* showed the second highest affinity for IgG, besides CD64, for CD16A and CD32A followed by CD32B, which partially reflect our observations. The differences concerning the interaction of IgG1 with human FcγR might due to different mAb, diverse test systems or variable read outs for the detection. Furthermore, distinct glycosylation patterns of the investigated mAb resulted in different observations, because it is known that sugar moieties could affect the interaction between IgG and FcγR [142, 253]. Furthermore it is known that CD4 is expressed on other cells like monocytes, macrophages and neutrophils as well. Therefore it is interesting to investigate the influence of BT-061 treatment on these cells. Initial studies on purified monocytes cultivated with soluble and coated BT-061 revealed that the treatment induced up-regulation of CD14 (data not shown).

We demonstrated that BT-061 is not designed as a blocking antibody. This conclusion was drawn, because the mAb needed to be cross-linked via its Fc portion to develop its function and CD4 was not detectable with a non-competing CD4 mAb upon BT-061 treatment. Blocking mAb sterically inhibit the binding of a ligand to its target. This mechanism does not require cross-linking and allows the detection of the target molecule upon mAb treatment. This fact implied that the function of BT-061 depended on the induction of signaling in the target cell that mediated CD4 down-modulation. Although CD4-specific mAb that induce the decrease of CD4 upon binding were described [194], the mechanisms underlying that effect have not been fully understood yet. Therefore, experiments were performed to clarify the signaling mechanism induced by BT-061 treatment in T cells. To investigate whether the treatment with BT-061 is beneficial for the improvement of clinical symptoms of RA and psoriasis patients, we were interested whether the administration of BT-061 favors the differentiation of naïve T cells into a special T cell subset, because the shifting of T helper cells (Th cells)1-Th 2-balance could be an option for the treatment of autoimmune diseases. Finally the obtained data should give deeper insights into the mode of action and the interaction with the human immune system. As we hypothesized, the interaction of BT-061 with CD4 expressed on T cells did not only induce down-modulation of CD4. The administration of the mAb caused additional effects within the target cell, which depended

on cross-linking of BT-061 by coating or the interaction with FcγR as well. These additional effects included the down-modulation of the surface molecules CD3, CD25, CD28 and CD69 on T cells (Figure 4.16) and increased numbers of transcripts for several transcription factors as well as the chemokine chemokine (C motif) ligand (XCL1) (Figure 4.18). The various effects cross-linked BT-061 causes in T cells support the assumption that a signal is triggered upon the interaction with CD4, albeit the induced pathway is not as obvious. The classical T cell receptor (TCR) signaling that requires engagement of the TCR by the respective antigen-major histocompabitility complex (MHC)-complex, induces various measurable modifications within the T cell. These checkpoints include for example activation of the sarcoma (Src) kinase lymphocyte-specific protein tyrosine kinase (Lck), phosphorylation of ζ-chain-associated protein kinase 70 (Zap-70) and phospholipase C-γ (PLC-γ), the influx of calcium $(Ca)^{2+}$, induction of proliferation or secretion of IL-2 (chapter 2.1.3). The therapeutic mAb OKT3 (also known as Orthoclone or Muromab) mediates the down-modulation of its target molecule CD3 on T cells upon binding [254]. The addition of OKT3 mimics antigen ligation with the TCR and activates T cells, but only in the presence of accessory cells like monocytes [217]. Therefore, the administration of OKT3 to human PBMC induces for example Ca^{2+}-influx [255], IL-2 secretion and proliferation [256-259]. These observations were also reported upon the treatment of T cells with the phorbol ester phorbol-12-myristate-13-acetate (PMA) (Figure 4.17C and D; [260, 261]) or the ionophor Ionomyicin (Figure 4.17C and D; [262]), whereas they trigger the previous mentioned pathway under exclusion of the TCR [263]. Interestingly, those mitogens induced a massive decrease of surface markers like CD4 and CD8 as well [264]. In spite of the parallels concerning the target down-modulation upon treatment between OKT3, PMA, Ionomycin and BT-061, BT-061 does not affect or depend on the modification of classical checkpoints affected upon TCR engagement. Initial analysis measuring Ca^{2+}-influx upon BT-061 treatment of T cells revealed no triggering of T cell activation on that level (data not shown). Although Ca^{2+}-flux was not detected, it is not clear whether the putative induced Ca^{2+}-flux was under the detection level. Therefore the usage of reagent to block Ca^{2+}-flux, or kinase activity could be taken into account. Furthermore neither release of IL-2 (Figure 4.17C and D) nor proliferation (Figure 4.19A, B and Figure 4.20A, B, C) were detected after treatment with BT-061. Additionally the status of activation markers like CD69 and CD25 was tested upon BT-061 treatment to investigate T cell activation on another level. The experiments revealed that BT-061 does not induce an up-

regulation of CD25 and CD69, which is observed following OKT3 treatment [265]. In spite of that, experiments using 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine (PP1) to block Lck activity in PBMC showed that the activation of Lck is partially required for CD4 down-modulation induced by BT-061 (Figure 4.14B). Concerning this data it is worth mentioning that the effective dosage of more than 10 µM to significantly reduce BT-061 mediated CD4 down-modulation clearly exceeded the effective amount reported by others [183]. Interestingly, Criado and Madrenas demonstrated that additionally to the conventional paradigm Lck plays also a critical role in the activation through the TCR, that Lck contributes to the down-regulation of T cell activation and cytokine production [266]. Therefore, the data indicating that BT-061 mediated CD4-down-modulation requires Lck activation are not oppositional to the observations that BT-061 induces no T cell activation. With respect to the previously mentioned data it is conceivable that the treatment of T cells with cross-linked BT-061 does not activate T cells. The data obtained with BT-061 are in line with the results reported by Morel *et al.*. Morel *et al.* concluded that the tested CD4-specific mAb BB14, BF8, BL4, 13B8.2 and F101-69 are not able to activate T cells, because proliferation and Ca^{2+}-flux of treated T cells were missing [196]. However, to further elucidate the triggered pathway(s) that is (are) involved in or crucial for CD4 down-modulation it would be interesting to investigate the phosphorylation status of variable components of T cell signaling. Western blot analysis or the cytometrically examination of the phosphorylation of the kinases like Zap-70, PLC-γ or mitogen-activated protein kinase (MAPK) or transcription factors like nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB) or nuclear factor of activated T cells (NFAT). Furthermore, the blockage of T cell signaling at different checkpoint or on variable levels might be helpful. Even though no influence of BT-061 on classical checkpoints of T cell activation was observed, the data obtained from other experiments indicated that BT-061 influences T cells. Microarray analysis showed a specific profile of genes regulated by BT-061 (Figure 4.18A and B) and furthermore down-modulated BT-061 the surface expression of CD3, CD4, CD25, CD28 and CD69 on T cells (Figure 4.16). The investigation of other mAb showed that these mAb do not activate T cells but influence T cells via suppression of T cell activation. Woods *et al.* demonstrated that the humanized CD4-targeing mAb YHB.46 caused an inhibition of up to 100% of the proliferation of purified $CD4^+$ T cells activated with coated OKT3 [267]. The addition of BT-061 did not influence the proliferation of T cells induced by OKT3 (Figure

4.19A and B). The absence of proliferation suppression indicated that BT-061 did not influence the pathway induced upon TCR stimulation by OKT3 to stimulate proliferation. Additionally, it was observed that BT-061 treatment of T cells co-cultured with allogenic DC did not affect the proliferation rate (Figure 4.20A, B and C), although CD4 expression was clearly reduced at day 7 (Figure 4.20B, left graph). This is in strong contrast to the results Merkenschlager *et al.* reported. Their studies demonstrated that 22 tested CD4-specific mAb inhibited T cell proliferation in a mixed lymphocyte reaction (MLR) [268]. A possible explanation for the variable results might be the different impact of the tested mAb on MHC class II-CD4-interaction, which seems to be crucial for the induction of T cell proliferation. The assumption that MHC class II-CD4-interaction is critical to induce proliferation is supported by the observation of Scheinecker *et al.*. Their data demonstrated that proliferation of T cells induced by highly purified cDC depends on high expression levels of MHC class II [269]. Furthermore Romain *et al.* described that the blockage of CD4 via mAb profoundly inhibits the auto-reactivity of T cells [270]. Therefore it was suggested that CD4 mAb inhibit proliferation within an MLR by sterically inhibiting the interaction between CD4 and MHC class II. Interestingly, investigations concerning the crystal structure of BT-061, MHC class II and CD4 revealed that the mAb does not interfere sterically with the binding of MHC class II to CD4 [222]. This observation might be an explanation for the missing suppressive effect of BT-061 on T cell proliferation within a MLR compared to other CD4 specific mAb. However, a clear CD4 down-modulation upon BT-061 treatment was observed, which seem to have no impact on T cell proliferation. Furthermore, it was shown that the expression of the co-stimulatory molecules B7-1 and B7-2 on human DC is crucial for the initiation of MLR [269]. These molecules interact with CD28 expressed on T cells [62]. Interestingly, the determination of CD28 surface expression revealed that this molecule was as well down-regulated upon BT-061 treatment (Figure 4.16). These data suggests that in spite of the down-regulation of CD28 induced by BT-061, the T cell proliferation within an MLR is not suppressed. Furthermore the obtained data revealed that BT-061 did not induce the differentiation of T cell subsets or triggers T cell subsets that were sufficient to suppress the proliferation induced in a MLR or by OKT3.

As mentioned before, the discrepancy between BT-061 and other CD4-specific mAb concerning the suppressive potential in an MLR or OKT3 induced proliferation might be due to the fact that variable epitope specificity causes different effects on T cell signal

transduction [267]. The different effects of CD4 mAb binding to variable epitopes of CD4 on T cells was demonstrated by comparing messenger ribonucleic acid (mRNA) expression induced by BT-061 and RPA-T4 (Figure 4.18A and B). BT-061 was shown to specifically bind to IgG like C2 type 1 domain of CD4 [222] and RPA-T4 to NH2- terminal D1 region of CD4 [271]. Further experiments demonstrated that these two mAb do not sterically inhibit each other, which supports the hypothesis that they might bind different epitopes of CD4 [222]. These non-competing mAb induced a different profile of regulated genes upon treatment (Figure 4.18A).

The results obtained with microarray analysis were additionally used to clarify whether BT-061 directs T cells towards Th1 or Th2 type responses. This shifting of the balance between Th1 and Th2 cells might be beneficial for the treatment of autoimmune diseases. The analysis of the transcriptome indicated that amongst others the mRNA for 4 transcription factors interferon regulatory factor (IRF)1, IRF4, early growth response protein (EGR)1 and (EGR3) as well as the mRNA for the chemokine XCL1 was significantly up-regulated compared to the other CD4-specific mAb RPA-T4 and soluble BT-061. However, the profile of genes regulated upon treatment of $CD4^+$ T cells with coated BT-061 indicates no definite polarization into Th1 or Th2. The administration of BT-061 induced the up-regulation of mRNA encoding for the transcription factors IRF1 [272] and IRF4 [273], which belong to the family of IRF. Whereas IRF1 is in general up-regulated upon stimulation of T cells with IFN I/II [272], IRF4 (also called lymphocyte specific IRF (LSIRF), NF.EM5 or PU.1-interaction partner (Pip)) is not activated by type I or type II IFN [274]. IRF1 seems to favor the differentiation of a Th1 response, because it was shown that this transcription factor represses IL-4 [275] and activates the promoter for Il12rb1, the gene encoding the IL-12 receptor β1 subunit [276]. In contrast, it was reported that IRF4 is associated with Th9 cell development *in vitro* as well as *in vivo* [277]. Furthermore, IRF4 regulates Th2 and Th17 cell differentiation [278]. In spite of this data, it was stated that it is not clear whether IRF4 acts as a master regulator of specific differentiation programs or serves more as an integrator of lymphocyte response [279]. Even the enhanced transcription of the zinc finger transcription factors EGR1 [280] and EGR3 [281] provided ambiguous information concerning the T cell commitment upon BT-061 stimulation. On the one hand Lohoff *et al.* reported that EGR1 is preferentially expressed in Th2 cells and is involved in acute transcription of the Th2 cytokine IL-4 upon T cell stimulation in human T cell lines [282]. On the other hand Safford *et al.* using microarray

analysis of murine cells demonstrated that EGR2 and EGR3 are key negative regulators of T cell activation and are associated in the induction of anergy upon TCR stimulation [283]. The induction of mRNA expression of the protein XCL1 (also called lymphotactin, chemokine-related cytokine (ATAC), single cysteine motif (SCM)-1) is a further evidence to suggest that BT-061 is beneficial for Th1 polarization, because the study of Nagai *et al.* indicated that differentiated Th1 cell from human cord blood cells highly express mRNA encoded for XCL1 upon PMA/Ionomycin stimulation compared to Th2 cells [284]. Furthermore it was shown that XCL1 might act via an autocrine positive feedback mechanism and thereby benefits to optimal functional maintenance of regulatory T cells (Treg cells) in asthma [285]. The data obtained using analysis of the transcriptome of $CD4^+$ T cell upon stimulation with BT-061 did not finally elucidate whether BT-061 stimulate $CD4^+$ T cells to differentiate into a Th1 or Th2 subset. The data measuring the release of Th1-specific cytokines IFN-γ support the assumption that BT-061 did not trigger a specific T effector cell subset, because no secretion was detected upon treatment (Figure 4.17B). However, it is reasonable to furthermore investigate whether BT-061 preferentially triggers a specific T cell subset or directs T cells towards the differentiation into a Th1 or Th2 type. Because it is described that BT-061 is able to selectively activate Treg cells by binding to a specific epitope of it is interesting to analyze the effect of BT-061 on various T cell subsets. Therefore it is possible to enrich T cell subsets like Th1, Th2 or Treg cells and to perform micro array analysis upon BT-061 treatment. The influence of BT-061 on T cell commitment to a specific subtype could be examined by analyzing the distribution of various T cell subsets in peripheral blood and the detection how the treatment with BT-061 changes the ratio between the investigated subsets. It would be interesting to measure the influence of BT-061 treatment on the distribution of variable T cells subsets under pro-inflammatory conditions, because these circumstances could prevail in the patient. Although the previously discussed data did not elucidate the pathway triggered or influenced by BT-061 treatment of T cells, they were extremely valuable to provide a risk assessment of BT-061. Although the potential of NK cells to interact with the Fc portion of BT-061 and to induce CD4 down-modulation was detected (Figure 4.3A and B), neither increased death of T cells (data not shown) nor the activation of NK cells was identified via measuring IFN-γ in BT-061 treated PBMC (data not shown). These data indicate that BT-061 does not deplete T cells via antibody dependent cell mediated cytotoxicity (ADCC), which is important to clarify, because T cell depletion is not the anticipated mode of

action for BT-061 to treat autoimmune diseases. Furthermore, this study showed that no side effects like a cytokine storm is expectable upon BT-061 application and are in line with observation obtained in preclinical trails. The additionally tested mAb OKT3 and TGN1412, which are known to induce a CRS in patients upon administration [160, 216], trigger T cells to secrete TNF-α and IL-2 (Figure 4.17A,C and D), whereas this was not detected after the treatment with BT-061 (Figure 4.17A,C and D). However, the data obtained with TGN1412 and OKT3 deviate from those described by other groups. They showed that TGN1412 induced TNF-α, IL-2 and additionally IFN-γ, IL-4, and IL-10 levels that varied significantly from those induced by controls. OKT3 induced the release of IL-2 and additionally IFN-γ, IL-4, and IL-10. These differences between the results might be due to the fact that they measured protein levels in a so called RL-PTE-100 module, consisting of PBMC, granulocytes, an endothelial cell monolayer grown on a collagen scaffold and 100% autologous platelet-poor plasma [286]. The data obtained analyzing the induction of mRNA expression gave no evidence that any of these cytokines, which are induced by known CDR stimulating mAb, are increased on mRNA level upon BT-061 treatment. Experiments with BT-061 added to cells, which were stimulated with OKT3 or allogenic imDC to induce proliferation, revealed that this mAb does not inhibit T cell expansion in that experiments. Therefore, it is imaginable that the treatment of patients with BT-061 does not lead to an immune suppressed status in the patient mediated by unresponsive T cells. Furthermore the obtained data revealed that the chosen isotype of BT-061 is suitable for the anticipated CD4 down-modulation. Because it was demonstrated that BT-061 needs to be cross-linked to be functional, the interaction with FcγR is important to develop efficacy. The data demonstrated that BT-061, which is designed as an IgG subtype, interacts with FcγR, but mediates no ADCC via the interaction of the Fc portion with FcγR.

6. Reference list

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286. Dhir, V., et al., *A predictive biomimetic model of cytokine release induced by TGN1412 and other therapeutic monoclonal antibodies.* J Immunotoxicol, 2012. **9**(1): p. 34-42.

7. Curriculum vitae

Personal data:

Date of birth	22.07.1982
Place of birth	Hamburg, Germany
Nationality	German

Education and Qualifications:

Since November 2008	Employed as Ph.D. student in the working group Experimental Infection Research of. Prof. Dr. Kalinke at the TWINCORE, Centre for Experimental and Clinical Infection Research in Hannover, Germany.
April 2008	Diplom (Biology)
February 2005	Vordiplom
May 2002	Abitur, Heisenberg-Gymnasium, Hamburg

Congress or course participation:

28.11. – 30.11.2011	**2nd EFIS-EJI Intensive Educational Course in Clinical Immunology**, Paris, France.
28.09. – 01.10.2011	**5th 2011 Joint Annual Meeting** of the Italian Society for Immunology, Clinical Immunology, Allergology (SIICA) and German Society of Immunology (DGfI), Riccione, Italy.
20.09. – 22.10.2011	**3rd TWINCORE-Symposium** with the title: "Establishment and Control of Chronic Infections", Hannover, Germany.
01.09. – 02.09.2011	**5th International VPM Days** (Vakzine Projekt Management GmbH) at the TWINCORE, Hannover, Germany.
02.09. – 03.09.2010	**4th International VPM Days** (Vakzine Projekt Management GmbH) at the TWINCORE, Hannover, Germany.

12.08. – 13.08.2010	**2nd TWINCORE-Symposium** with the title: "Antimicrobials and Vaccines", Hannover, Germany.
09.05. – 16.05.2010	**5th ENII EFIS/EJI Immunology Summer School**, Capo Caccia, Italy (Poster presentation).
In 2010	**9th workshop dendritic cells** at the Medical School Hannover, Germany.
12.11. – 14.11.2009	**Masterclass of Virology** at the Medical School Hannover, Germany (Poster presentation).
09. – 10.09.2009	**1st TWINCORE-Symposium** with the title: "Infection Research and Beyond", Hannover, Germany. **2009 8th workshop dendritic cells** at the TWINCORE with the title: "Innate regulation of DC function", Hannover, Germany.
13.09. – 16.09.2009	**2nd Congress of Immunology**, Berlin, Germany.
03.09. – 07.09.2007	Congress of Botany, Hamburg, Germany (Poster presentation).

Poster presentation:

09.05. – 16.05.2010	**5th ENII EFIS/EJI Immunology Summer School**. Title of the poster: "A new approach to study interactions between the constant moiety of IgGs and human Fcγ-receptors".
12.11. – 14.11.2009	**Masterclass of Virology at the Medical School**. Title of the poster: "Towards a better understanding of the interaction of the constant antibody moiety with human immune cells".
03.09. – 07.09.2007	**Congress of Botany**. Title of the poster: "Detection of putative biotic factors involved in the Dieback disease of sissoo (*Dalbergia sissoo* Roxb.) in Bangladesh" by Nayuv Valedez, Stephanie Vogel, Imdadul Hoque and Hanny Tantau.

Studies abroad:

19.05. – 17.07.2008	Two-month **internship** in the working group of Prof. Dr. Dickinson at the University of Oxford. Oxford, United Kingdom. Title of the work: „**Epigenetic regulation and function of plant imprinted genes**“.
02.04. – 16.05.2008	Six-week **internship** in the working group of Prof. Dr. Palukaitis at the Scottish Crop Research Institute, Dundee, Scotland. Title of the work: “**Investigation of the interaction of a protein involved in inhibiting virus replication**“.
Mar. 2007 – Feb. 2008	**Scientific assistant** for the student courses for genetics and microbiology
Mar. 2007	Four-week **internship** in the working group of Prof. Dr. Mühlbach at the University of Hamburg. Title of the work: “Investigations in *Rubus spec.* as potential host of EMARAV“.
Mar. 2007 – Feb. 2008	**Scientific assistant** for the student courses for genetics and microbiology
Jan. 2006	Six-week **internship** in the working group „molecular parasitology“ of Dr. Bruchhaus at the Bernhard-Nocht-Institute for tropical medicine (Hamburg). Title of the work: “Investigations in RNA interference on *Entamoeba histolytica*“.
Oct. 2004 – Apr. 2006	**Scientific assistant** in the working group of Prof. Dr. Sand in the institute of microbiology of the University of Hamburg

8. Publication list

Published:

S Vogel, H Tantau, N Mielke-Ehret, MI Hoque, RH Sarker, ML Saha, SK Shamimul Alam, MS Khan, HP Mühlbach 2011. "Detection of virus particles and double-stranded RNA in dieback affected *Dalbergia sissoo* Roxb. from Bangladesh". *Bangladesh Journal of Botany.* 40(1): 57-65.

Manuscript submitted:

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