AUS DEM LEHRSTUHL
FÜR IMMUNOLOGIE
FRAU PROF. DR. DANIELA MÄNNEL
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

EFFECTS OF TNF RECEPTOR SIGNALLING ON THE FUNCTION OF SUPPRESSIVE IMMUNE CELLS

Inaugural – Dissertation
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Für meine Eltern

Abstract

Regulatorische T-Zellen und myeloide Suppressorzellen sind Immunzellen, deren Hauptfunktion nicht in der Abwehr von Pathogenen, sondern in der Regulation von Immunantworten liegt. Die Fehlfunktion dieser immunregulatorischen Zellen kann eine Vielzahl von fatalen Folgen haben. Falls die immunregulatorische Funktion geschwächt ist, kann dies zu einer fehlenden Hemmung von autoreaktiven Zellen führen. Diese überschießende Aktivität von Immunzellen gegen das köpereigene Gewebe kann zu der Entstehung von Autoimmunerkrankungen beitragen. Andererseits können die suppressiven Zellen auch verstärkt aktiv sein. Die gesteigerte Inhibition von Immunzellen kann in diesem Fall eine adäquate Immunreaktion gegen Krebszellen verhindern und begünstigt damit die Entwicklung und Ausbreitung von Tumoren. Neben einer Vielzahl von Mediatoren wird auch der Tumor Nekrose Faktor (TNF) als ein wichtiger Modulator jener immunsuppressiven Zellen gesehen. In vorausgehenden Experimenten in unserem Labor konnte gezeigt werden, dass bestimmte Agonisten von dem TNF-Rezeptor Typ 2 (TNFR2) die Suppression von $CD4^+$ und $CD8^+$ T-Zellen durch regulatorische T-Zellen hemmen. Ziel dieser Arbeit war es, die Auswirkungen von dem spezifischen TNFR2-Agonisten TNCscTNF80 auf die Suppressionsfähigkeit von regulatorischen T-Zellen und myeloiden Suppressorzellen zu bestimmen. Es konnte gezeigt werden, dass TNCscTNF80 die Suppression von T-Zellen durch diese regulatorischen Immunzellen hemmt. Des Weiteren wurde untersucht, welche Veränderungen TNCscTNF80 in regulatorischen T-Zellen auf molekularer Ebene auslöst. TNCscTNF80 führte zu einer verminderten Expression von bestimmten Oberflächenmolekülen, die charakteristisch für regulatorische T-Zellen sind und die zu deren Suppressorfunktion beitragen sollen. Es kann daher spekuliert werden, dass Agonisten am TNFR2 die Funktion von regulatorischen T-Zellen hemmen, indem sie die Zusammensetzung der Oberflächenmoleküle verändern. Außerdem wurde überprüft, ob TNCscTNF80 einen Einfluss auf intrazelluläre Signalkaskaden in regulatorischen T-Zellen hat. Dafür wurde die Aktivität von zwei Signalmolekülen untersucht; dem signal transducer and activator of transcription 5 (Stat5) und dem ζ-chain-associated protein of 70kD (ZAP70), welche in Folge der Stimulation von dem T-Zell Rezeptor (TCR) bzw. dem IL-2 Rezeptor (IL-2R) hochreguliert werden. TNCscTNF80 hatte jedoch keinen deutlichen Einfluss auf die Aktivierung dieser Signalmoleküle in regulatorischen T-Zellen.

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List of abbreviations

Abbreviation	Explanation
$A_{2A}R$	A_{2A} receptor
AF488	Alexa fluor 488
AF647	Alexa fluor 647
AICD	Activation-induced cell death
Akt	Serine-threonin kinase
AMP	Adenosine monophosphate
AP-1	Activator protein-1
APC	Allophycocyanin
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Cbf-β	Core-binding factor β
γc	γ-chain
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Conserved non-coding sequences
CS	Control supernatant
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
Con A	Concanavalin A
DAG	Diacylglycerol
DC	Dendritic cell
DPBS	Dulbecco’s phosphate-buffered saline
Ebi-3	Epstein-Barr-virus induced gene 3
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assays
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting

FADD	Fas-associated death domain
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein P3
g	Gravitational acceleration
GITR	Glucocorticoid-inducible tumor necrosis factor receptor family-related gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRP	Horseradish Peroxide
H_2O_2	Hydrogen Peroxide
hTNF	Human tumor necrosis factor
IBD	Inflammatory bowel disease
ICER	Inducible cAMP early repressor
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IL-2R	IL-2 receptor
IL-2Rα	IL-2 receptor α-chain
IL-2Rβ	IL-2 receptor β-chain
iNos	Inducible nitric oxide synthase
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance
IP_3	Inositol 1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motifs
iT_{reg}	Inducible regulatory T cell
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAT	Linker for activation of T cells
LAG-3	Lymphocyte-activated gen 3
LR	Lower right
mAB	Monoclonal antibody
MACS	Magnetic cell separation

MAPK	Mitogen activated protein kinase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
mg	Milligram
ml	Millilitre
mTNF	Membrane-bound tumor necrosis factor
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometre
NK cells	Natural killer cells
ns	Non-stimulated
nT_{reg}	Natural regulatory T cell
PAMP	Pathogen-associated molecular patterns
PE	Phycoerythrin
PerCP	Peridinin chlorophyll-A protein
pg	Picogram
pH	p[H] value
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC-θ	Protein kinase C-θ
PLCγ1	Phospholipase C-γ1
PRR	Pattern-recognition receptors
pStat5	Phosphorylated signal transducer and activator of transcription 5
PTEN	Phosphatase and tensin homolog
RIP	Receptor-interacting protein
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RT	Room temperature
Runx1	Runt-related transcription factor 1
scTNF	Single chain tumor necrosis factor
SHC	SRC-homology-2-domain-containing transforming protein C

SLP-76	SH2-domain-containing leucocyte phosphoprotein of 76kDa
SP	Single positive
Stat5	Signal transducer and activator of transcription 5
sTNF	Soluble tumor necrosis factor
TACE	Tumor necrosis factor converting enzyme
T_{conv}	Conventional T cell
TCR	T cell receptor
T_{eff}	Effector T cell
TGF-β	Transforming growth factor-β
Th	T helper
TNC	Tenascin C
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
$TNFR1^{-/-}$	Tumor necrosis factor receptor 1-deficient
TNFR2	Tumor necrosis factor receptor 2
$TNFR2^{-/-}$	Tumor necrosis factor receptor 2-deficient
TRADD	Tumor necrosis factor receptor-associated death domain
TRAF2	Tumor necrosis factor receptor-associated factor 2
T_{reg}	Regulatory T cell
TSDR	T_{reg}-specific demethylation region
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
Tyr	Tyrosine
U	Enzyme activity unit
UL	Upper left
UR	Upper right
ZAP-70	ζ-chain-associated protein of 70kD
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

1 Introduction

1.1 The immune system

An advanced immune system has emerged in highly developed species in order to provide efficient host defence against bacterial, viral, fungal or parasitic infections. This system is divided into two functionally different components, the innate (natural) and acquired (adaptive) immune system (1).

The innate immune system is comprised of both cells and soluble factors, which are activated at the beginning of an infection to fight the invading pathogens. Innate immune responses are initiated following the detection of evolutionary conserved pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRR) (2). Cells capable of phagocytosis, e.g. neutrophils, monocytes or mastocytes, or of producing proinflammatory factors, e.g. basophiles, mast cells or eosinophiles, and natural killer (NK) cells constitute the cellular compartment, whereas complement factors, acute-phase proteins and cytokines, including interleukins (IL) and interferons (IFN), represent the molecular section. In contrast to the adaptive immunity, innate immune responses are not directed against a specific antigen and do not improve upon reencounter with pathogens that have previously invaded the organism because no immunological memory remains (3).

The adaptive immune system, however, is capable of forming memory cells, which initiate a quicker and more specific response upon reencounter with an antigen. T- and B-lymphocytes represent the effector cells of the adaptive immune system, which express receptors that are specific for a certain antigen. A large repertoire of those receptors is necessary in order to recognize a great variety of antigens. Therefore, extensive recombination of the T cell receptor (TCR) during the T cell development in the thymus and of the antibodies that form part of the B-cell receptor (BCR) is required. The TCR consists of α- and β-chains or γ- and δ-chains and non-covalently binds to the cluster of differentiation (CD) 3 complex, which is composed of four different polypeptides, the γ-, δ-, ε- and ζ-chains. The co-receptors CD4 or CD8 are additionally associated with the TCR complex. This receptor complex recognizes peptides that have been processed from antigen proteins and are subsequently presented on cell surfaces by the major histocompatibility complex (MHC). Activation of $CD4^+$ cells is induced by the interaction of the TCR with peptide antigens presented by MHC II, which are especially found on antigen-presenting cells (APC), e.g. dendritic cells (DC), activated macrophages and B cells (1, 4). $CD8^+$ cells, on the other hand, bind to MHC I, which are ubiquitously expressed on most nucleated cells and which present

cytosolic proteins. Cells that are virally infected or malignantly transformed can, thereby, present their intracellular antigens to $CD8^+$. As $CD8^+$ are cytotoxic, they can eliminate those pathogenic cells (1, 4, 5).

1.2 $CD4^+$ T helper cells

In contrast to $CD8^+$ T cells that are directly cytotoxic, $CD4^+$ T helper cells predominantly secret cytokines and activate other cells of the immune system (5). In order to be activated, naïve $CD4^+$ T cells need to receive two signals, the interaction of a MHC/peptide complex with their TCR and additional co-stimulatory signals. For instance, the co-stimulatory receptor CD28 is bound by the ligands CD80/CD86, which are expressed on the cell surface of mature APC (6). Thus, in order to initiate an adaptive immune response, those APC, such as macrophages or DC, need to recognize a foreign antigen, undergo maturation, including the upregulation of CD80/CD86, and migrate to the lymph nodes, where they present the processed antigen peptide on their MHC II. T cells that express the TCR specific for that certain antigen recognize the MHC/antigen complex, which, together with co-stimulatory signals, induces the activation and differentiation of T cells (7). Once activated, T cells secret cytokines and initiate the activation of B cells, thus, enabling somatic hypermutation of the BCR and the immunoglobulin class switch (5).

1.2.1 T helper cells: Th1, Th2, Th17

Depending on the cytokine milieu and factors that are present during the activation, $CD4^+$ cells can develop into different T helper (Th) subtypes (8). Based on the profile of cytokine secretion, those $CD4^+$ Th cells can be divided into three major groups, i.e. Th1, Th2 and Th17 cells, as seen in Figure 1 (9). Cytokines produced by Th1 cells, e.g. IL-2 and IFN-γ promote the initiation of a cellular immune response, which includes the activation of macrophages and cytotoxic T cells to fight intracellular pathogens. Th2-speficic cytokines, e.g. Il-4, IL-5, IL-6 and IL-10, on the other hand, initiate the humoral immune response by inducing the differentiation of B cells into antibody-producing plasma cells (5, 10). Th17 cells produce the proinflammatory cytokines IL-17 and IL-22, which induce the secretion of other immunostimulatory cytokines, e.g. IL-6, IL-1β and tumor necrosis factor (TNF), and growth factors that promote the recruitment of neutrophils. Therefore, it is suggested that Th17 cells play an important role in the early defence against extracellular pathogens that are not efficiently cleared by Th1 or Th2 cells (9, 10).

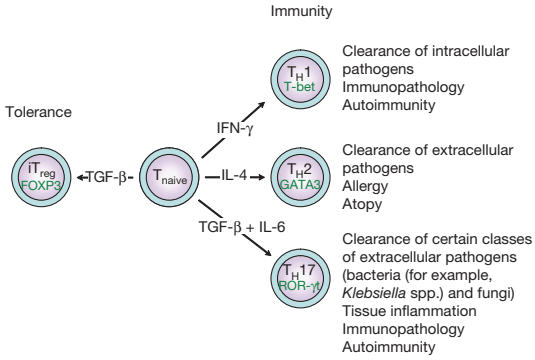

Figure 1: T helper cell subpopulations. Naïve $CD4^+$ cells can develop into different T helper cells subsets (Th1, Th2, Th17) or inducible regulatory T cells (iT_{reg}) depending on the cytokines present during the initial activation. Each sub-population expresses characteristic transcription factors (T-bet, GATA3, ROR-γt, FOXP3) (figure from Bettelli *et al.*, 2008, p.1051 (9)).

1.3 Control over inflammation

A major question in immunology is how the immune system distinguishes between self and non-self. In order to effectively eliminate pathogenic antigens while inhibiting autoimmunity, various mechanisms have evolved to ensure self-tolerance. A key mechanism is the central tolerance established in the thymus. During negative selection, lymphocytes are eliminated if they bear TCR that have high affinities for self-peptides presented by medullary thymic epithelial cells or DC. But this mechanism is not perfect, as autoreactive T cells escape the negative selection if they express a TCR with low affinity for self-antigens or with specificities for self-antigens that are not sufficiently presented in the thymus (11, 12). Furthermore, the early development of conventional T cells in the neonatal period occurs faster than in adults due to a compromised negative selection, which results in a higher chance of autoreactive T cells being released into the periphery (13). Thus self-recognizing T cells are circulating in the periphery, which implies that further control mechanisms are necessary in order to reinforce self-tolerance and prevent destruction of healthy tissue. One way to stop possible autoimmune processes is the establishment of immunological privileged tissues. In the absence of inflammation, potential autoreactive T cells are excluded from those tissues, limiting the contact with tissue-restricted antigens and thus the occurrence of autoimmunological processes in those organs (12). Moreover, for full activation of T cells co-stimulatory signals are required in addition to the TCR stimulation, e.g. the interaction of CD28 with its ligands CD80/CD86 expressed by activated APC. Yet, APC are only completely activated after recognizing external pathogens; self-peptide presenting APC therefore do not upregulate CD80/CD86 on their surface. Thus, autoreactive T cells

recognizing MHC/self-peptide complexes do not receive co-stimulation via CD28 and subsequently become anergic. The necessity of co-stimulation represents an elegant mechanism, with which clonal activation of T cells in response to self-antigens is prevented (14). Self-reactive T cells are additionally controlled by the initiation of the activation-induced cell death (AICD), which is mediated via the interaction of the death receptor Fas (CD95) with its ligand (FasL/CD95L) or via mitochondrial proteins. CD28, however, rescues T cells from AICD, posing another way of how co-stimulatory signals are utilized to ensure the balance between self-tolerance and efficient pathogen control (15).

1.3.1 Regulatory $CD4^+CD25^+$ cells

In addition to the mechanisms acting directly on the T cells, T cell extrinsic mechanisms also promote peripheral tolerance. In this regard, peripheral lymphocytes themselves contribute to self-tolerance. A specific $CD4^+$ cell subset, the $CD4^+CD25^+$ regulatory T cells (T_{reg}), which express the high affinity IL-2 receptor α-chain (IL-2Rα), also referred to as CD25, and forkhead box protein P3 (Foxp3), exert a homeostatic control over the immune system. As early as 1970, the existence of suppressive immune cells, which could not only promote but also moderate immune responses, was proposed (16). While the existence of those suppressive T cells was doubted again during the 1980s, mostly due to the lack of specific markers, they once more became the focus of investigation in the 1990s. Sakaguchi *et al.* convincingly showed that a distinct subset of lymphocytes, the $CD4^+CD25^+$ T cells, was required to maintain self-tolerance *in vivo* (17). Further it was demonstrated that those cells responded in a hypoproliferative manner upon TCR stimulation and effectively suppressed $CD4^+CD25^-$ cells *in vitro* (18). In contrast to the conventional $CD4^+$ effector T cells (T_{eff}), which exhibit an important function in the adaptive immune response by activating cytotoxic $CD8^+$ T cells, B cells or macrophages, those regulatory T cells are of importance in the control and suppression of immune reactions. Depletion of these cells leads to various autoimmune disorders, e.g. inflammatory bowel disease (IBD), autoimmune gastritis, autoinflammatory Type 1 diabetes or thyroiditis (17, 19). Nevertheless, T_{reg} also have negative effects as they strongly contribute to the pathogen persistence during chronic infections and the evasion of tumor cells from the immune control (20, 21).

The $CD4^+CD25^+$ T_{reg} constitute 5-10 % of the $CD4^+$ T cells in the periphery of rodents, whereas in humans estimates vary from only 1-2 % to 7-8 % depending on the markers used (21, 22). Although T_{reg} constitutively express CD25 and are usually purified based on their $CD4^+CD25^+$ phenotype, conventional T cells (T_{conv}) are also known to

upregulate CD25 upon activation via their TCR (23–25). Thus, CD25 cannot be used as an exclusive and reliable marker for T_{reg}. Yet, an additional and more specific marker, the transcription factor Foxp3, which is essential for both the development and the function of $CD4^+CD25^+$ T_{reg}, was identified (26). A defect in the Foxp3 gene is responsible for the lethal over-proliferation of $CD4^+$ cells in scurfy mice (27), while it causes the immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX) syndrome in humans, which is characterized by IBD, allergies and severe autoimmune diseases in various organs (28). As Foxp3 promotes the expression of T_{reg}-associated cell surface molecules and acts as an initiator of the T_{reg} specific genetic program, it is considered to be the master regulator for the phenotype, differentiation and lineage stability of T_{reg}. Further, Foxp3 also inhibits the production of the T cell growth factor IL-2 (29) and of other proinflammatory cytokines, such as IL-4, IL-17, IL-21, IFN-γ and TNF (30). The expression of Foxp3 is specific for murine $CD4^+CD25^+$ T_{reg} and is not upregulated upon TCR stimulation in naïve T cells (26). In humans however, a fraction of former $CD4^+CD25^-$ cells gains transient FOXP3 expression upon stimulation via their TCR (31, 32). Apart from the expression of Foxp3, T_{reg} also share other characteristic markers, e.g. cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-inducible tumor necrosis factor receptor family-related gene (GITR), and lymphocyte-activated gen 3 (LAG3) (33). Like CD25, both CTLA-4 and GITR are constitutively expressed on T_{reg} but also show upregulation on T_{eff} upon activation (34, 35). Furthermore, a variety of new markers for T_{reg} has been described, e.g. the ectonucleoside triphosphate diphosphorhydrolase-1 CD39, ecto-5-nucleotidase CD73, latency-associated peptide, the homing-associated cell adhesion molecule CD44, CD103, chemokine receptors such as CCR6, members of the galectin family and the transcription factor Helios (36).

1.3.2 Myeloid-derived suppressor cells

In addition to regulatory T cells, myeloid-derived suppressor cells (MDSC) also represent an important type of immunoregulatory cells that are characterized by the capacity to suppress T and NK cell activity. Rather than being a specific subpopulation, MDSC are constituted of a heterogeneous group of immature myeloid cells that fail to fully differentiate into mature cells during pathological processes. Under physiological conditions, maturation of bone marrow (BM) cells into DC, macrophages or mature granulocytes is readily achieved. However, in pathologic conditions, such as cancer, microbial infections, chronic inflammation and trauma, there is a delay of cell maturation and immature myeloid cells expand, become

activated and acquire suppressor function (37). As MDSC suppress T cell activation especially in the cancer patients, they contribute to an impaired lymphocyte-mediated antitumor immunity and thereby promote tumor progression. Thus, great effort is put into finding strategies to decrease the number and suppressor function of MDSC (38).

In mice, MDSC are defined by the simultaneous surface expression of Gr-1 and CD11b. Gr-1 was later identified to be a common epitope of two distinct surface molecules, Ly6G and Ly6C. Hence, MDSC can be further divided into two subsets, the granulocytic ($CD11b^{+}Ly6G^{+}Ly6C^{low}$) and monocytic ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) phenotype (39). Expansion and activation of the MDSC is achieved by a variety of factors, i.e. cyclooxigenase-2, prostaglandin E2, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor, IL-6, IL-1β and vascular endothelial growth factor (40). *In vitro*, GM-CSF most accurately simulates the *in vivo* expansion of DC under inflammatory conditions (41). Conversely, it has been shown that *in vitro* stimulation with low-dose GM-CSF can induce the generation of suppressive MDSC after eight to ten days, while under high-dose conditions only three to four days are required (42). MDSC utilize various mechanisms of suppression, which include the upregulation of arginase-1 or of the inducible nitric oxide synthase (iNOS), enzymes that both metabolize the amino acid arginine, and increase the production of nitric oxide and reactive oxygen species (ROS) (43). Other reports also suggest that suppression is mediated via the induction of T_{reg} (44), secretion of transforming growth factor-β (TGF-β) (45) or depletion of cysteine (46).

1.4 Intracellular signalling in effector and regulatory T cells

1.4.1 Signalling via the TCR in conventional T cells

As shown in Figure 2 (47), TCR engagement initiates different signalling pathways that result in the activation of three major transcription factors that are important for the promotion of T cell proliferation and function, i.e. nuclear factor of activated T cell (NFAT), nuclear factor kappa B (NF-κB) and the activator protein-1 (AP-1) (48). Following the activation of the TCR, tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs), located in the TCR-associated CD3-chains, are phosphorylated by Src family kinases Lck and Fin, resulting in the binding of ζ-chain-associated protein of 70kDA (ZAP70). After being recruited to the receptor complex, ZAP70 is activated through phosphorylation by Lck or via autophosphorylation. Active pZAP70 in turn phosphorylates the adapter molecules linker for activation of T cells (LAT) and SH2-domain-containing leucocyte phosphoprotein of 76kDa (SLP-76) (49, 50). This leads to the recruitment of

downstream signalling factors and the activation of the phospholipase C-γ1 (PLCγ1) pathway, which is necessary for the activation of both NFAT and NF-κB. PLCγ1 splits phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 increases calcium (Ca^{2+}) release from the endoplasmatic reticulum and the extracellular space into the cytosol. Ca^{2+} binds calmodulin, which activates the phosphatase calcineurin (51). Activated calcineurin dephosphorylates NFAT, enabling it to translocate into the nucleus, where it acts as a transcriptional activator for various cytokines, e.g. IL-2, IL-4, IL-5, IFN-γ and TNF (52). Although NFAT alone is sufficient to promote transcription, it can also form complexes with other transcription factors. As such, the NFAT:AP-1 complex binds to specific sites at the DNA enhancing the transcription of the aforementioned cytokines (47). Further, the PLCγ1-induced generation of DAG is necessary for the induction of the NF-κB signalling pathway. In resting cells, NF-κB is associated with the inhibitor of κB (IκB) in the cytoplasm, which impedes the translocalization of NF-κB into the nucleus. DAG activates protein kinase C-θ (PKC-θ), which facilitates CARMA-1, BCL-10 and MALT to form a complex, allowing them to phosphorylate and thus activate the IκB kinase (IKK) complex. The activated IKK complex then in turn phosphorylates IκB, resulting in its polyubiquinitation and proteosomal degradation. Upon dissociation from its cytosolic inhibitor NF-κB is able to translocate into the nucleus and act as a transcriptional activator (47, 53). The third signalling pathway, the mitogen activated protein kinase (MAPK) cascade, leads to the activation of the transcription factor AP-1. The activation of various MAPKs, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase, is triggered by DAG and eventually results in the expression and activation of c-Fos and c-Jun, which together form AP-1 (47).

In order to be fully activated, T cells do not only require stimulation via their TCR but also through co-stimulatory receptors, which enhance the calcium influx and activation of NFAT, NF-κB or AP-1 respectively. For instance, the CD28-CD80/CD86 interaction leads to an increased cytoplasmic Ca^{2+} concentration via a phosphatidylinositol 3-kinase (PI3K)-mediated PLCγ1 activation and is also necessary for DAG/PKC-θ-induced NF-κB activation (52). Moreover, via the CD28-induced PI3K pathway phosphatidylinositol 3,4,5-triphosphate (PIP_3) is generated, activating downstream targets such as mammalian target of rapamycin (mTOR) and the serine-threonin kinase (Akt), which are inductors of a variety of genes related to cell survival and proliferation (54).

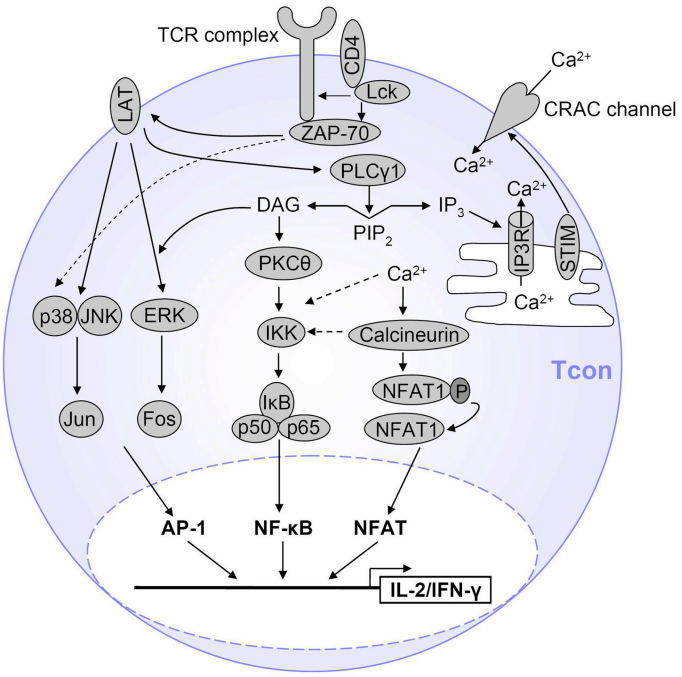

Figure 2: T cell receptor signalling pathways. Three main transcription factors, i.e. AP-1, NFAT and NF-κB are induced upon engagement to the TCR. The activation of the MAPK pathway leads to AP-1 activation. The PKCθ-induced proteosomal degradation of IκB and increase in Ca^{2+} levels promote the NF-κB up-modulation. Ca^{2+}-dependent activation of calcineurin induces the nuclear translocation of NFAT (figure from Schmidt *et al.*, 2012, p.8 (47)).

1.4.2 Signalling via the TCR in T_{reg}

In order to become suppressive, T_{reg} themselves need to be stimulated via their TCR initially. Once activated, they can readily suppress T_{eff} in an antigen-nonspecific manner (55). However, T_{reg} remain in a hyporesponsive and anergic state when activated via their TCR *in vitro* (17, 56). Only a combination of high amounts of IL-2 and optimal TCR signalling can break this anergy (56, 57). Furthermore, the antigen concentrations, which are required for T_{reg} stimulation, are much lower in comparison to concentrations needed for T_{eff} activation (18). Thus, it must be expected that there are differences in TCR signal pathways between $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T_{eff}. It has been proposed that the proximal signalling in T_{reg} is altered compared to T_{conv}. It was shown that the phosphorylation of ITAMs in the TCR-CD3 complex was lower, which resulted in the inability of ZAP70 to bind to the CD3ζ chain and in an attenuated activation of SLP-76, a key substrate of ZAP70. As a consequence, VAV, a molecule essential for actin remodelling, was not recruited and the formation of an actin cytoskeleton, which normally facilitates the accumulation of various signalling molecules, was perturbed leading to a suboptimal functioning of the immunological synapse between APC and T cells (58). A reduction of activated ERK upon TCR stimulation on T_{reg}

was also shown, indicating that the activation of MAPK pathway is inhibited (57, 58). Others have reported impairment of Akt activation upon CD3/CD28 co-stimulation (59). Further studies have demonstrated that the activity of PLCγ1 is markedly reduced in T_{reg} upon TCR stimulation, especially in the presence of co-stimulatory signals via CD28. As PLCγ1 catalyses the generation of DAG and IP_3, those second messengers are also diminished in T_{reg} cells. Lack of DAG results in a lower level of activation of the MAPK/AP-1 and PKCθ/NF-κB pathways, which contributes to the inability of T_{reg} to produce IL-2 upon activation via their TCR (60). Deficiency of IP_3 leads to a decreased Ca^{2+} influx, hindering the activation of calcineurin and thus the dephosphorylation of NFAT, which subsequently limits the nuclear translocation of NFAT (60, 61).

In addition, Foxp3 has been shown to modulate the function of different transcription factors. For example, Foxp3 binds to the DNA in a complex with NFAT, outcompeting NFAT:AP-1 complexes at binding sites for NFAT-target genes such as IL-2, IL-4 or CTLA-4 (62, 63). Furthermore, Foxp3 forms a complex with the runt-related transcription factor 1 (Runx1), which together with the co-factor Core-binding factor β (Cbf-β) binds to the promoters of Il2 and Ifng. This not only impedes the production of IL-2 and IFN-γ but also promotes anergy and suppressor function of T_{reg} cells (64). Further, the Foxp3-Runx1-Cbf-β complex enhances Foxp3 expression and thus plays a crucial role in maintaining T_{reg} stability (65). Foxp3 also inhibits the transcription of NF-κB-mediated proinflammatory genes through direct interaction with NF-κB (66).

1.4.3 Signalling via the TCR in T_{conv} under T_{reg}-mediated suppression

Several studies have revealed alterations in the TCR signalling in T_{conv} under suppression. Although there is no alteration in proximal signalling in the course of suppression, the activation and nuclear translocation of NF-κB and NFAT is attenuated (48, 67). Upon encounter with T_{reg}, T_{conv} fail to deplete their intracellular Ca^{2+} stores. As calcineurin activity and IKK degradation are dependent on Ca^{2+}, Ca^{2+} is crucial for the activation of NFAT and NF-κB, whereas the activation of AP-1 is mostly Ca^{2+}-independent (47, 48).

1.4.4 Signalling via the IL-2 receptor in T_{conv}

IL-2 is an immunoregulatory cytokine, which together with IL-4, IL-7, IL-9 IL-15 and IL-21 belongs to the common γ-chain (γc) cytokine family. Next to its importance for the proliferation and survival of activated T cells, IL-2 also is also required for T_{reg} development, peripheral maintenance and function (13, 68). The IL-2 receptor consists of three subunits:

IL-2Rα (CD25), IL-2Rβ (CD122) and the common γc (CD132). The β- and γ-chains together only have an intermediate affinity, while combination with the α subunit leads to the formation of the high affinity IL-2R. Upon ligation by IL-2, the IL-2Rβ and IL-2Rγ chains heterodimerize and the tyrosine kinases Janus kinase 1 (JAK1) and JAK 3 are activated, leading to the phosphorylation of tyrosine residues in the IL-2R chains. Subsequently, adaptor proteins are recruited, which, as shown in Figure 3 (69), activate the MAPK and PI3K pathways, as well as the signal transducer and activator of transcription 5 (Stat5) (13, 70). Stat5 forms dimers and directly translocates into the nucleus where it positively regulates various cell cycle proteins and, importantly, also upregulates the IL2ra transcription (71). Thus, the expression of CD25 on T cells is increased upon stimulation with IL-2 (72). The initiation of the MAPK pathway eventually leads to the activation of the transcription factor AP-1 (71). Similar to CD28, signalling via IL2R also activates the PI3K pathway resulting in the activation of downstream targets such as Akt and mTOR (73). Thus, via Stat5, PI3K and MAPK IL-2 promotes cell cycle progression, survival and growth in T cells.

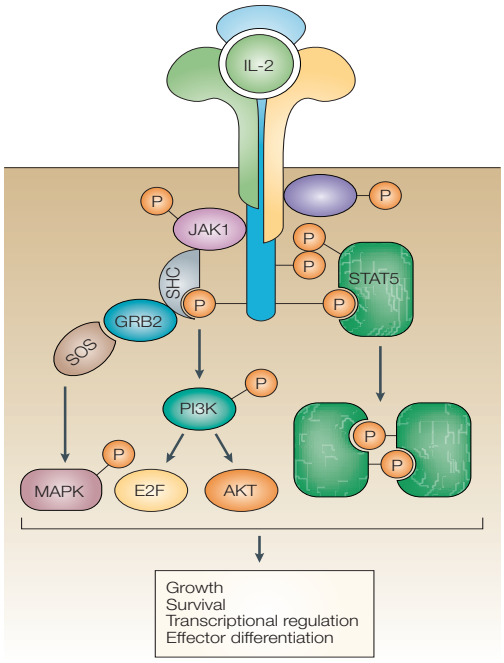

Figure 3: IL-2 receptor signalling in T cells. IL-2 induces three major signalling cascades in T cells, i.e. the MAPK, the PI3K/AKT and the STAT5 pathway. Following the stimulation of the high affinity IL-2R complex, the JAK1 and JAK3 are activated, which in turn leads to the phosphorylation of tyrosine residues in the receptor chains and JAK molecules themselves. This induces the recruitment of STAT5 and SHC (SRC-homology-2-domain-containing transforming protein C). SHC mediates the activation of the PI3K/AKT and MAPK pathways. STAT5 is also phosphorylated at certain tyrosine residues, which allows it to dimerize and translocate into the nucleus. Together these transcription factors promote T cell proliferation and function (figure from Malek et *al.*, 2004, p.666 (69)).

1.4.5 Signalling via the IL-2 receptor in T_{reg}

As Foxp3 induces the downregulation of IL-2 production, T_{reg} are highly dependent on paracrine IL-2 for their survival and expansion (13, 26, 29). Yet, T_{reg} remain hyporesponsive upon IL-2 stimulation in spite of constitutively expressing the high affinity IL-2R (56). Although IL-2 is important for the survival of both T_{conv} and T_{reg}, it does not promote proliferation of T_{reg} suggesting that the intracellular signals induced by IL-2 must differ between both cell lines (74). Strikingly, while the activation of the Stat5 pathway remains intact, the PI3K/Akt pathway is not activated in T_{reg}. Stat5 is responsible for the upregulation of cell cycle-associated and anti-apoptotic proteins, thus enhancing both cell cycle progression and survival in T_{reg} (74). Further, Stat5 induces the upregulation of Foxp3 and the IL-2Rα (75, 76). Foxp3 in turn enhances the expression of the IL-2Rα but also inhibits IL-2 production. Thus, via the IL-2-dependent Stat5 activation the characteristic T_{reg} phenotype is maintained (77). Signalling via the PI3K/Akt pathway, however, is impaired in T_{reg} due to the strong expression of the phosphatase and tensin homolog (PTEN) that negatively regulates PI3K signalling (74, 78). Downstream targets of PI3K, such as Akt, mTOR and cell cycle regulators, are therefore inactive in T_{reg}, explaining the hypoproliferative response towards IL-2 stimulation (74). Yet, when T_{reg} encounter a combination of TCR and high dose IL-2 stimuli, they break their anergic state (56, 79). At least in part this can be explained by the TCR-induced inactivation of PTEN, which results in the reactivation of the PI3K pathway and restoral of IL-2R signalling (74).

1.5 Development of regulatory T cells

1.5.1 Thymic development

T cells develop in the thymus by passing through a positive and negative selection. During the positive selection double positive cells expressing both CD4 and CD8 are rescued from apoptosis if their TCR recognizes MHC expressed on thymic DC. If a double positive cell associates with MHC II, it will become a single positive (SP) $CD4^+$ cell; recognizing MHC I, however, leads to the development of SP $CD8^+$ cells. During negative selection, SP cells are eliminated if they bind to MHC that present self-peptides with high affinities (80). In addition to $CD4^+$ and $CD8^+$ cells, 90 % of T_{reg} also develop in the thymus (77). Thus, those natural T_{reg} (nT_{reg}) constitute a separate thymus-derived population of T cells, which unlike other $CD4^+$ T cell subsets do not differentiate from T_{conv} in the periphery. In the course of thymic differentiation, T_{reg} require TCR-MHC engagement (62). Because thymocytes are normally eliminated when they recognize self-peptides, T_{reg} must somehow evade the

negative selection. The current understanding of thymic T_{reg} selection is based on the assumption that the TCR affinity is crucial for the fate of the developing thymocytes. Initiation of T_{reg} differentiation requires an intermediate affinity of their TCR towards self-peptides presented by MHC, while lower TCR signal strength towards self-antigens results in the positive selection of T_{conv} and stronger signals lead to programmed cell death of self-reacting precursor T cells (14, 81). Further, several lines of evidence have shown that the development of T_{reg} cells is a two-step process. According to this model, stimulation of developing T_{reg} via TCR/CD28 leads to the generation of $CD25^{+}Foxp3^{-}$ precursor cells, which strongly upregulate CD25 and thus show a high responsiveness towards paracrine IL-2. In a second TCR-independent step cytokines sharing the common γc, mainly IL-2, induce those precursor cells to completely differentiate into T_{reg} (82, 83). Stimulation with IL-2 leads to phosphorylation and activation of Stat5, which in turn binds to the Foxp3 promoter and thus induces Foxp3 transcription (84). Nevertheless, the exact molecular mechanism of how T_{reg} differentiation is initiated is still controversial. It has been found that in response to TCR/CD28 signalling the transcription factor c-Rel, a NF-κB family member, initiates T_{reg} development in the thymus through epigenetic changes (85–87). The Foxp3 locus contains three evolutionary conserved non-coding sequences (CNS) with binding sites for a variety of transcription factors that function as Foxp3 enhancers, out of which CNS3 within the intronic region of Foxp3 is thought to be essential for the induction of Foxp3 expression during thymic T_{reg} development (88). CNS3 is bound by c-Rel, which facilitates the demethylation of a CpG-rich island located within this site. Thereby, the chromatin structure is opened up and hence made more accessible for other transcription factors (88, 89).

1.5.2 Peripheral differentiation

T_{reg} do not only derive from the thymus, but are also induced in the periphery. As the organism is chronically exposed to antigens even under non-inflammatory conditions, by encountering commensal bacteria, food and allergens for instance, the generation of T_{reg} with TCR specific for those antigens is indispensable for the prevention of overshooting immune responses against non-pathogenic antigens. The inducible T_{reg} (iT_{reg}) therefore pose a key factor for the maintenance of peripheral tolerance (81, 90). Unlike thymus-derived nT_{reg}, whose TCR repertoire shows specificity for self-antigens, the TCR expressed by iT_{reg} recognize antigens the organism is constitutively exposed to. As an essential factor, TGF-β promotes iT_{reg} differentiation upon antigenic stimulation of naïve $CD4^{+}$ (91, 92). Smad3, a downstream effector molecule of the TGF-β signalling pathway, binds to a conserved region

within the Foxp3 promoter, the CNS1, modifies the acetylation of histones and subsequently regulates the transcription of Foxp3 (93). Moreover, IL-2 is also required for the peripheral transformation (94). During TGF-β-mediated iT_{reg} induction, TGF-β downmodulates SOCS3, an inhibitor of the Stat5. IL-2-induced Stat5 in turn binds to CNS2, which harbours a CpG island, the so-called T_{reg}-specific demethylation region (TSDR), whose methylation status is associated with the stability of Foxp3 expression (88). Because Stat5 binds CNS2 independently of its methylation status, it is thought to contribute to the opening of this enhancer region. CNS2 is then bound by other transcription factors in a demethylation-dependent manner that are important for T_{reg} function and Foxp3 expression (95–97). Additionally, retinoic acid released by specialized $CD103^+$ DC in the gut can further facilitate the generation of iT_{reg} (98). The gut-associated lymphoid tissue and mesenteric lymph nodes are sites of high prevalence of iT_{reg}, since those sites serve as environmental interfaces where the immune system is in constant contact with commensal bacteria, food and environmental antigens (99). Underlining the importance of different factors contributing to the peripheral iT_{reg} development is the notion that only together they can promote proper iT_{reg} induction and inhibit differentiation into effector T cells. TGF-β induces the expression of Foxp3 but also RORγt, which is an essential transcription factor for the differentiation of naïve T cells into Th17 cells. The transcription of Foxp3 is favoured in the presence of IL-2 or retinoic acid. Foxp3 then directly interacts with RORγt and thus inhibits the generation of Th17 cells (100). Stimulation with TGF-β and IL-6, a cytokine that is released during the course of inflammation, on the other hand, results in the preferential generation of Th17 cells that can potentially promote autoimmunity through induction of tissue inflammation (101-103).

1.6 Mechanisms of regulatory T cells

Although T_{reg} have been in the centre of attention for a long time, still little is known about the exact mechanisms that are used by T_{reg} to suppress immune responses. Currently, as shown in Figure 4 (47), three major mechanisms are thought to be essential for the suppressor function exerted by T_{reg}: cell-contact dependent suppression of T cells and APC, deprivation of growth factors and release of immunosuppressive mediators.

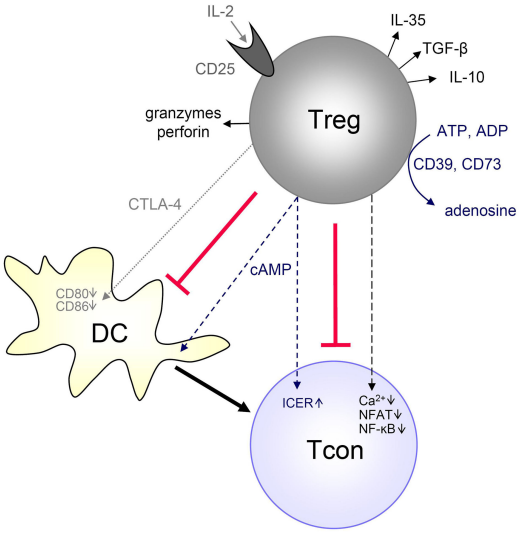

Figure 4: Mechanisms of T_{reg}-mediated suppression. T_{reg}-mediated suppression is thought to be due to three major mechanisms. (A) T_{reg} cells can mediate suppression of T_{conv} through cell-contact dependent mechanisms: The induction of apoptosis in neighbouring cells via granzymes and perforines, the increase of cAMP in T_{conv} through the transfer via gap junctions, the reduction of cytosolic Ca^{2+} concentration in T_{conv} leading to direct suppression of important transcription factors, such as NFAT and NF-κB. Moreover, T_{reg} can inhibit APC function through the CTLA-mediated downregulation of the co-stimulatory molecules CD80/CD86 and through increasing intracellular cAMP in those cells via gap junctions. (B) T cell suppression can be induced through preferential consumption of IL-2, thereby depriving T_{conv} of a cytokine important for cell proliferation. (C) Suppression can be also mediated through the release of soluble factors: The secretion of immunomodulatory cytokines such as IL-10, IL-35 and TGF-β or the generation of adenosine via CD73 and CD39, which can further enhance cytosolic cAMP levels in T_{eff} (figure from Schmidt *et al.*, 2012, p.8 (47)).

1.6.1 Cell-contact dependent suppression

1.6.1.1 CTLA-4: An immunosuppressive receptor

CTLA-4 is a membrane receptor on T cells that binds to the co-stimulatory surface molecules CD80/CD86 in competition with CD28 (104). It is constitutively expressed only on $CD4^+CD25^+$ T cells, but not on $CD4^+CD25^-$ T cells (33). Upon activation, however, the expression of CTLA-4 is upregulated on both T_{reg} and T_{eff} (105). CTLA-4 is an immunosuppressive molecule, which is important for the prevention of systemic autoimmunity (106). Several mechanisms of CTLA-4-mediated suppression are described. CTLA-4 is thought to inhibit CD28 co-stimulation by outcompeting CD28 for the binding to the shared ligands CD80/CD86 as it shows much higher affinity towards those ligands than CD28 (107). It also appears to directly suppress T cell proliferation and cytokine production through negative signalling into these cells (108). Yet, CTLA-4 is important for T_{reg} suppressive function as well, which is shown by the notion that CTLA-4-deficiency in T_{reg} results in a diminished suppressive capacity and severe autoimmune disorders (109). CTLA-4

expressed on T_{reg} induces the production of indoleamine 2,3-dioxygenase (IDO) by APC, which, being a tryptophan-catabolizing enzyme, depletes T cells of this essential amino acid resulting in the inhibition of T cell proliferation (110). Furthermore, it has been described recently that the expression of CD80/CD86 on APC is reduced due to trans-endocytosis of CD86 into CTLA-4 expressing T_{reg}, which limits the number of available ligands for CD28 and thus attenuates CD28-induced co-stimulation (111, 112). CTLA-4 also induces an increased motility of T_{conv} cells, which shortens the contact time with APC. T_{reg}, however, are resilient to CTLA-4-induced motility and are, thereby, able to outcompete T_{conv} over the binding to APC. Thus, the effector cells receive less co-stimulatory signals and T_{reg} can further downregulate the expression of CD80/CD86 (113).

1.6.1.2 LAG-3: In competition with CD4

LAG3, a CD4 homologue, binds to MHC II on APC in competition with CD4. It is expressed on activated T_{eff} and T_{reg}, but shows a more stable and enhanced expression on the latter and has been found to play an important role in T_{reg}-mediated suppression (114). Upon ligation with MHC II it suppresses the maturation and activity of APC, presumably through reverse signalling via MHC II (115). As MHC II molecules are expressed by a variety of immune cells, e.g. DC, B cells, monocytes, and macrophages, the inhibition through MHC II is antigen-independent and therefore explains how T_{reg} can suppress immune responses in an antigen non-specific manner.

1.6.1.3 Granzyme and perforin: Induction of Apoptosis

Inducing apoptosis is another mechanism of suppression. Human nT_{reg} mediate immune regulation via granzyme A and perforin, whereas iT_{reg} conduct cytotoxicity in a granzyme B- and perforin-dependent manner (116). Murine T_{reg}, on the other hand, only require granzyme B, but not perforin, to efficiently suppress a variety of immune cells (117). Granzymes are serine proteases that cleave caspases in their target cells and thus lead to apoptosis. Cytotoxic NK or $CD8^+$ cells usually utilize granzyme- and perforin-mediated apoptosis to kill virus-infected or tumor cells. On the other hand, tumor cells induce the expression of granzyme B in T_{reg}, enhancing the T_{reg}-induced suppression of immune cells in the tumor environment and thereby inhibit efficient tumor cell clearing (118).

1.6.1.4 cAMP: An immunosuppressive secondary messenger

As a secondary messenger, cyclic AMP (cAMP) can lead to various intracellular responses and can mediate a great diversity of cellular functions. In the immune systems, one

important role of cAMP is to limit T cell proliferation, differentiation and cytokine production (119). In the course of T_{reg}-mediated suppression T_{eff} experience an increase in intracellular cAMP, resulting in an enhanced nuclear translocation of the inducible cAMP early repressor (ICER), which acts a potent suppressor of genes such as IL-2, TNF, IL-4 and IL-13 (120). Moreover, cAMP also augments the expression of the immunosuppressive receptor CTLA-4 on T cells (121, 122). Underlining the importance of cell contact in the course of suppression, it has been shown that T_{reg} promote cAMP upregulation in suppressed T cells via gap junctions (123). In addition to the effects cAMP exhibits in suppressed T cells, it also conveys negative signals into DC through the transfer via gap junctions. Accumulation of cAMP in the cytosol of DC results in the downregulation of co-stimulatory molecules, such as CD80/CD86 and MHCII (124).

1.6.2 Deprivation of IL2

T_{reg} constitutively express the high affinity IL-2R and their survival is strongly dependent one paracrine IL-2 (29, 77, 125). Because they are not capable of producing IL-2 themselves, it has been suggested that the competition over IL-2 between T_{reg} and T_{eff} is a key mechanism of suppression (126–128). T_{eff} deprived of the IL-2 stimulus cannot undergo proliferation or differentiation and enter apoptosis (128). Yet, this seems inconsistent with the observation that close proximity is required for suppression (56). Nevertheless, the effects of cytokines are restricted to a microenvironment around the cells, which can also be disturbed by physically separation (129). However, it has been repeatedly demonstrated that instead of IL-2 consumption T_{reg} rather mediate suppression by abolishing IL-2 production in suppressed T_{conv} (18, 21, 56, 130). A possible explanation for the controversial results is that the consumption of IL-2 might only be important in physiological situations when T_{reg} exert control over immune homeostasis. In the course of inflammation, however, IL-2 production is enhanced and those high amounts of IL-2 can overcome the suppressive capacity of T_{reg} (18, 21, 127, 128). Through the inhibition of T_{reg}-mediated suppressor function an efficient immune response against the invading pathogens is enabled and other mechanisms of T_{reg} suppression may be favoured.

1.6.3 Soluble Mediators of Suppression

1.6.3.1 Secretion of IL-10, TGF-β and IL-35

Although the suppressor activity of T_{reg} seems to be cell-contact dependent and cytokine-independent in *in vitro* studies (18, 56, 131), immunosuppressive cytokines such as

IL-10 and TGF-β may at least play a supportive role in suppressing immune cells *in vivo* (132–135). IL-10 is known to be an anti-inflammatory cytokine and has long been viewed as a key cytokine for T_{reg} suppression. IL-10 represses the production of proinflammatory cytokines, such as IL-1, TNF, IL-12 and IFN-γ, and also downregulates the expression of MHCII and CD80/CD86 on APC (136, 137). IL-10 can directly suppress the proliferation of T cells as well as indirectly through the inhibition of IL-2 production by activated T cells (138). While IL-10 does not seem to be necessary for overall repression of immune responses, it is important for the control over intestinal inflammation (132, 139, 140). The IL-10-producing $CD4^+Foxp3^+$ T_{reg} are necessary in the prevention of excessive inflammation in tissues that constitute natural barriers towards the environment, i.e. the skin, the colon or airways (134). Furthermore, IL-10 itself can initiate differentiation of T cells into IL-10-producing regulatory cells or induce IL-10 expression in preformed T_{reg} (141–143). Another soluble molecule that is often associated with T_{reg}-mediated suppression is TGF-β. However, it remains to be elucidated, if TGF-β is solely necessary for the peripheral induction and maintenance of T_{reg} or if it contributes to T_{reg} suppressor function (144). There is controversial data regarding this, but it appears that TGF-β is at least partially responsible for the suppressive effects on T_{eff} cells (135, 145, 146). TGF-β especially seems to be essential for the T_{reg}-mediated suppression of tumor specific cytotoxic $CD8^+$ and NK cells (147, 148). A novel interleukin, IL-35, which is a heterodimer consisting of the Epstein-Barr-virus induced gene 3 (Ebi3) and IL-12α, was found to be preferentially produced by murine T_{reg} and appears to be essential for maximal T_{reg}-mediated inhibitory activity (149). Yet, in human $FOXP3^+$ T_{reg} expression or upregulation of IL-35 upon activation could not be demonstrated, whereas activated T_{eff} showed elevated levels of both Ebi3 and IL-12α (150). Other data, however, showed that during the course of T_{reg}-mediated suppression, IL-35 converts both human and murine T_{conv} into inducible T_{reg} that produce IL-35. Those cells acquire a hyporesponsive phenotype and are highly suppressive, although they do not upregulate the expression of Foxp3 (151).

Those anti-inflammatory cytokines appear to be important especially after the acute phase of inflammation, when T_{reg} have to regain their suppressor function in order to protect the host from exaggerated immune responses that would be resulting in tissue damage and/or auto-immunological diseases. As T_{reg} upregulate the production of IL-10, TGF-β and IL-35 during highly inflammatory states they cannot only inhibit T_{eff} directly, but also induce conversion of T_{conv} into suppressing T cells. Through this positive feedback loop, T_{reg} themselves contribute to the expansion of regulatory cells that release anti-inflammatory

cytokines in situations when the immune system is strongly activated and is posing a potential threat to the healthy tissue (143, 151–153).

1.6.3.2 Production of adenosine

In addition to the transfer of cAMP via gap junctions, cAMP levels are also increased by adenosine, which induces a de novo synthesis of cAMP in suppressed T_{eff} (154). T_{reg} highly express CD39 and CD73, two ectonucleotidases that hydrolyse adenosine triphosphate (ATP) into adenosine (155–157). Expression of CD39 and CD73 is not limited to the T_{reg} subset, since those surface molecules are expressed by a large amount of different cells. The highest proportions of $CD39^+$ cells among lymphocytes are found in the $B220^+$ B cell compartment, but also Langerhans cells, NK cells or macrophages express CD39 (155, 157). T_{eff} do not constantly express CD39, but also upregulate it upon activation (158). CD73 is found on a variety of cells, such as DC, epithelial cells or fibroblasts (159). In the T cell compartment, however, only $CD4^+Foxp3^+$ T_{reg} show coexpression of CD39 and CD73 (155). As ATP induces various proinflammatory responses, e.g. release of IL-1β by monocytes, maturation of DC, enhanced IL-2 production and proliferation of T cells, the sole degradation of ATP into adenosine can account for the anti-inflammatory effects of the ATP-hydrolysing enzymes (158, 160). Adenosine, however, is also a well-known anti-inflammatory agent (161–163). Regarding the T cell compartment, adenosine preferably mediates immunosuppressive effects via the A_{2A} receptor ($A_{2A}R$), which is expressed constitutively on T_{reg} and on activated T_{eff} (155, 164). In T_{eff}, adenosine enhances cytosolic cAMP leading to an impaired cell proliferation and a reduced production of proinflammatory cytokines, such as IFN-γ, IL-2 and TNF (154, 156, 164). Moreover, adenosine indirectly inhibits T cell function, as it limits cytokine production by APC and their maturation (165). Signalling via $A_{2A}R$ on T_{reg}, on the other hand, results in their expansion and increase of suppressor capacity. Strikingly, the adenosine receptor is upregulated only in the late phase of the immune response. At this stage the concentration of ATP is the highest due to cell destruction and hypoxia, which in turn can lead to an increase in local adenosine (155). By limiting only the late phase of immune activation, adenosine-mediated suppression of effector cells, therefore, might pose an elegant mechanism to balance out pathogen control and protection against tissue damaging immune responses.

1.7 Potential modulation of immunosuppression by TNF

1.7.1 TNF: a cytokine with multiple function

TNF is a pleiotropic cytokine that was first described in 1975 as a glycoprotein, which was released by macrophages in response to endotoxin and which showed necrotic effects on tumor cells (166). Initially it was suggested that TNF could be beneficial as an anti-tumor therapy, but efforts of using TNF to target cancer cells were detained due to the strong side effects caused by its systemic administration and the observation that TNF also plays a role in the genesis of certain tumors (166, 167). In addition to its necrotic effects on cancer cells, TNF is a key mediator in inflammatory processes and plays a role in the induction of fever, septic shock, infection-driven coagulation, cell proliferation and differentiation (167, 168). Moreover, TNF is important for host defence against several pathogens, especially viruses and intracellular bacteria. Since it is important for the formation of granulomas, control over mycobacterial infection is also dependent on TNF (169, 170). TNF is produced by a variety of hematopoietic cells including monocytes, B and T lymphocytes, mast cells, but also non-hematopoietic cells such as fibroblasts, endothelial cells and micro glia cells (168, 170-172).

1.7.2 Signalling pathways induced by TNF

TNF signals via two structurally related but functionally distinct receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (170, 173). Whereas TNFR1 is ubiquitously expressed in most tissues, TNFR2 is predominantly found on lymphocytes (174). TNF can mediate its effects both in its membrane-bound (mTNF) and soluble (sTNF) form. sTNF is cleaved off from mTNF by the TNF converting enzyme (TACE) (169). Yet, in contrast to TNFR1, which is stimulated equally well by both sTNF and mTNF, the TNFR2 is more efficiently stimulated by mTNF (175).

1.7.2.1 Signalling via TNFR1

Signalling via TNFR1 mediates the proinflammatory, cytotoxic and apoptotic effects that are characteristically associated with TNF (170, 176). TNFR1 contains a TNF receptor-associated death domain (TRADD) in its cytoplasmic tail, which can initiate different signalling cascades, resulting in apoptosis or proinflammatory signals. Firstly, TRADD binds adaptor proteins like the Fas-associated death domain (FADD) and subsequently leads to caspases-mediated apoptosis (169, 176). However, as illustrated in Figure 5 (174), TRADD also recruits TNF receptor-associated factor 2 (TRAF2) and

receptor-interacting protein (RIP), adaptor proteins that mediate the induction of important proinflammatory signalling pathways, i.e. the JNK/c-Jun, IKK/NF-κB and p38-MAPK cascades (177–179). The MAPK cascade also mediates the activation of JNK, which eventually leads to the induction of c-Jun. c-Jun, as a heterodimer with c-Fos, forms the proinflammatory transcription factor AP-1 (170). TNF-driven activation of the transcription of NF-κB-associated genes requires the degradation of the NF-κB inhibitor I-κB facilitating nuclear translocation of NF-κB (170, 178). Activation of NF-κB-dependent transcription does not only promote proinflammatory stimuli but also inhibits the TNF-induced apoptosis. Thus, upon TNF-TNFR1 interaction immunoregulatory and anti-apoptotic effects are preferably induced instead of apoptosis (169, 170, 177).

1.7.2.2 Signalling via TNFR2

The TNF-induced effects on lymphocytes are mainly mediated via TNFR2, which functions as a co-stimulatory receptor for TCR-induced cell activation by lowering the threshold for T cell activation and promoting survival during the early phase of antigen-driven immune response (180, 181). While, in contrast to TNFR1, TNFR2 lacks a death domain, it can directly interact with adaptor proteins such as TRAF2 (182). Subsequently, the proinflammatory transcription factors NF-κB and AP-1, the latter through the activation of the MAPK/JNK/c-Jun cascade, are induced (174, 178). Moreover, it has been shown that TNFR2 signalling promotes cell survival and IL-2 induction during TCR/CD28-induced immune responses through the enhanced activation of Akt and the anti-apoptotic molecule Bcl-$_{xL}$ (183). The stimulation of the Akt pathway poses a link between TNFR2 and CD28 co-stimulation, as CD28 signalling also activates Akt as key mediator for IL-2 induction (184). Yet, TNFR2 signalling does not only induce cell proliferation, cytokine release and anti-apoptotic effects but also increases TNFR1-induced apoptosis (185–187). Upon co-ligation of TNFR1 and TNFR2, TRAF2 is subject to proteosomal degradation. Thus, the TRAF2-dependent activation of NF-κB and AP-1 downstream of TNFR1 are attenuated and the TRADD/FADD-mediated induction of apoptosis is preferentially promoted (187, 188).

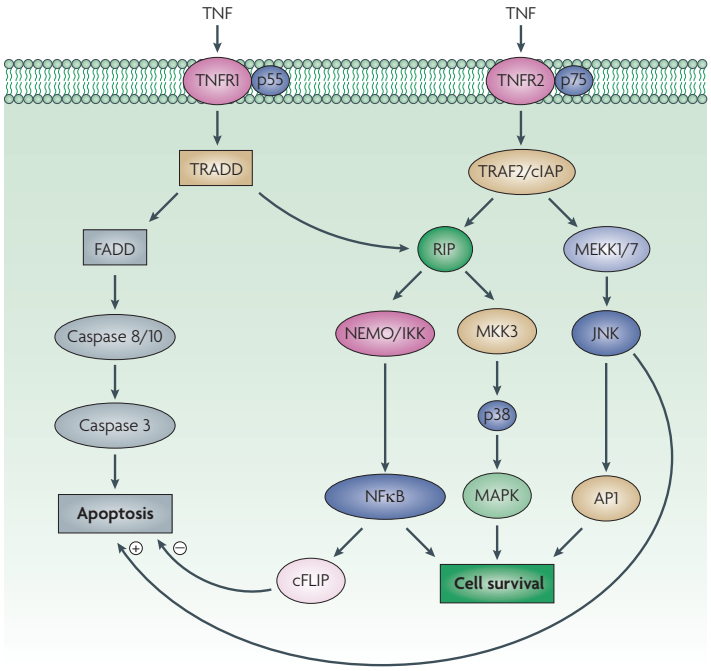

Figure 5: Signalling pathways in response to TNFR1 and TNFR2 signalling. Ligation to TNFR1 leads to the recruitment of TRADD, which either initiates apoptosis via the FADD/caspase pathway or induces proinflammatory signals via the recruitment of the adaptor protein TRAF2. TRAF2 is also recruited to the TNFR2. This eventually results in the activation of NF-κB, MAPK and AP-1, transcription factors that promote cell survival and proliferation. Moreover, NF-κB activation also results in the inhibition of the FADD-induced apoptotic pathway (figure from Faustman *et al.*, 2010, p.483 (174)).

1.7.3 TNF: An important mediator in autoimmunity

Several lines of evidence have shown that TNF plays a crucial role in the development of autoimmune disorders (171, 189–192). Accordingly, TNF blockade is beneficial in a variety of autoinflammatory diseases, e.g. rheumatoid arthritis, IBD, juvenile idiopathic arthritis, psoriatic arthritis or ankylosing spondylitis (193–196). Nevertheless, in some individuals anti-TNF treatment results in the induction of inflammatory disorders (194, 197). It has been reported that patients develop or show enhancement of psoriatic skin lesions, systemic lupus erythematodus or lupus-like conditions and inflammatory demyelination of the central nervous system similar to multiple sclerosis while being treated with TNF blockers (198–200). Thus, although neutralizing TNF has shown promising results in many patients suffering from autoimmune diseases, it can also aggravate inflammatory processes. The outcome for individual patients is therefore not always predictable. Great effort has been put into revealing the distinct proinflammatory and immunoregulatory functions of TNF. Nevertheless, still little is known about how TNF influences the activity of different immune cell subsets. Similarly, it remains to be clarified if TNF has enhancing or inhibiting effects on immunosuppressive cells like T_{reg} and MDSC.

1.7.3.1 TNF and T_{reg}: promoting or interrupting suppressor capacity?

As both T_{reg} and TNF play a vital role in the development of auto-inflammatory disorders, a link between them has long been suspected. T_{reg} numbers are not reduced in the peripheral blood of patients suffering from autoimmune diseases when compared to healthy individuals and in some cases T_{reg} cells can even be enriched at the site of inflammation. Yet, T_{reg} show impaired suppressor function and fail to suppress tissue inflammation in various types of autoimmunity (201–204). Thus, not an actual deficiency of T_{reg} numbers but diminished T_{reg} function, caused by factors such as TNF, may contribute to the defective T_{reg}-induced suppression of autoreactive T cells. Correspondingly, it has been shown that anti-TNF therapy increased T_{reg} numbers and restored their compromised function in patients with rheumatoid arthritis (205, 206). Moreover, patients suffering from IBD also benefit from treatment with anti-TNF agents (193), which has been attributed to increased T_{reg} numbers and function (207–209). Thus, it has been proposed that TNF has inhibitory effects on T_{reg} function. Yet, there is conflicting data on the interaction of T_{reg} and TNF. While various reports have shown that TNF decreases T_{reg} suppressor capacity (210–215), several lines of evidence, on the other hand, suggest that TNF has enhancing effects on T_{reg} proliferation and suppressor function (216–223).

1.7.3.2 TNF and MDSC: promoting suppressive function?

MDSC have a great capacity to suppress antitumor immune responses as explained before (refer to 1.3.2). Therefore, downregulation of their suppressor function could provide an effective anti-cancer treatment. Strong efforts have been made in order to reveal factors that modulate the accumulation and suppressor capacity of MDSC (38). It appears that TNF does not only influence the function of T_{reg} but also of immunosuppressive MDSC. It could be demonstrated that TNF promotes MDSC survival through inducing anti-apoptotic signals via TNFR2 (224) and increases MDSC-induced suppression of T cell proliferation (225, 226). Thus, it is suggested that blocking TNF inhibits the expansion of MDSC and, thereby, the suppression of antitumor immunity. Therefore, anti-TNF therapy seems to be a promising way to limit tumor progression (224).

1.7.4 TNF mutants with increased specificities for TNFR1 or TNFR2

Previous studies have revealed that TNF-induced effects on effector lymphocytes and T_{reg} are mainly mediated via the TNFR2 (180, 211, 216–218). Yet, especially regarding the T_{reg} and MDSC compartments little is known about the potential effects of TNFR1 and TNFR2 signalling on their suppressor function. In order to study to which extend MDSC and

T_{reg} function is influenced by ligation to those receptors, specific agonistic TNF mutants are required.

1.7.4.1 TNCscTNF80: a TNFR2-specific agonist

Single chain TNF (scTNF) consists of three TNF monomers that are connected via a dipeptide linker forming a trimeric construct. This scTNF trimer shows greater stability and binds TNFR1 and TNFR2 with higher affinities than sTNF (227). Selectivity for the TNFR2 is achieved by introducing mutations of two amino acids in scTNF (sc(mu)TNF80 D134/A145R) (231). However, although TNFR2 is bound by both sTNF and mTNF, it is only fully stimulated by the membrane-bound form (175). Thus, the TNF variant needs to form aggregates that resemble mTNF (227). Therefore, the Flag-tagged trimerization domain of Tenascin C (TNC) is linked to scTNF80. As illustrated in Figure 6 (228), this allows the self-assembly of three trimeric scTNF80 molecules. This TNF mutant, i.e. sc(mu)TNF80-FLAG-TNC (TNCscTNF80), simulates mTNF and thereby efficiently activates TNFR2 (228).

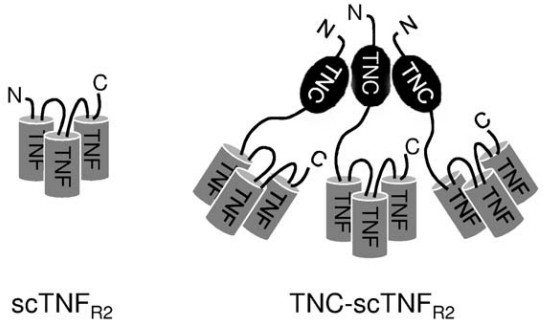

Figure 6: Structure of TNCscTNF. By connecting three TNF monomers a trimeric TNF construct, scTNF, is formed. Linking scTNF to the trimerization domain of TNC allows the self-assembly of three of those scTNF constructs, resulting in an oligomeric structure that simulates mTNF (figure from Fischer *et al.*, 2011, p.2 (228)).

1.7.4.2 TNCwtTNF

TNCwtTNF binds to both TNFR1 and TNFR2, as there are no mutations introduced. Yet, in order for it to fully activate TNFR2, the trimerization of wtTNF is required, which is achieved by linking it to TNC.

1.7.4.3 Human TNF: a TNF agonist that specifically binds mouse TNFR1

Human TNF (hTNF) can only signal via murine TNFR1 but not via murine TNFR2 (173, 229). Thus, any effect hTNF shows in mouse models must be mediated via TNFR1, which potentially allows excluding TNFR2 involvement (229).

1.8 Aim of the thesis

Previous studies in our laboratory had shown that TNFR2 signalling reduced the T_{reg}-mediated inhibition of $CD4^+$ and $CD8^+$ proliferation. The aim of this thesis was to study the specific functions of TNFR2 on effector lymphocytes and suppressive immune cells, i.e. regulatory T cells and myeloid-derived suppressor cells. To this end, the effect of different TNFR agonists on the proliferation and cytokine production of T cells was examined. Moreover, in order to shed some light on the impact of TNFR2 signalling on the suppressor capacity of immunoregulatory cells, we analysed to what extend different TNFR agonists changed the function, phenotype and intracellular signalling events in those cells, with focus on regulatory T cells. The analysis of TNFR1- and TNFR2-deficient cell populations complemented these studies.

2 Materials and Methods

2.1 Materials

2.1.1 Instrumentation

Item	Manufacturer	City (Country)
Centrifuge 5417R	Eppendorf	Hamburg (GER)
Centrifuge 54810	Eppendorf	Hamburg (GER)
CO_2-Incubator	Heraeus	Hanau (GER)
CO_2-Incubator	Integra Bioscience	Zizers (CH)
Flow Cytometer BD FACSCalibur	BD Biosciences	Heidelberg (GER)
Flow Cytometer BD LSRII	BD Biosciences	Heidelberg (GER)
MACS Milteny Chill (15,50)	Miltenyi Biotec	Bergisch-Gladbach (GER)
MACS Multistand	Miltenyi Biotec	Bergisch-Gladbach (GER)
Microscope Axiostar plus	Zeiss	Oberkochen (GER)
Microscope CK2	Olympus	Hamburg (GER)
Multichannel Pipette (100, 300 µl)	Eppendorf Research	Hamburg (GER)
MS2 mini shaker	IKA	Staufen (GER)
Neubauer hemocytometer	Brandt	Wertheim (GER)
Neubauer hemocytometer	HBG	Giessen (GER)
Pipette Discovery (1000 µl)	Abimed	Langenfeld (GER)
Pipette Labmate (10, 200 µl)	Abimed	Langenfeld (GER)
Pipette (2, 10, 100, 200, 1000 µl)	Eppendorf	Hamburg (GER)
Pipette (200 µl)	Glison	Middleton (USA)
Pipette (2, 20, 200, 1000 µl)	Thermo Scientific	Langenselbold (GER)
Pipette Controller accu-jet® pro	Brandt	Wertheim (GER)
Tissue culture hood HERAsafe	Thermo Scientific	Langenselbold (GER)
Water bath TW20	Julabo	Seelbach (GER)

2.1.2 Consumables

Item	Manufacturer	City (Country)
FACS Tubes	BD Biosciences	Heidelberg (GER)
Cell Strainer (40 µm)	BD Biosciences	Heidelberg (GER)
Centrifuge tube (15, 50 ml)	Sarstedt	Nümbrecht (GER)
MACS Separation Columns	Miltenyi Biotec	Bergisch-Gladbach (GER)
Microtubes original eppendorf®	Eppendorf	Hamburg (GER)
Minisart ® Syringe Filters (0.2 µm)	Satorius	Göttingen (GER)
Multiwell Plate (6-, 24-, 96-well)	BD Falcon™	Heidelberg (GER)
Petri dish	BD Biosciene	Heidelberg (GER)
Pipette Tips (10, 20, 200, 1000 µl)	Sarstedt	Nümbrecht (GER)
Serological Pipettes (2, 5, 10, 25 ml)	Sarstedt	Nümbrecht (GER)
Syringe (2 ml)	BD Discardit™	Heidelberg (GER)

2.1.3 Chemicals and Reagents

Item	Manufacturer	City (Country)
Acetone	Merck	Darmstadt (GER)
Albumin from bovine serum	Sigma-Aldrich	München (GER)
Anti-mouse $CD4^+CD25^+$ isolation microbeads	Milteny Biotec	Bergisch-Gladbach (GER)
ConA	Sigma-Aldrich	München (GER)
DPBS	PAN Biotech GmbH	Aidenach (GER)
Formaldehyde (36.5-38 %)	Sigma-Aldrich	München (GER)
Foxp3 Staining Buffer Set	eBioscience	Frankfurt (GER)
H_2O_2 (30 %)	Merck	Darmstadt (GER)
IL-2	Immunotools	Friesoyethe (GER)
Recombinant mouse IL-3	Immunotools	Friesoyethe (GER)
Methanol	Merck	Darmstadt (GER)
Penicillin/Streptomycin	Invitrogen	Darmstadt (GER)
RPMI 1640 PAA	PAN Biotech GmbH	Aidenach (GER)

Streptavidin-HRP	R&D Systems	Minneapolis (USA)
Streptavidin-AF488 (1:200)	Invitrogen	Darmstadt (GER)
Streptavidin-APC (1:200)	BD Biosciences Pharmingen	Heidelberg (GER)
Streptavidin-FITC (1:300)	BD Biosciences Pharmingen	Heidelberg (GER)
Streptavidin-PE (1:200)	Dianova	Hamburg (GER)
Streptavidin-PerCP (1:250)	BD Biosciences Pharmingen	Heidelberg (GER)
Substrate Solution A (ELISA)	OptEIA™ BD Biosciences	Heidelberg (GER)
Substrate Solution B (ELISA)	OptEIA™ BD Biosciences	Heidelberg (GER)
Triton X-100	Serva Feinbiochemica	Heidelberg (GER)
Tween® 20	AppliChem	Darmstadt (GER)

2.1.4 TNF receptor agonists

Generously provided by Harald Wajant, University of Würzburg (GER). The compounds were provided as cell culture supernatants of producer cell lines, without further purification.

Item	**Concentrations**
sc(mu)TNF80-Flag-TNC	3.5 µg/ml, 2.7 µg/ml
TNCsc(mu)TNF(wt)Flag	4.6 µg/ml
Human TNF	10 µg/ml
Control Supernatant (CS)	Used in equal volumes as sc(mu)TNF80-Flag-TNC

2.1.5 Buffers and Solutions

Item	**Composition**
ACK (red cells lysis buffer)	Na_2EDTA, pH 7.4
Antibody Diluent Buffer	DBPS, 1 % FCS, 0.3 % Triton X-100
Blocking Buffer (intracellular staining)	DPBS, 5 % FCS, 0.3 % Triton X-100
Buffer for extracellular staining (FACS buffer)	DPBS, 1 % FCS
Buffer for magnetic cell separation (MACS buffer)	DPBS, 0.5 % BSA, 2mM EDTA
Cell culture medium	RPMI, 10 % FCS, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, 100 µM ß-mercaptoethanol

Cell culture medium for MDSC preparation	RPMI, 10% FCS, 10% GM-CSF supernatant, 100 U/ml Penicillin, 0.1mg/ml Streptomycin, 50 µM ß-mercaptoethanol
ELISA Washing Buffer (IFNγ, IL-10)	DPBS, 0.05 % Tween® 20
ELISA Blocking Buffer (IFNγ)	DPBS, 1 % BSA
ELISA Reagent Diluent (IFNγ)	Tris-buffer Saline, 0.1 % BSA, 0.05 % Tween® 20
FC-Block anti-CD16/CD32	20 µg/ml αmFcyR$^{II/II}$-antibody in FACS buffer
Stop Solution for HRP (ELISA)	2N H_2SO_4

2.1.6 Antibodies

Antibody	Clone (Dilution)	Manufacturer	City
Anti-mouse B220-PE	RA3-6B2 (1:1000)	BD Biosciences	Heidelberg (GER)
Anti-mouse B220-PerCP	RA3-6B2 (1:600)	eBioscience	Frankfurt (GER)
Anti-mouse CD3ε	145-2C11	BD Biosciences	Heidelberg (GER)
Anti-mouse CD4-AF488	GK1.5 (1:1000)	Biolegend	Fell (GER)
Anti-mouse CD4-AF647	RM 4-5 (1:1000)	eBioscience	Frankfurt (GER)
Anti-mouse CD4-PE	RM 4-5 (1:1000)	eBioscience	Frankfurt (GER)
Anti-mouse CD4-PercP	SK3 (1:1000)	BD Biosciences	Heidelberg (GER)
Anti-mouse CD8a-AF647	53-67 (1:1000)	BioLegend	Fell (GER)
Anti-mouse CD8a-PerCP	53-6.7 (1:800)	eBioscience	Frankfurt (GER)
Anti-mouse CD25-APC	PC61 (1:200)	BD Biosciences	Heidelberg (GER)
Anti-mouse CD25-Biotin	PC61 (1:400)	eBioscience	Frankfurt (GER)
Anti-mouse CD25-FITC	7D4 (1:100)	BD Biosciences	Heidelberg (GER)
Anti-mouse CD25-PE	PC61 (1:100)	BD Biosciences	Heidelberg (GER)
Anti-mouse CD45.1-PE	A20 (1:50)	BD Biosciences	Heidelberg (GER)
Anti-mouse CD45.2-PerCP	104 (1:200)	eBioscience	Frankfurt (GER)
Anti-mouse FoxP3-PE	FJK-16s (1:100)	eBioscience	Frankfurt (GER)

Anti-mouse FoxP3-FITC	FJK-16s (1:100)	eBioscience	Frankfurt (GER)
Anti-hamster IgG-Biotin	cocktail	BD Biosciences	Heidelberg (GER)
Anti-rabbit IgG Cy™-5	1:500	Dianova	Hamburg (GER)
Rabbit mAB pStat5 Tyr694	D47E7 (1:600)	Cell Signaling	Danvers (USA)
Rabbit mAB pStat5 Tyr694	C71E5	Cell Signaling	Danvers (USA)
Rabbit mAB pZap 70	Tyr319/Syk Tyr352 (1:100)	Cell Signaling	Danvers (USA)

2.1.7 Kits

Item	Manufacturer	City (Country)
$CD4^+CD25^+$ Regulatory T cell Isolation Kit	Milteny Biotec	Bergisch-Gladbach (GER)
DuoSet ELISA Development Kit IFNγ	R&D System	Minneapolis (USA)
Mouse IL-10 ELISA Ready-Set-GO!	eBioscience	Frankfurt (GER)

2.1.8 Software

Item	Manufacturer	City (Country)
CellQuest™ Pro	BD Biosciences	Heidelberg (GER)
GraphPad Prism 5	GraphPad Software	San Diego (USA)
Microsoft Office 2011	Microsoft	Redmond (USA)

2.1.9 Mouse strains

Strain	Characteristics
C57BL/6	wild type (WT) mouse strain
$TNFR2^{-/-}$	C57BL/6 background; genetic knock-out of TNF receptor 2
$TNFR1^{-/-}$	C57BL/6 background; genetic knock-out of TNF receptor 1
CD45.1	C57BL/6 background; carries the CD45.1 isoform of the CD45 gene. This can be used to identify hematopoietic cells based on their expression of CD45.1 instead of the isoform CD45.2 that is expressed by the parental C57BL/6 line.

2.2 Preparation of cell cultures

2.2.1 Cell culture conditions

The preparation of cell cultures was performed in the laminar flow cabinet HERAsafe® using sterile reagents, pipettes and consumables. The specific mediums and reagents are listed in 2.1.5. Cells were cultured in multiwell cell culture plates at the following conditions: 37 °C, 5 % CO_2 and 95 % humidity.

2.2.2 Isolation and purification of spleen cells

Following anaesthesia with ether, the mice were killed by cervical dislocation. The fur was sterilized with 70% ethanol. A small skin cut was made, the peritoneum opened and the spleen was extracted. The spleen was placed in 5 ml cell culture medium. From this point forward the work was performed under a laminar flow cabinet. The spleen was pressed out with a 2 ml syringe plunger and the cell suspension was transferred into a 15 ml centrifuge tube. After letting the debris settle down, the suspension was transferred into a new 15 ml centrifuge tube. The cell suspension was centrifuged with 300 g for 5 minutes at 4 °C and the supernatant was discarded. In order to remove erythrocytes from the whole splenocyte preparations, the cells were resuspended in 5 ml ACK buffer, pelleted with 300 g for 5 minutes at 4 °C and the supernatant was discarded. The cells were then resuspended in 10 ml cell culture medium, centrifuged with 300 g for 5 minutes at 4 °C and the supernatant was discarded.

2.2.3 Preparation of bone marrow cells

Myeloid-derived suppressor cells were generated from bone marrow cells by applying a previously described method (230). For this purpose E67L2 mice were used. The mice were killed by cervical dislocation after anaesthesia with ether. 70 % ethanol was sprayed onto the fur for sterilization. The femora and tibiae were extracted and cleaned with a paper towel. The following steps were performed under a laminar flow cabinet. The bones were placed into 70 % ethanol for two minutes in order to sterilize them, dried by gentle tapping them on a petri dish and then transferred into DPBS. After incubation in DPBS for one minute, they were dried again and the epiphyses were removed with scissors. The bone marrow was rinsed with 5 ml cold DPBS using a 20G cannula. The cell suspension was collect in a cold 50 ml centrifuge tube. After the resuspension, a centrifugation step (300 g, 10 min, 4 °C) followed and the supernatant was decanted. Then, the cells were picked up in 5 ml cell culture medium

that contained 10 % GM-CSF supernatant, which is referred to as GM-CSF medium throughout the work with MDSC.

2.2.4 Determination of cell numbers

Cell numbers were determined with a Neubauer hemocytometer. Therefore, a small volume was taken from the cell suspension. This was resuspended in adequate volumes of Turck staining solution or cell culture medium to create a 1:5 dilution. 10 µl of this dilution were placed in between the hemocytometer and the glass microscope slide. A total of 100 to 200 cells were counted in four different big squares and the mean value was determined. The total cell number was calculated by applying Formula 1.

$$\left(\text{cells in a big square}\right) \times \text{dilution factor} \times 10^4 = \text{cells/ml}$$

Formula 1 Calculation of cell number using a Neubauer hemocytometer.

2.2.5 CFSE labelling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining was utilized to measure proliferation of $CD4^+$ and $CD8^+$ cells. The colourless CFSE contains two acetate groups that allow the passive diffusion into cells, where intracellular esterases quickly remove the acetate groups. The resultant molecule carboxyfluorescein shows green fluorescence and reduced permeability for plasma membranes. Moreover, the succinimidyl ester reacts with amines of cytosolic proteins forming stable conjugates. Those dye-protein adducts are also formed with low turnover proteins. Thus, they are stably retained within the cells during cell division and do not diffuse into adjacent cells ensuring high stability of fluorescence (231, 232). However, the carboxyfluorescein-tagged molecules are divided between daughter cells upon cell division. With each cell cycle progression the fluorescence intensity is thereby progressively halved, which allows the monitoring of lymphocyte proliferation by flow cytometry (233). Up to eight cell divisions can be measured with CFSE before the dye dilutes to levels below the autofluorescence of unlabelled cells (234).

For the CFSE labeling, splenocytes were isolated and lysed as described in 2.2.2. Before staining with CFSE cells were washed twice DPBS. Each wash step included resuspension in 5 ml DPBS, centrifugation (300 g, 5 min, 4 °C) and removal of the supernatant. After the first wash step, the cell numbers were determined using Formula 1. Following the second wash step, cells were resuspended in a DBPS solution containing 1 % of foetal calf serum (FCS) and 2 µM CFSE, and incubated in a water bath at 37 °C for 10 minutes. 1 ml of the CFSE-solution was used for 10^7 cells. The reaction was stopped by

adding four volumes of 5 % FCS-DPBS and centrifuging with 300 g for 5 minutes at 4 °C. The supernatant was removed and the cells were washed twice in 5 ml 5 % FCS-DPBS. For cultivation, the cell pellet was resuspended in 5 ml cell culture medium. Then, cells were counted again as explained above.

2.2.6 MACS separation of $CD4^+CD25^+$ regulatory T cells

In order to separate $CD4^+CD25^+$ regulatory T cells from whole splenocyte preparations, magnetic cell separation (MACS) was performed with the Regulatory T cell Isolation Kit listed in 2.1.7. Cells and the MACS buffer were kept cold throughout this protocol. For this procedure the spleen was placed in a cell strainer over 5 ml MACS buffer, pressed out with a 2 ml syringe plunger and the cell suspension was transferred into a 15 ml centrifuge tube. This cell suspension was then pelleted (300 g, 5 min, 4 °C) and the supernatant was decanted. Then, cells were then resuspended in 10 ml MACS buffer and the total cell number was determined using Formula 1. A cell suspension volume containing 10^8 cells was used for the subsequent separation process. Cells were centrifuged with 300 g for 5 minutes at 4 °C, the supernatant was removed carefully and the pellet was picked up in 400 µl MACS buffer. 50 µl biotin-antibody cocktail were added and the suspension was incubated at 4 °C for 10 minutes in the dark. Then 300 µl MACS buffer, 100 µl anti-biotin beads and 50 µl CD25-PE were added, the mixture was well resuspended and incubated for 15 minutes at 4 °C in the dark. To stop the reaction 13 ml MACS buffer were added and the suspension was centrifuged with 300 g for 5 minutes at 4 °C. Then, the supernatant was removed and the cell pellet was resuspended in 500 µl MACS buffer. In order to deplete cells that were not $CD4^+$, the cell suspension was run over a LD-Column. To remove any particles remaining in the cell suspension, a cell strainer (40 µl) was placed over the opening of the LD column. The cell suspension was transferred completely onto the column, which was then rinsed twice with 1 ml MACS buffer. The first rinse was performed over the cell strainer, whereas the buffer was pipetted directly into the column for the second rinse. The cells that were not of interest, i.e. all non-$CD4^+$, had been marked by the biotin-antibody cocktail and thus bound to the column. Therefore, all cells collected in the effluent were $CD4^+$. The effluent was then transferred into a 15 ml centrifuge tube and centrifuged with 300 g for 5 minutes at 4 °C, the supernatant was removed carefully and the cells were resuspended in 900 µL MACS buffer. 50 µl anti-PE beads were added and the suspension was incubated in the dark for 15 minutes at 4 °C. The reaction was stopped by the addition of 13 ml MACS buffer and subsequent centrifugation with 300 g for 5 minutes at 4 °C. The supernatant was

discarded and cells were resuspended in 500 µl MACS buffer. For the positive selection of $CD25^+$ cells the cell suspension was run over a MS column. All cells that had been marked by the anti-PE beads, i.e. the $CD25^+$ cells, bound to the column and could therefore be collected when cells were extracted from the column. The MS column containing the cell suspension was rinsed three times with 500 µl MACS buffer. Then, it was placed over a new 15 ml centrifuge tube, rinsed with 1 ml MACS buffer and flushed out by firmly applying the plunger. This suspension was run over a second MS column, which was also rinsed with 500 µl MACS buffer three times. The second column was placed over a 15 ml centrifuge tube, rinsed with 1 ml MACS buffer and flushed out by firmly applying the plunger. This cell suspension, containing only $CD4^+CD25^+$ cells, was centrifuged with 300 g for 5 minutes at 4 °C and the supernatant was discarded. The cells were resuspended in 500 µl cell culture medium and cell numbers were acquired by applying Formula 1.

2.3 Cultivation of cells

2.3.1 Cultivation of bone marrow derived dendritic cells

Bone marrow-derived cells were obtained as described in 2.2.3. After resuspending the cells in 5 ml GM-CSF medium, they were pelleted with 300 g for 10 minutes at 4 °C and the supernatant was discarded. The cell pellet was resuspended in 1 ml GM-CSF medium and the suspension was transferred into a new centrifuge tube using a cell strainer (40 µl) to remove remaining particles. To determine the total cell number, 50 µl were taken off the suspension and diluted in Turck solution at a ratio of 1:1. Cells were counted using Formula 1. Then, 2×10^5 cells were cultivated for four days in 6-well cell culture plates containing 2 ml GM-CSF medium. Half of the wells were additionally treated with 10 ng/ml TNCscTNF80. On day three, the cells were fed with 2 ml GM-CSF medium, again 10 ng/ml TNCscTNF80 was added to half of the wells.

2.3.2 Suppression assays with MDSC

For the MDSC suppression assays, CFSE-labelled splenocytes and bone marrow-derived MDSC were co-cultured. In order to generate the MDSC, bone marrow cells were cultured with GM-CSF as described in 2.3.2. Following the generation, the MDSC were harvested using a 5 ml pipette. This cell suspension was transferred into a 15 ml centrifuge tube, the cells were pelleted (300 g, 5 min, 4 °C) and the supernatant was discarded. Following resuspension in 1 ml normal cell culture medium, cells were counted as indicated in Formula 1. Splenocytes were prepared as described in 2.2.2 and labelled with CFSE as

described in 2.2.5. The cell numbers were calculated using Formula 1 and per well 2 x 10^5 CFSE-labelled splenocytes were cultured in 96-well culture plates. The MDSC were added to the cultures at ratios to the splenocytes of 1:1, 1:2, 1:4 and 1:8. Thus, 2 x 10^5, 1 x 10^5, 0.5 x 10^5 and 0.25 x 10^5 MDSC were placed into the wells respectively. All wells apart from the negative control got stimulated with 1 µg/ml anti-CD3ε antibody alone or a combination of 1 µg/ml anti-CD3ε antibody and 0.5 µg/ml anti-CD28 antibody. Additional cell culture medium was placed into the wells beforehand so that each well had a final volume of 200 µl.

2.3.3 Proliferation assays

In the proliferation assays CFSE-labelled splenocytes were cultivated and stimulated with different reagents for up to 72 hours. Splenocytes were prepared and labelled with CFSE as described before. Cell numbers were determined using Formula 1 and 1.5 x 10^5 cells were placed into each well. For cell stimulation 0.5 µg/ml anti-CD3ε antibody was added per well. Some wells were additionally treated with 10 ng/ml TNCscTNF80 or TNCwtTNF. When Control Supernatant (CS) was used, it was added in equivalent volumes to 10 ng/ml TNCscTNF80. 96-well cultures plates were used and the final volume per well was 200 µl.

2.3.4 Suppression assays with $CD4^+CD25^+$ regulatory T cells

For the T_{reg} suppression assays CFSE-labelled splenocytes from TNFR2-deficient ($TNFR2^{-/-}$) mice were co-cultured with MACS-isolated $CD4^+CD25^+$ T_{reg} from WT mice. Splenocytes from $TNFR2^{-/-}$ mice were prepared and labelled with CFSE as described before, cell numbers were determined using Formula 1 and a total number of 1.5 x 10^5 cells was transferred into each well. $CD4^+CD25^+$ cells were purified from whole splenocyte preparations of WT mice by using MACS as shown in 2.2.6. The separated $CD4^+CD25^+$ cells were cultivated with the CFSE-labelled splenocytes from the $TNFR2^{-/-}$ mice at ratios of 1:3, 1:6 and 1:12. Thus, 0.5 x 10^5, 0.25 x 10^5 or 0.125 x 10^5 $CD4^+CD25^+$ cells were added to the wells. For activation 0.5 µg/ml anti-CD3ε antibody was given into each well. Some wells were additionally treated with 10 ng/ml TNCscTNF80 or TNCwtTNF.

2.3.5 Cell culture for the analysis of phosphorylated Stat5

In order to analyse the expression of phosphorylated Stat5 (pStat5) and extracellular markers, monocultures of splenocytes were stimulated for various periods of time. The splenocytes were prepared as described in 2.2.2 and then washed in 10 ml cell culture medium. After pelleting and discarding the supernatant, the cells were resuspended in 5 ml cell culture medium. Total cell numbers were determined using Formula 1 and 2 x 10^6 cells

were cultivated in 6-well culture plates that had a total volume of 1000 µl. Except for the negative controls, 0.5 µg/ml anti-CD3ε antibody was added to all wells to stimulate cells. Cells that were activated with anti-CD3ε antibody alone served as a positive control. For the short-term studies, the cell cultures were additionally treated with various combinations of 10 ng/ml IL-2 and TNFR agonists at a concentration of 10 ng/ml for either 30 or 60 minutes. Long-term studies with incubation times of up to 72 hours were also performed. For this purpose, the cells were cultivated in the following manner: non-stimulated control, activated with 0.5 µg/ml anti-CD3ε antibody, activated with 0.5 µg/ml anti-CD3ε antibody and 10 ng/ml of either TNCscTNF80 or hTNF. Those cell cultures plates were incubated for three, 24, 48 and 72 hours at 37 °C and analysed by flow cytometry.

In one experiment, $CD4^+CD25^+$ were purified from CD45.2 mice with MACS and then co-cultured with splenocyte preparations from CD45.1 mice. In this experiment 1.5 x 10^5 $CD45.1^+$ cells were cultured with various numbers of $CD4^+CD25^+$ from CD45.2 mice in presence of 0.5 µg/ml anti-CD3ε antibody and different TNFR agonists at concentrations of 10 ng/ml. The stimulation was performed in 96-well culture plates for three up to 72 hours.

2.3.6 Cell culture for the analysis of phosphorylated ZAP70

Splenocytes were prepared as described in 2.2.2 and resuspended in 10 ml cell culture medium. The cell suspension was centrifuged (300 g, 5 min, 4 °C), the supernatant was discarded and the cell pellet was resuspended in 10 ml cell culture medium. Cell numbers were determined using Formula 1 and 2 x 10^6 cells were cultured in fluorescence-activated cell sorting (FACS) tubes with a total volume of 1000 µl. For stimulation cells were treated with 0.01 % H_2O_2 with or without additional 10 ng/ml TNCscTNF80. The tubes were placed in a water bath at 37 °C during this activation process. To stop the reaction the tubes were put on ice and 1 ml DPBS was added immediately. This was followed by a centrifugation step with 300 g at 4 °C for 5 minutes. For the subsequent flow cytometric analysis, cells were placed in FACS tubes that had been prefilled with 1000 µl DPBS. Throughout this procedure, tubes, reagents and solutions were kept on ice as far as possible.

2.3.7 Harvesting of cells

Before the harvesting of the cells, the multiwell plates were examined in a microscope in order to ensure that no contamination had taken place. FACS tubes were prefilled with 1 ml FACS buffer or DPBS, depending on the buffer that was used subsequently in the staining process for flow cytometric analysis. Then, for a number experiments, supernatants were

collected and stored at -20 °C. In order to harvest cells, cell suspensions were resuspended in the multiwell culture plates and then transferred into the prepared FACS tubes.

2.4 Flow cytometry analysis

2.4.1 Staining of surface markers for flow cytometry analysis

The FACS tubes, which contained the cell suspensions in 1 ml FACS buffer, were centrifuged with 300 g for 5 minutes at 4 °C and the supernatant was decanted. Then, the tubes were repeatedly tapped on paper towels to remove the remaining fluid. In order to block unspecific binding, 1 µg of the αmFcyR$^{II/II}$-antibody was added to each tube. The tubes were centrifuged with 300 g for 1 minute at 4 °C, shaken to resuspend the cell pellet and incubated at room temperature (RT) in the dark for 5 minutes. Antibody master mixes were prepared using antibodies in the recommended dilutions, which are listed in 2.1.6. Following the incubation with the αmFcyR$^{II/II}$-antibody, the antibody mixes were added. The tubes were centrifuged with 300 g for 1 minute at 4 °C, shaken and incubated at RT for 5 minutes or at 4 °C for 10 minutes. A wash step was performed that included the addition of 1 ml of FACS buffer, centrifugation (300 g, 5 min, 4 °C) and discharge of the supernatant. If a biotin-labelled antibody had been used in the antibody master mix, cells were additionally treated with fluorochrome-conjugated streptavidin, which was followed by centrifugation with 300 g for 1 minute at 4 °C, shaking of the tubes and incubation for 5 minutes at RT. Then the wash step was repeated. Cells were fixed with a 2 % formaldehyde solution, which had been prepared in FACS buffer, and stored at 4 °C until FACS analysis. Additional FACS buffer was added before the measurements in the flow cytometer.

2.4.2 Staining of pStat5 and pZAP70 for flow cytometry

The FACS tubes, which contained the harvested cell in 1 ml DPBS, were centrifuged with 300 g for 5 minutes at 4 °C. The supernatant was decanted and remaining drops were dried by repeatedly tapping the tubes on paper towels. The cells were fixed by incubation in 500 µl of a 4 % formaldehyde solution at RT for 15 minutes. After a centrifugation step (300 g, 5 min, 4 °C), the supernatant was discarded and cells were washed three times. These wash steps included resuspension in 1 ml DPBS, centrifugation (300 g, 5 min, 4 °C), discharge of the supernatant and tapping the tubes on paper towels. After the last wash step, the supernatant was aspirated completely instead of being decanted. Cells were resuspended in 1 ml 100 % methanol, which had been stored at -20 °C, and incubated for 5 minutes at -20 °C. After the incubation, cells were centrifuged, the supernatant was discarded and two

additional wash steps followed. The cells were incubated with 1 ml blocking buffer, as listed in 2.1.5, for 60 minutes at RT. After incubation, a centrifugation step followed (300 g, 5 min, 4 °C) and the supernatant was removed. In order to block unspecific binding, 1 µg of αmFcyR$^{II/II}$-antibody was added per tube. The tubes were centrifuged with 300 g for 1 minute at 4 °C, shaken and incubated for 10 minutes at RT. Antibody master mixes were prepared using antibodies against both intra- and extracellular markers at the same time, including the monoclonal antibodies (mAB) against pStat5-Tyr694 or pZAP70. Cells were resuspended with the antibody master mix and incubated overnight at 4 °C. After the incubation, a wash step in 1 ml DPBS followed. Then, the secondary antibody anti-rabbit IgG Cy^{TM}-5 was added. If a biotin-labelled primary antibody had been used previously, fluorochrome-conjugated streptavidin was also added in this step. The dilutions of the secondary antibody and streptavidin were prepared in DBPS, cells were resuspended in those solution and incubated for 60 minutes at 4 °C. Following the incubation, the cells were resuspended in 1 ml DPBS, pelleted with 300 g for 5 minutes at 4 °C and the supernatant was decanted. Between 200 and 250 µl of 1 % FCS-DBPS were added for FACS analysis.

2.5 Detection of cytokine secretion in the supernatants of proliferation and suppression assays with ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed to detect cytokines in the supernatant of proliferation and suppression assays. Therefore, the DuoSet ELISA Development kit IFNγ and the mouse IL-10 ELISA Ready-SET-Go!® Kit, which are listed in 2.1.7, were used. All additional buffers that were required are listed in 2.1.5. First, each well of a 96-well plate was coated with 50 µl of an unlabelled capture antibody. The plate was firmly sealed with plastic wrap to prevent evaporation and incubated overnight at RT for IFN-γ detection or at 4 °C for IL-10 detection. After the overnight incubation, the capture antibody solution was removed and remaining drops were dried by firmly blotting the plate onto paper towels. Then the plate was washed by adding 400 µl wash buffer per well, discarding the buffer and drying the plates as described above. This procedure is referred to as the wash step throughout this protocol. This wash step was repeated twice for a total of three repetitions. Then, 300 µl of the blocking buffer (IFN-γ ELISA) or the Assay Diluent (IL-10 ELISA) was added per well, the plate was sealed and incubated for 60 minutes at RT. Afterwards, the blocking buffer was discarded, the plate was dried and three wash steps were performed. After blocking the plate, the standards and the samples were added. First, the standards needed to be prepared. Therefore, they were diluted in Reagent Diluent

(IFN-γ ELISA) or Assay Diluent (IL-10 ELISA) to a final concentration of 2000 pg/ml or 4000 pg/ml respectively. Then, two-fold serial dilutions were prepared in Reagent diluent (IFN-γ ELISA) or Assay Diluent (IL-10 ELISA) in order to obtain an eight point standard curve. 50 µL of those standard dilutions were transferred as duplicates into the wells. In addition, 50 µl of the samples were given into the other wells. Some samples were used in their original concentration, while other samples were used as a 1:3 dilution in Reagent diluent (IFN-γ ELISA) or Assay Diluent (IL-10 ELISA). Following the addition of the standards and samples, the plate was sealed and incubated for two hours at RT. After the incubation, the liquid was removed and three wash steps were performed. Then 50 µl of the detection antibody was added to each well. The plate was sealed and incubated at room temperature for two hours (IFN-γ ELISA) or 60 minutes (IL-10 ELISA). The detection antibody was removed and the plate was dried. Three wash steps followed. 50 µl of horseradish-peroxidase (HRP) conjugated to streptavidin (IFN-γ ELISA) or Avidin-HRP (IL-10 ELISA) were added per well. The plate was covered and incubated at room temperature for 20 minutes (IFN-γ ELISA) or 30 minutes (IL-10 ELISA). Streptavidin-HRP and Avidin-HRP were removed, the plate was dried and washed three times. Then, 50 µl Substrate Solution was transferred into each well. For the detection of IFN-γ a 1:1 mixture of Substrate Solution A and Substrate Solution B was used, whereas an undiluted Substrate Solution was provided in the kit for IL-10 detection. For the incubation process, the plate was placed in the dark for 15 minutes, which was terminated by the addition of 25 µl Stop Solution (2 N H_2SO_4). Then, the intensity of fluorescence was analysed using a microplate reader set to 450 nm. Wavelength correction was set to 570 nm to correct optical imperfections.

3 Results

3.1 Proliferation of $CD4^+$ and $CD8^+$ lymphocytes in responses to the TNFR2 agonist TNCscTNF80

TNF is known to be a proinflammatory mediator in the immune system, which exerts its effect via two receptors, TNFR1 and TNFR2 (170). Previous studies have shown that TNF promotes T cell proliferation through TNFR2 (181, 183). Yet, the effect on T_{reg} proliferation and function remains to be revealed. Throughout this work, TNCscTNF80, an agonist that binds specifically to TNFR2 with a high bioactivity (228), was used to determine the effect of TNFR2 signalling on T_{reg} function. In order to study how this specific TNFR2 agonist interferes with $CD4^+$ and $CD8^+$ cell expansion, TNCscTNF80 was added to T cell proliferation assays. Those assays were performed using both lymphocytes from wild type (WT) and TNFR2-deficient ($TNFR2^{-/-}$) mice in order to control for interactions of TNCscTNF80 and TNFR1.

3.1.1 Comparison of the behaviour of wild type and $TNFR2^{-/-}$ lymphocytes

3.1.1.1 Proliferation of $CD4^+$ cells from wild type and $TNFR2^{-/-}$ mice

In this experiment the behaviour of $CD4^+$ cells from WT and $TNFR2^{-/-}$ mice was compared. The relative proportions of proliferating out of all $CD4^+$ cells were determined after lymphocytes had been cultured with anti-CD3ε antibody for 72 hours. In general, $CD4^+$ cells from the wild type mice proliferated more than $CD4^+$ cells from the $TNFR2^{-/-}$ mice. If a higher concentration of anti-CD3ε antibody was used for stimulation, the proportion of $CD4^+$ cells that were proliferating increased. TNCscTNF80 further enhanced the proliferation of WT $CD4^+$ cells. This statistically significant effect was seen after stimulation with 0.06 µg/mL (Figure 7A) and 0.5 µg/mL anti-CD3ε antibody (Figure 7B). The addition of control supernatant (CS), however, did not increase the proliferation of WT $CD4^+$ cells. $CD4^+$ cells from the $TNFR2^{-/-}$ mouse did not show an increased proliferation when TNCscTNF80 had been added to the culture.

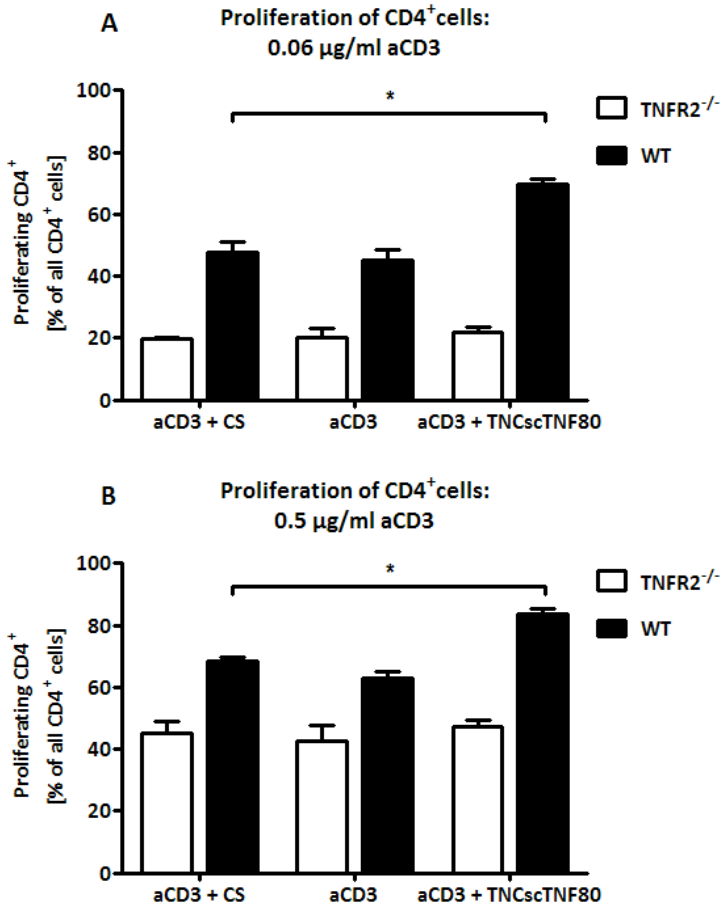

Figure 7: Impact of TNCscTNF80 on proliferation of CD4$^+$ cells from WT and TNFR2$^{-/-}$ mice. CFSE-labeled naïve splenocytes from WT (black bars) or TNFR2$^{-/-}$ (white bars) mice were cultured alone, with 10 ng/ml TNCscTNF80 or with CS used in equivalent volume in the presence of 0.06 µg/ml anti-CD3ε (A) or 0.5 µg/ml anti-CD3ε (B) for 72 hrs. The data is derived from a representative of two experiments, shown is the mean + SD of three technical replicates. * indicates a statistically significant difference of the indicated groups. Statistical analysis was done by two-way ANOVA with Bonferroni post-test.

3.1.1.2 Proliferation of CD8$^+$ cells from wild type and TNFR2$^{-/-}$ mice

The relative proportions of proliferating out of all CD8$^+$ cells were also determined. Corresponding to the findings in the CD4$^+$ cell subset, the TNFR2$^{-/-}$ CD8$^+$ cells were proliferating less than the WT CD8$^+$ cells after stimulation with 0.06 µg/ml anti-CD3ε. Additional stimulation with TNCscTNF80 significantly enhanced the proliferation rate of WT CD8$^+$ cells (Figure 8A). This effect was far less pronounced when compared to the CD4$^+$ cells. In the repeat of this experiment, however, TNCscTNF80 increased the proliferation of CD8$^+$ to a stronger extend (data not shown). The addition of TNCscTNF80 did not considerably enhance the proliferation of TNFR2$^{-/-}$ CD8$^+$ cells. Stimulation with 0.5 µg/mL anti-CD3ε antibody resulted in a strong proliferation of CD8$^+$ cells from both WT and

TNFR2$^{-/-}$ mice. TNCscTNF80 could not further enhance the proliferation in this case (Figure 8B).

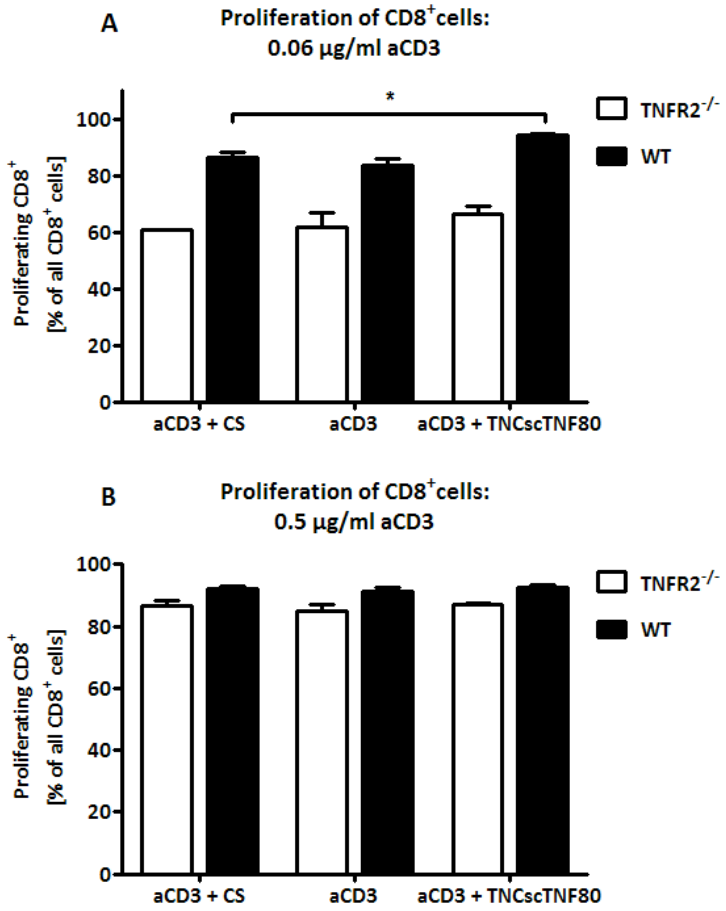

Figure 8: Impact of TNCscTNF80 on proliferation of CD8$^+$ cells from WT and TNFR2$^{-/-}$ mice. CFSE-labeled naïve splenocytes from WT (black bars) or TNFR2$^{-/-}$ (white bars) mice were cultured alone, with 10 ng/ml TNCscTNF80 or with CS used in equivalent volume in the presence of 0.06 µg/ml anti-CD3ε (A) or 0.5 µg/ml anti-CD3ε (B) for 72 hrs. The data is derived from a representative of two experiments, shown is the mean + SD of three technical replicates. * indicates a statistically significant difference of the indicated groups. Statistical analysis was done by two-way ANOVA with Bonferroni post-test.

3.1.2 Serial dilution of anti-CD3ε antibody: Proliferation of T cells

A serial dilution of the anti-CD3ε antibody was performed and lymphocyte cultures were stimulated with the different concentrations of anti-CD3ε antibody for 72 hours in order to determine if the anti-CD3ε concentration influences the effect of TNCscTNF80 on the proliferation rate of CD4$^+$ and CD8$^+$ cells.

3.1.2.1 Proliferation of CD4$^+$ cells depending on anti-CD3ε concentration

As shown in Figure 9, following stimulation with anti-CD3ε antibody, the proliferation of CD4$^+$ cells increased in a concentration-dependent manner. Additionally, lymphocytes were treated with TNCscTNF80, which enhanced proliferation of CD4$^+$ cells

even further. The correlation between the level of $CD4^+$ proliferation and the concentration of the anti-CD3ε antibody could also be observed if the cells were treated with TNCscTNF80. Nevertheless, the proliferation reached a plateau after stimulation with 0.25 µg/ml anti-CD3ε antibody and TNCscTNF80; a higher concentration of anti-CD3ε antibody did not result in an increased proliferation anymore. The addition of CS did not enhance the level of proliferation of $CD4^+$ cells.

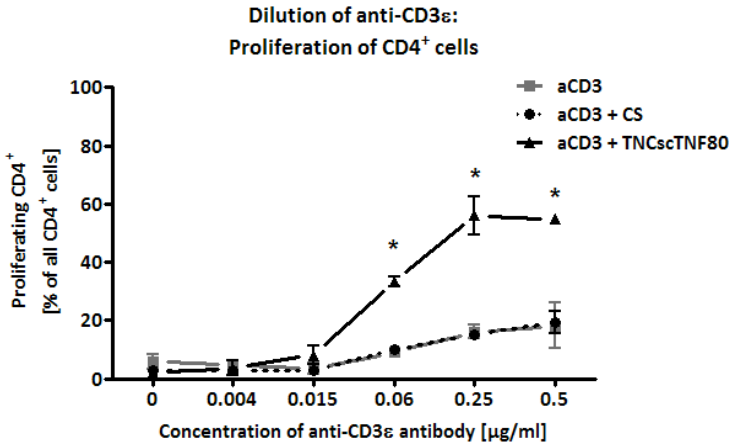

Figure 9: Proliferation of $CD4^+$ cells with increasing concentrations of anti-CD3ε antibody. CFSE-labeled splenocytes from WT mice were cultured alone (grey line, squares), with 10 ng/ml TNCscTNF80 (black line, triangles) or with CS used in equivalent volume (dotted line, circles) in the presence of increasing concentrations of anti-CD3ε antibody for 72 hrs. The data represents three individual experiments, shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.1.2.2 Proliferation of $CD8^+$ cells depending on anti-CD3ε concentration

The same effect, which was shown for the $CD4^+$ cells, was also observed in the $CD8^+$ cell population (Figure 10). The $CD8^+$ cells generally proliferated to a higher extend than the $CD4^+$ cells. With increasing concentrations of anti-CD3ε antibody the proliferation enhanced in a dose-dependent manner. Addition of TNCscTNF80 could further augment the proliferation, while addition of CS in equivalent amounts to TNCscTNF80 could not. The proliferation of $CD8^+$ reached a plateau if the lymphocytes had been stimulated with 0.25 µg/mL anti-CD3ε antibody and TNCscTNF80. Further increasing the concentration of anti-CD3ε antibody did not elevate the level of proliferation anymore.

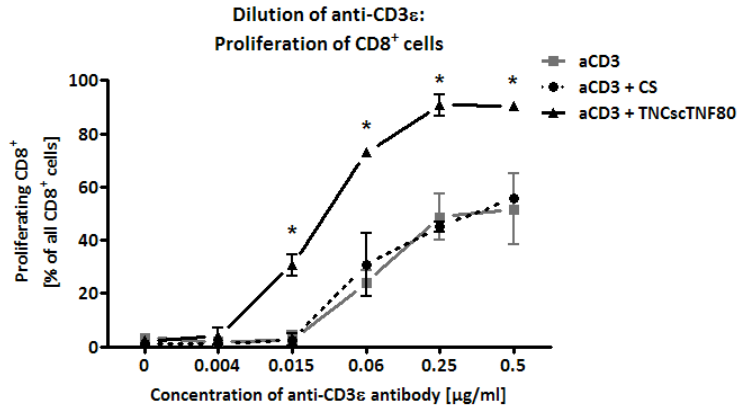

Figure 10: Proliferation of $CD8^+$ cells with increasing concentrations of anti-CD3ε antibody. CFSE-labeled splenocytes from WT mice were cultured alone (grey line, squares), with 10 ng/ml TNCscTNF80 (black line, triangles) or with CS used in equivalent volume (dotted line, circles) in the presence of increasing concentrations of anti-CD3ε antibody for 72 hrs. The data represents three individual experiments, shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.1.3 Serial dilution of TNCscTNF80: Proliferation of T cells

Throughout this work, TNCscTNF80 was utilized in a concentration of 10 ng/mL, according to the TNF concentration used in previous studies with T_{reg} (216). The objective of the following experiments was to assess if changing the concentration of TNCscTNF80 alters the enhancing effect it has on T cell proliferation.

3.1.3.1 Proliferation of $CD4^+$ cells depending on TNCscTNF80 concentration

As seen in Figure 11, TNCscTNF80 enhanced the proliferation of activated $CD4^+$ cells in a dose-dependent manner. While the proliferation could not be increased with 0.001 ng/ml or 0.01 ng/mL TNCscTNF80, it was steadily increasing when TNCscTNF80 was added to the culture in a concentration of 0.1 ng/mL or higher. No effect on the proliferation of $CD4^+$ cells could be observed if the CS had been given into the culture in equivalent volumes to TNCscTNF80.

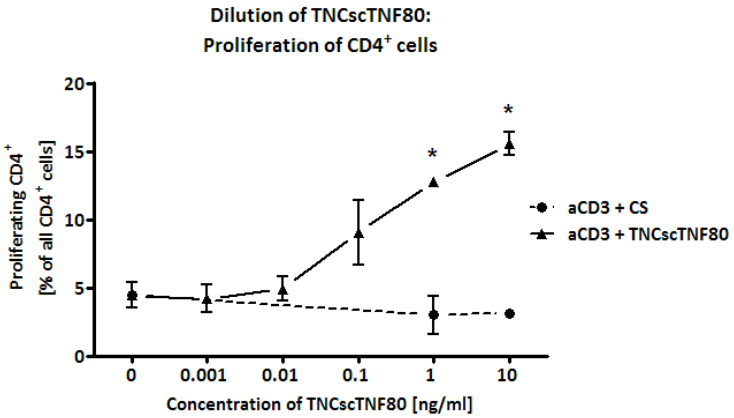

Figure 11: Proliferation of CD4$^+$ cells with increasing concentrations of TNCscTNF80. CFSE-labelled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (black line, triangles) or increasing amounts of CS used in equivalent volumes (dotted line, circles) in the presence of 0.5 µg/ml anti-CD3ε for 72 hrs. The data originates from a representative of three experiments, shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.1.3.2 Proliferation of CD8$^+$ cells depending on TNCscTNF80 concentration

Adding higher concentrations of TNCscTNF80 also increased the proliferation of activated CD8$^+$ cells (Figure 12). While treatment with up to 0.01 ng/mL TNCscTNF80 only slightly augmented the CD8$^+$ proliferation, treatment with 0.1 ng/ml, 1 ng/ml and 10 ng/mL TNCscTNF80 increased the proliferation in a concentration-dependent manner. Corresponding to the CD4$^+$ cell population, CD8$^+$ cells did not proliferate to a stronger extend if CS was present in the culture.

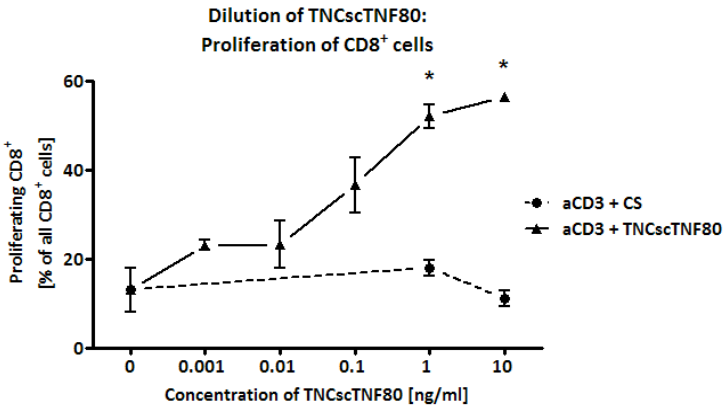

Figure 12: Proliferation of CD8$^+$ cells with increasing concentrations of TNCscTNF80. CFSE-labelled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (black line, triangles) or increasing amounts of CS used in equivalent volumes (dotted line, circles) in the presence of 0.5 µg/ml anti-CD3ε for 72 hrs. The data originates from a representative of three experiments, shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.1.4 Effect of TNFR agonists on the proliferation of lymphocytes

TNCscTNF80, which has been used in this work so far, is specific for the TNFR2. TNCwtTNF, on the other hand, can activate both TNFR1 and TNFR2. Although several studies have suggested that TNFR2 is predominately responsible for the co-stimulatory effect TNF has on lymphocyte proliferation (180, 181, 183), signalling via TNFR1 might also contribute to enhanced cell activation. Thus, the proliferative response of $CD4^+$ and $CD8^+$ cells towards the TNFR1 and TNFR2 agonist TNCwtTNF was compared to effect of the TNFR2-specific agonist TNCscTNF80.

3.1.4.1 Proliferation of lymphocytes in response to stimulation with TNFR agonists for 48 hours

Figure 13A shows that the proliferation of $CD4^+$ cells was only marginally increased by TNCscTNF80 and only if used in higher concentrations, whereas TNCwtTNF had an enhancing effect on the proliferative response of $CD4^+$ cells. The proliferation rate of $CD8^+$ was promoted by both TNCwtTNF and TNCscTNF80; TNCwtTNF, however, influenced the proliferative response of $CD8^+$ cells to a higher extend (Figure 13B). Nevertheless, the overall proliferation of $CD8^+$ and especially $CD4^+$ cells following stimulation for 48 hours was uncommonly low. The 48 h time-point is therefore too early to adequately detect an impact of TNFR agonists on T cell proliferation.

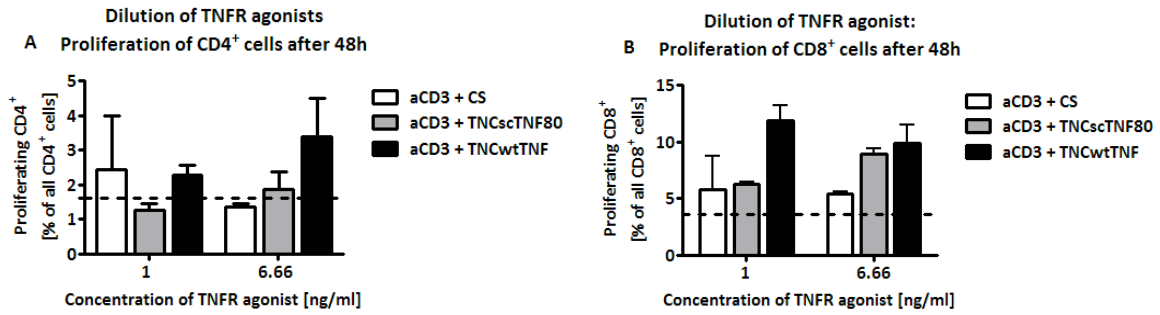

Figure 13: Proliferation of $CD4^+$ (A) and $CD8^+$ (B) cells with increasing concentrations of TNFR agonists after 48h of stimulation. CFSE-labeled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (grey bars), TNCwtTNF (black bars) or increasing amounts of CS utilized in equivalent volumes as TNCscTNF80 (white bars) in the presence of 0.5 µg/ml anti-CD3ε for 48 hrs. The dotted line shows the proliferation rate of $CD4^+$ cells that were stimulated with 0.5 µg/ml anti-CD3ε alone. Shown is the mean + SD of two technical replicates.

3.1.4.2 Proliferation of lymphocytes in response to stimulation with TNFR agonists for 72 hours

The proliferative response of both $CD4^+$ and $CD8^+$ cells was generally higher following stimulation for 72 hours, as demonstrated in Figure 14. TNCwtTNF and

TNCscTNF80 only slightly enhanced the proliferation of $CD4^+$ (Figure 14A) and $CD8^+$ (Figure 14B) cells. However, no difference could be seen between the effect of TNCscTNF80 and TNCwtTNF. CS had only marginal effects on both cell populations.

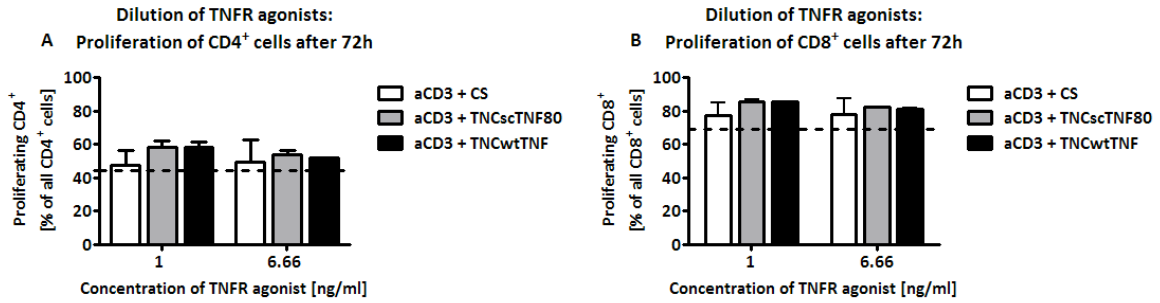

Figure 14: Proliferation of $CD4^+$ (A) and $CD8^+$ (B) cells with increasing concentrations of TNFR agonists after 72h of stimulation. CFSE-labeled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (grey bars) and TNCwtTNF (black bars) or increasing amounts of CS utilized in equivalent volumes to TNCscTNF80 (white bars) in presence of 0.5 µg/ml anti-CD3ε for 48 hrs. The dotted line shows the proliferation rate of $CD4^+$ cells that were stimulated with 0.5 µg/ml anti-CD3ε alone. Shown is the mean + SD of two technical replicates.

3.2 Cytokine production in the course of proliferation

Cytokines play an important role in mediating immune responses. IL-10 functions as an anti-inflammatory factor reducing the proliferation of different immune cells and the production of proinflammatory cytokines (137). It is secreted by a variety of immune cells, including $CD4^+$ regulatory T cells (153). IFN-γ, on the other hand, is an immunostimulatory cytokine that is mainly released by Th1 cells (235). As TNF, via TNFR2, promotes T cell proliferation, it was of interest if TNF-TNFR2 interaction could also alter the secretion of IL-10 or IFN-γ by T cells. While IFN-γ was detectable in the cell culture supernatants of various experiments, IL-10 was not found at all (data not shown).

3.2.1 Production of IFN-γ during proliferation assays in the presence of TNCscTNF80

First of all, it was assessed if the presence of the TNFR2 agonist TNCscTNF80 could alter the production of IFN-γ during the course of T cell proliferation. IFN-γ was detectable in the cell culture supernatants following T cell activation for 72 hours with 0.5 µg/ml anti-CD3ε antibody. Addition of TNCscTNF80 resulted in a sharp increase of IFN-γ in those supernatants, whereas CS did not have any effect on the level of production (Figure 15). When cells were activated with only 0.06 µg/ml anti-CD3ε antibody, no IFN-γ was found in the supernatants, not even in the presence of TNCscTNF80 (data not shown).

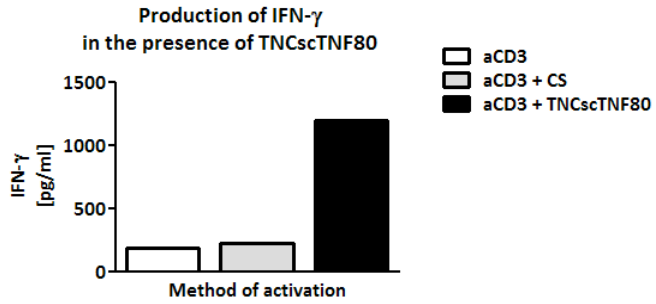

Figure 15: Production of IFN-γ depending on the presence of TNCscTNF80. CFSE-labeled naïve splenocytes from WT mice were cultured alone (white bar), with CS (grey bar) or with TNCscTNF80 (black bar) in the presence of 0.5 µg/ml anti-CD3ε antibody for 72 hrs. The supernatants of three technical replicates were pooled and the concentration of IFN-γ was measured using ELISA.

3.2.2 Production of IFN-γ depending on the concentration of anti-CD3ε antibody

It was observed in the previous experiment that the activation with 0.06 µg/ml anti-CD3ε antibody failed to induce IFN-γ production. Thus, it was examined if increasing the concentrations of the stimulatory antibody has an effect on the secretion of IFN-γ by T cells. There was no IFN-γ detectable in cultures that had received 0.004 µg/ml, 0.015 µg/ml or 0.06 µg/ml anti-CD3ε antibody for activation, neither with nor without additional TNCscTNF80 (data not shown). Nevertheless, a dose-dependent increase of IFN-γ was observed when cells had been activated with 0.25 µg/ml or 0.5 µg/ml in the presence of TNCscTNF80 (Figure 16).

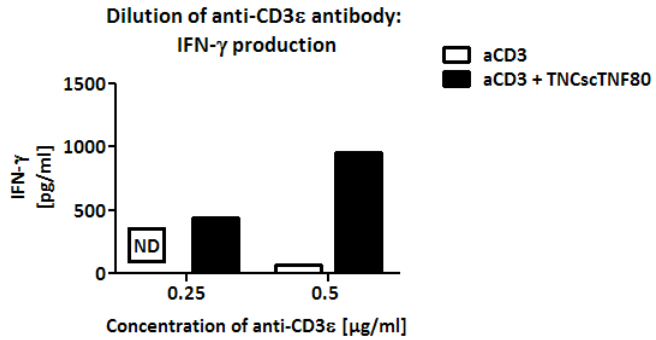

Figure 16: Increased production of IFN-γ due to higher concentration of anti-CD3ε antibody. CFSE-labeled naïve splenocytes from WT mice were cultured alone (white bars), or with TNCscTNF80 (black bars) in the presence of increasing concentrations of anti-CD3ε antibody for 72 hrs. The supernatants of two technical replicates were pooled and the concentration of IFN-γ was measured using ELISA. ND: IFN-γ was not detectable.

3.2.3 Production of IFN-γ depending on the concentration of TNCscTNF80

In addition to the effect of increasing concentration of anti-CD3ε antibody, the impact of enhancing TNCscTNF80 concentration was also studied. If the cell cultures were treated

with more TNCscTNF80, the concentration of IFN-γ in the cell culture supernatants increased in a dose-dependent manner (Figure 17). The addition of CS equivalent in volume to 10 ng/ml of TNCscTNF80, however, only resulted in a limited secretion of IFN-γ, which was comparable to the concentration found in the absence of TNCscTNF80.

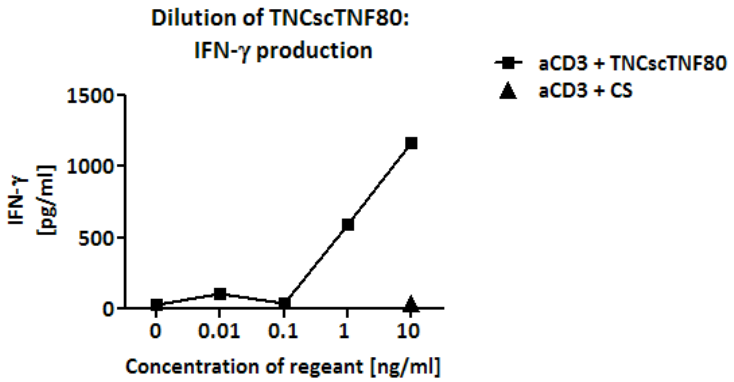

Figure 17: Increased production of IFN-γ due to higher concentration of TNCscTNF80. CFSE-labeled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (black line, squares) or CS used in equivalent amounts to 10 ng/ml of TNCscTNF80 (triangle) in the presence of 0.5 µg/ml anti-CD3ε for 72 hrs. The supernatants of two technical replicates were pooled and the concentration of IFN-γ was measured using ELISA.

3.2.4 Effect of different TNFR agonists on the production of IFN-γ

In the previous experiments it was demonstrated that the TNFR2 specific agent TNCscTNF80 increased the ability of T cells to produce IFN-γ. Yet, signalling via TNFR1 might also influence IFN-γ secretion. Therefore, the level of IFN-γ in cell culture supernatants of activated lymphocytes in the presence of TNCwtTNF, which activates both TNFR1 and TNFR2, was measured and compared to the amount of IFN-γ present in the cultures that were treated with TNCscTNF80. As illustrated in Figure 18, stimulation with TNCwtTNF for 72 hours generally increased the concentration of IFN-γ in the cell culture supernatants to a higher extend than TNCscTNF80. The production of IFN-γ was also dependent on the concentration of TNCwtTNF, which is in line with the previous observations made for TNCscTNF80. If cell cultures were stimulated for 48 hours in the presence of high-dose TNCwtTNF, the amount of IFN-γ found in the supernatant was even greater than after treatment with TNCwtTNF for 72 hours. Yet, stimulation with TNCscTNF80 for 48 hours failed to induce IFN-γ production. In the presence of CS no IFN-γ production was detectable either (data not shown).

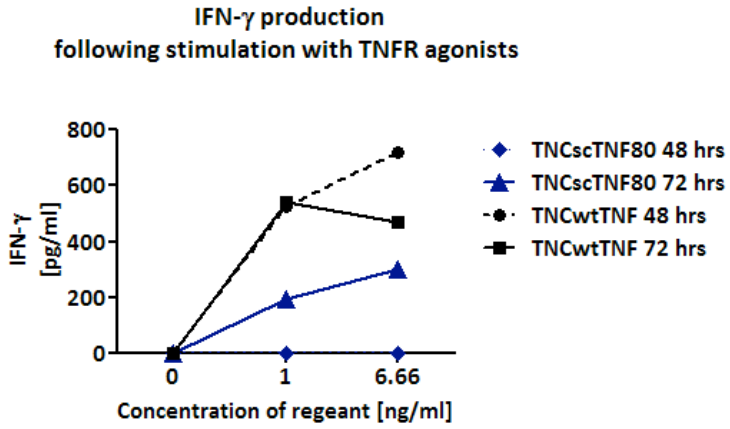

Figure 18: Increased production of IFN-γ in the presence of different TNFR agonists. CFSE-labeled naïve splenocytes from WT mice were cultured with increasing concentrations of TNCscTNF80 (blue symbols) and TNCwtTNF for (black symbols) in the presence of 0.5 µg/ml anti-CD3ε for 48 hrs (dotted lines) and 72 hrs (solid lines). The supernatants of two technical replicates were pooled and the concentration of IFN-γ was measured using ELISA.

3.3 Suppression assays: Proliferation of T_{eff} under the suppression of T_{reg}

$CD4^+CD25^+$ T_{reg} are important for the maintenance of immune tolerance as they suppress the proliferation and function of T_{eff} and thus inhibit overshooting immune responses (18). It has been found that T_{reg} express the TNFR2 on their surface to a stronger extend than T_{eff} (216). Further, it has been suggested that TNF promotes proliferation and suppressor capacity of T_{reg} cells (217). Yet, other groups have reported that TNF has inhibitory effects on T_{reg} function (210, 211). Thus, the goal of the following experiments was to determine how TNF-TNFR2 signalling affects the T_{reg}-mediated suppression of effector T cells.

3.3.1 Effect of TNCscTNF80 on the suppressive activity of $CD4^+CD25^+$ cells

In this study, the suppression assays were performed in the absence or presence of the TNFR2-specific agent TNCscTNF80 in order to examine the effect of TNFR2 signalling on the suppressive capacity of $CD4^+CD25^+$ cells. TNCscTNF80 cannot signal via TNFR1 (236) and thus does not have any effects on TNFR2-deficient lymphocytes. This has been demonstrated in the proliferation assays with splenocytes from $TNFR2^{-/-}$ mice that were performed for this thesis (refer to 3.1.1). To ensure that TNCscTNF80 only modulates T_{reg} function without influencing T_{eff} cells, lymphocytes from $TNFR2^{-/-}$ mice were cultivated with MACS sorted $CD4^+CD25^+$ T_{reg} cells from WT mice. Changes in the proliferative response of $TNFR2^{-/-}$ $CD4^+$ and $CD8^+$ cells must, therefore, be due to direct effects of TNCscTNF80 on the suppressive activity of the $CD4^+CD25^+$ cells.

As expected, $CD4^+CD25^+$ T_{reg} suppressed the proliferation of $CD4^+$ (Figure 19A) and to a lesser extend also of $CD8^+$ (Figure 19B) cells from $TNFR2^{-/-}$ mice. In the presence of T_{reg}, the average proliferation rate of anti-CD3-stimulated $CD4^+$ cells was reduced to 77.80 % of its initial level. The mean proliferation level of $CD8^+$ cells was reduced to 91.26 % respectively. In the presence of TNCscTNF80, however, the proliferation of $TNFR2^{-/-}$ $CD4^+$ cells under T_{reg}-mediated suppression was markedly higher. If TNCscTNF80 was added, the average proliferation rate of $CD4^+$ cells that were co-cultured with $CD4^+CD25^+$ T_{reg} was enhanced to 102.06 % of the initial level found in the absence of T_{reg} (Figure 19A). Likewise, the proliferation of $CD8^+$ cells under T_{reg}-induced suppression was enhanced to 94.74 % of the initial rate in the absence of T_{reg} (Figure 19B). Thus, it could be shown that TNF, through signalling via TNFR2 on $CD4^+CD25^+$ T_{reg}, reduced the suppressive capacity of T_{reg} over $CD4^+$ and $CD8^+$ lymphocytes. CS in equivalent volumes as TNCscTNF80 did not reduce the suppressive activity of T_{reg}, it even slightly enhanced the T_{reg}-mediated suppression of $CD4^+$ and especially $CD8^+$ cells.

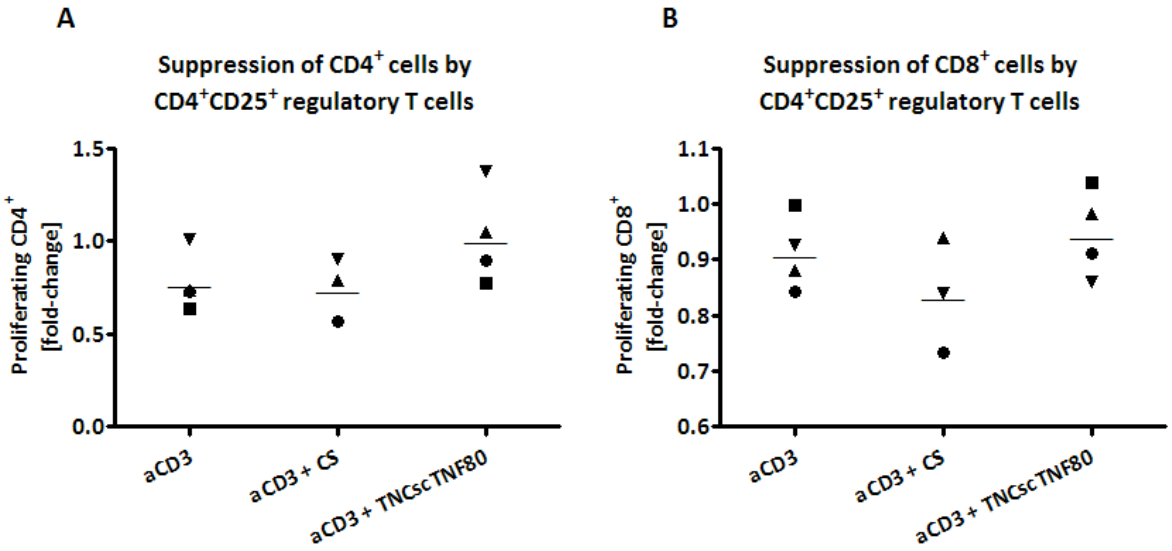

Figure 19: Impact of TNCscTNF80 on the suppressive capacity of $CD4^+CD25^+$ cells over the proliferation of $CD4^+$ (A) and $CD8^+$ (B) cells. CFSE-labeled naïve splenocytes from $TNFR2^{-/-}$ mice were cultured together with MACS-sorted $CD4^+CD25^+$ cells from WT mice at ratios of 3:1 either alone, with 10 ng/ml TNCscTNF80 or with CS used in equivalent volume in the presence of 0.5 µg/ml anti-CD3ε antibody for 72 hrs. The proliferation of $TNFR2^{-/-}$ cells that were stimulated with 0.5 µg/ml anti-CD3ε in the absence of $CD4^+CD25^+$ cells served as a control. The data was then normalized to those controls; the proliferation levels of these controls are defined as 100% and the proliferation rate of the $CD4^+$ and $CD8^+$ cells under suppression is shown as the fold change in comparison to the controls. The data originates from four different experiments; every group of dots represents one individual experiment. Every dot shows the mean of two technical replicates; the mean values for all the experiments are additionally shown (black lines).

3.3.2 Comparison of the effect of TNCscTNF80 on $CD4^+CD25^+$ cells from $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice

Whereas the majority of cells express TNFR1, lymphocytes mainly express TNFR2 (170). Moreover, it is generally believed that TNF preferentially mediates its effects on lymphocytes through TNFR2 (183). The TNF-associated effects on T_{reg} cells have also been attributed to TNFR2 (211, 217). Since the TNF mutant TNCscTNF80 is supposed to be specific for TNFR2, the objective of this experiment was to verify that TNCscTNF80 did not have any effect on the function of $CD4^+CD25^+$ T_{reg} that was mediated via a receptor other than TNFR2. Therefore, it was assessed if incubation with TNCscTNF80 could also change the suppressive activity of $CD4^+CD25^+$ from $TNFR2^{-/-}$ mice. $CD4^+CD25^+$ from TNFR1-deficient ($TNFR1^{-/-}$) mice served as a control. As seen in Figure 20, $CD4^+CD25^+$ cells from both $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice suppressed the proliferation of $CD4^+$ and $CD8^+$ cells, although the inhibitory effect of $TNFR1^{-/-}$ $CD4^+CD25^+$ cells on T cell proliferation was relatively low. Nevertheless, TNCscTNF80 completely reversed the suppressive activity of $CD4^+CD25^+$ cells from TNFR1-deficient mice. In the presence of TNCscTNF80, the proliferation of $CD4^+$ and $CD8^+$ cells in co-cultures with $TNFR1^{-/-}$ T_{reg} was higher than in the controls without T_{reg}. However, TNCscTNF80 did not reduce the suppressive activity of $TNFR2^{-/-}$ $CD4^+CD25^+$ cells. The effect of TNCscTNF80 on the $TNFR1^{-/-}$ T_{reg}-mediated suppression must be due to direct effects on T_{reg}, because the suppressed T_{eff} were TNFR2-deficient and therefore could not respond to TNCscTNF80. Thus, it can be excluded that TNCscTNF80 inhibits T cell suppression by reducing the susceptibility of T_{eff} towards T_{reg}-mediated suppression. Taken together, it could be shown that the attenuation of T_{reg}-induced T cell suppression by TNCscTNF80 is mediated through TNFR2 and not TNFR1 on T_{reg} cells.

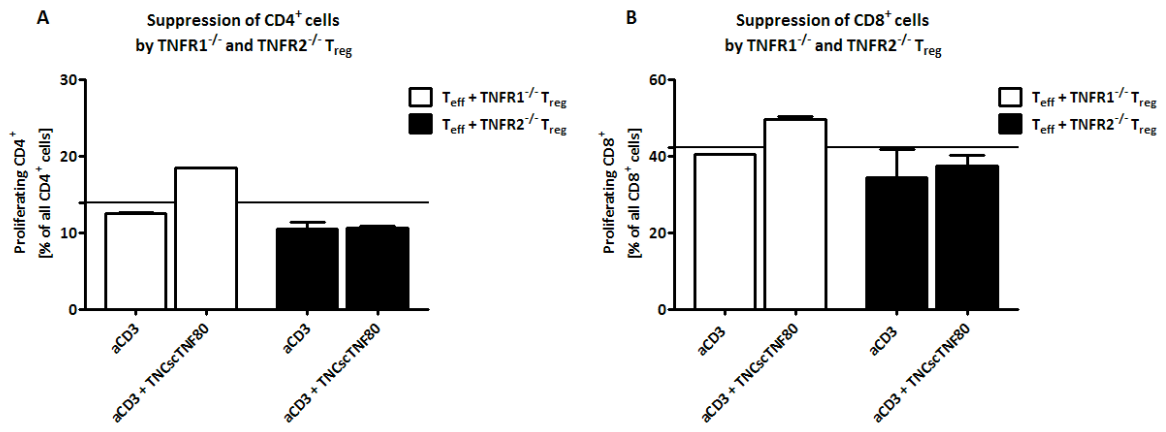

Figure 20: Effect of TNCscTNF80 on the suppressive activity of TNFR1$^{-/-}$ and TNFR2$^{-/-}$ CD4$^+$CD25$^+$ T$_{reg}$ over the proliferation of CD4$^+$ (A) and CD8$^+$ (B) cells. CFSE-labeled TNFR2$^{-/-}$ splenocytes were cultured with MACS-sorted CD4$^+$CD25$^+$ cells from TNFR1$^{-/-}$ (white bars) and TNFR2$^{-/-}$ (black bars) mice at ratios of 3:1 and activated with 0.5 µg/ml anti-CD3ε antibody with or without 10 ng/ml TNCscTNF80 for 72 hrs mice. Shown is the mean + SD of two technical replicates. The mean value for the proliferation of TNFR2$^{-/-}$ splenocytes that were cultured without purified CD4$^+$CD25$^+$ cells in the presence of 0.5 µg/ml anti-CD3ε is shown as a solid line.

3.3.3 Change of the suppressive activity of CD4$^+$CD25$^+$ cells: Comparison of TNCwtTNF and TNCscTNF80

It was shown in the previous experiment that TNCscTNF80 inhibits T$_{reg}$ suppressor capacity exclusively through signalling via the TNFR2. Yet, T$_{reg}$-induced suppression might also be influenceable by TNFR1 signalling. In order to reveal if the additional activation of TNFR1 can interfere with T$_{reg}$ function, suppression assays were performed in the presence of the combined TNFR1 and TNFR2 agonist TNCwtTNF as well. The proliferation of TNFR2$^{-/-}$ CD4$^+$ and CD8$^+$ cells in presence of WT CD4$^+$CD25$^+$ T$_{reg}$ and either TNCwtTNF or TNCscTNF80 was compared. When T$_{reg}$ were co-cultured with splenocytes at ratios of 1:3, they suppressed the anti-CD3ε-induced proliferation of CD4$^+$ (Figure 21A) and CD8$^+$ (Figure 21B). Both TNCscTNF80 and TNCwtTNF reduced the T$_{reg}$-mediated suppression of anti-CD3ε-activated CD4$^+$ and CD8$^+$ cells. The effect of TNCscTNF80, however, was less pronounced. TNCwtTNF was able to reverse the suppression induced by T$_{reg}$, while TNCscTNF80 reduced the T$_{reg}$-induced suppression but did not completely abrogate it. Treatment with CS, on the other hand, did not decrease the suppression but enhanced T cell suppression to a certain degree instead. Taken together, it could be shown that additional activation of TNFR1 did not reverse the TNFR2-induced reduction of T$_{reg}$ suppressive function but rather enhanced it.

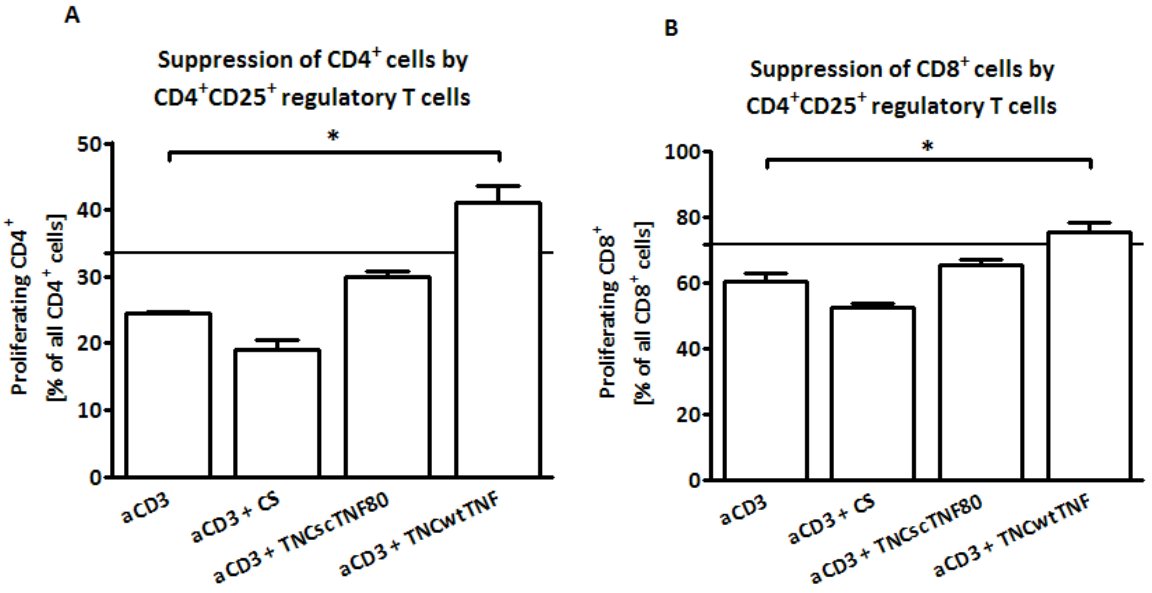

Figure 21: Impact of different TNFR agonists on the suppression of $CD4^+$ (A) and $CD8^+$ (B) cells by T_{reg}. CFSE-labeled $TNFR2^{-/-}$ splenocytes were cultured with MACS-sorted $CD4^+CD25^+$ cells from WT mice at ratios of 3:1 for 72 hrs and stimulated either with 0.5 µg/ml anti-CD3ε antibody alone or with anti-CD3ε antibody together with 10 ng/ml TNCwtTNF, 10 ng/ml TNCscTNF80 or CS used in equivalent volumes as TNCscTNF80. Shown is the mean + SD of two technical replicates. The mean value for the proliferation of $TNFR2^{-/-}$ splenocytes that were cultured without purified $CD4^+CD25^+$ cells in the presence of 0.5 µg/ml anti-CD3ε is shown as a solid line. * indicates a statistically significant difference of the indicated groups as determined by one-way ANOVA with Bonferroni post-test.

3.3.4 Serial dilution of TNCscTNF80: Effect on the suppression by T_{reg}

In the suppression assays throughout this work, TNCscTNF80 was used at a concentration of 10 ng/ml. Previous reports that had assessed the effects of TNF on T_{reg} suppressor function also used TNF at concentrations of 10 ng/ml (216). Yet, the TNFR2-specific agonist TNCscTNF80 has a higher affinity for its receptor (228), and might, therefore, mediate stronger effects when used in equal concentrations. Thus, it was of interest, how much the suppressive activity of WT $CD4^+CD25^+$ cells is changed when the concentrations of TNCscTNF80 is reduced. TNCscTNF80 decreased the T_{reg}-induced suppression of $TNFR2^{-/-}$ $CD4^+$ (Figure 22A) and $CD8^+$ (Figure 22B) cells in a dose-dependent manner. However, in contrast to previous experiments (refer to 0), 10 ng/ml TNCscTNF80 completely reversed T_{reg} suppression of $CD4^+$ cells. The proliferation was higher in this case when compared to the proliferation of $CD4^+$ cells in the absence of T_{reg} (Figure 22A). Addition of CS to the co-culture did not have any effect on the suppressive capacity of the $CD4^+CD25^+$ cells on $CD4^+$ or $CD8^+$ cells (data not shown).

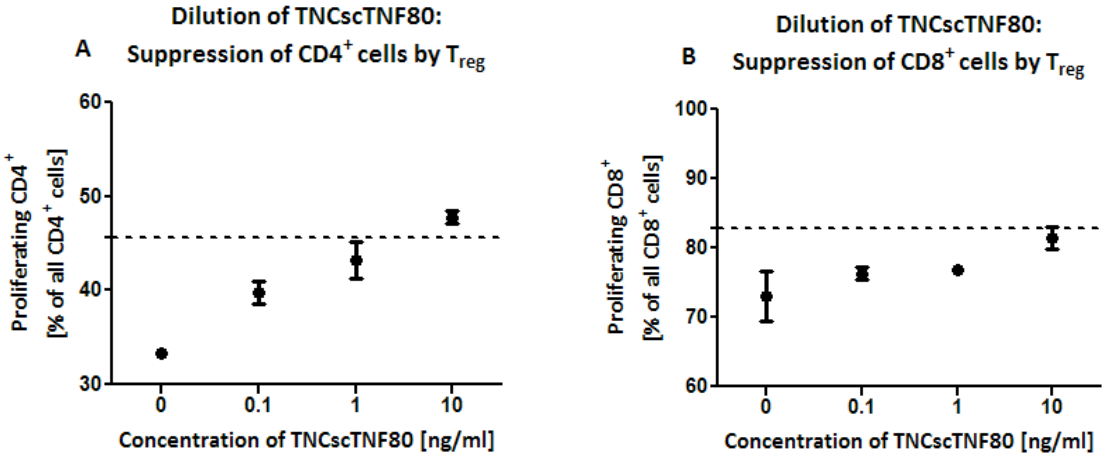

Figure 22: Effect of the concentration of TNCscTNF80 on the T_{reg}-mediated suppression of $CD4^+$ (A) and $CD8^+$ (B) cells. CFSE-labeled $TNFR2^{-/-}$ splenocytes were culture with MACS-sorted WT $CD4^+CD25^+$ cells in the presence of 0.5 µg/ml anti-CD3ε antibody and increasing concentrations of TNCscTNF80. Shown is the mean + SD of two technical replicates. $TNFR2^{-/-}$ splenocytes that were cultured alone in the presence of 0.5 µg/ml anti-CD3ε served as a control (dotted line).

3.4 Surface markers

There are a variety of surface molecules, which are preferentially expressed by resting and activated T_{reg} cells but which are only found on conventional T cells upon activation (33). In the previous experiments it was demonstrated that the TNFR2 agonist TNCscTNF80 limits the T_{reg}-mediated suppression of T lymphocytes. Because some of the surface markers are also associated with T_{reg} function (36), downregulation of those characteristic molecules could lead to an abrogation of T_{reg} suppressor capacity. This might pose a possible mechanism for the observed TNF-induced attenuation of T_{reg} function. Thus, it was assessed if TNCscTNF80 influences the expression of a selection of typical surface markers, i.e. CD25, CD39, CD73, OX40, GITR and CTLA-4. Therefore, splenocytes were activated with anti-CD3ε antibody for up to 72 hours in the absence or presence of TNCscTNF80 and the expression levels of those markers were measured on lymphocytes that were gated on $FoxP3^+$.

As seen in Figure 23A, the expression of CD25 after incubation for three hours was equally high for both the non-stimulated and the activated cells and there was no alteration observed when TNCscTNF80 was present in the culture. An increase in CD25 was noticed following anti-CD3ε-induced stimulation for 24 hours or more. Addition of TNCscTNF80 reduced CD25 expression after 48 and 72 hours. The majority of $Foxp3^+$ cells were $CD39^+$, regardless of stimulation or not. Yet, TNCscTNF80 lead to a sharp decrease of CD39 expression after 48 and 72 hours, while addition of CS did not alter the expression at all

(Figure 23B). The level of CD73 was constantly high, almost all cells were positive for CD73, ranging from 97.50 % to 99.71 %, over the whole time span independently of additional TNCscTNF80 or CS (Figure 23C). Generally, the expression of OX40 was relatively high and was enhanced further when cells were activated with anti-CD3ε, reaching a plateau after 48 hours. Yet, there was only a slight difference when TNCscTNF80 was present in the culture (Figure 23D). CTLA-4 was the surface marker that was least expressed by T_{reg}, only between 52.08 % and 55.56 % of $Foxp3^+$ cells showed this receptor on their surface. While the non-stimulated cells maintained similar levels of CTLA-4 over the whole incubation time, stimulation with anti-CD3 for 48 and 72 hours resulted in a strong decrease of CTLA-4 expression regardless of additional TNCscTNF80 or CS (Figure 23E). GITR was found on the majority of $Foxp3^+$ cells, both on activated and non-stimulated cells. Yet, the addition of TNCscTNF80 led to a decline of its expression after 48 and 72 hours (Figure 23F). In conclusion, TNCscTNF80 did not considerably alter the expression of CD73, OX40 and CTLA-4, while it decreased the expression of CD39, GITR and to a smaller extend also of CD25.

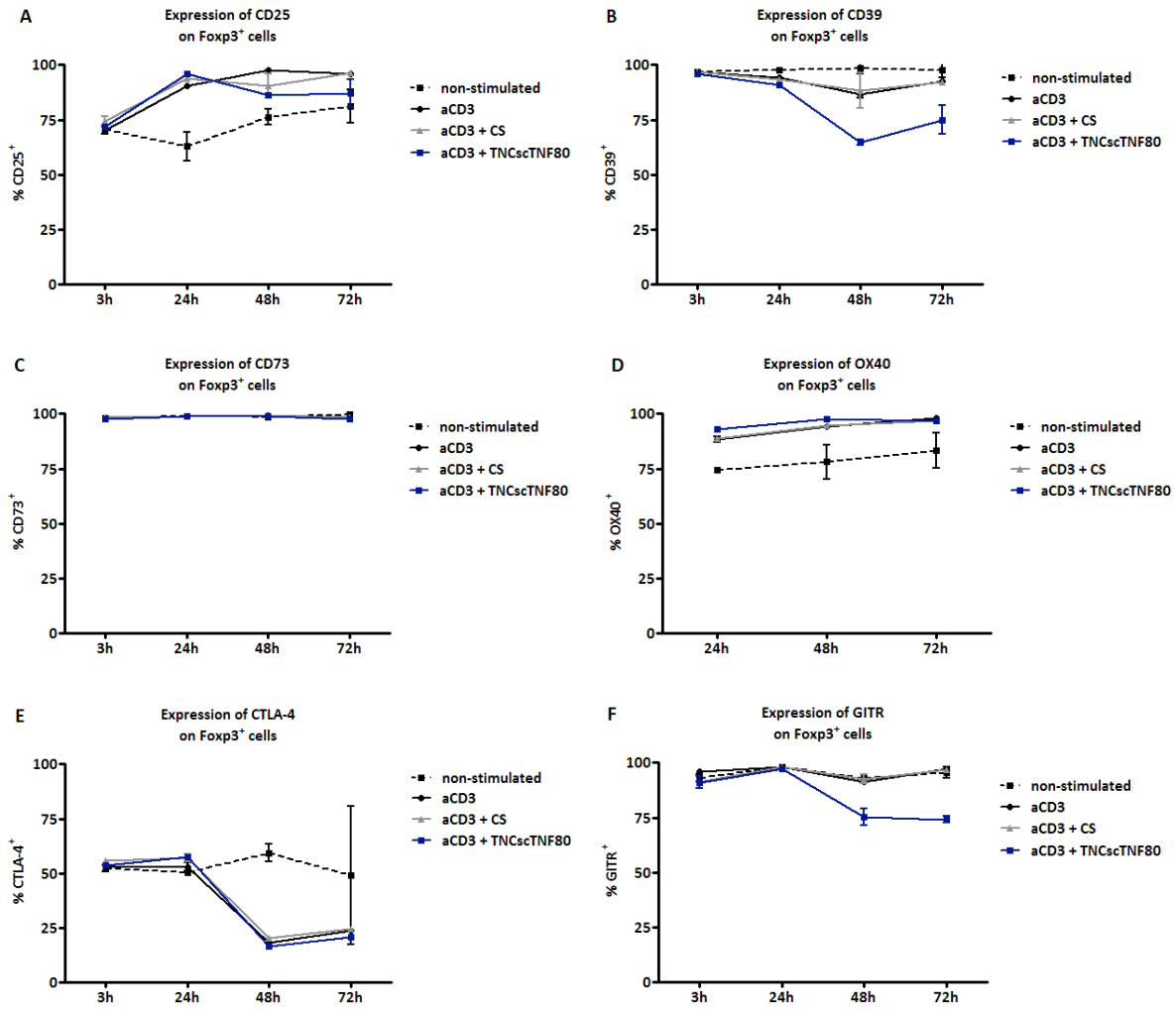

Figure 23: Expression of surface markers on Foxp3$^+$ cells. Naïve splenocytes were cultured alone (black line, circles), with CS (grey line, triangles) or TNCscTNF80 (blue line, squares) in the presence of 0.5 µg/ml anti-CD3ε antibody for up to 72 hrs. Non-stimulated cells (dotted line, circles) served as a negative control. The percentages of cells positive for the surface markers were obtained in dot plots gated on Foxp3$^+$ cells. Shown is the mean + SD of two technical replicates.

3.5 Establishment of a method for intracellular staining of pStat5

We demonstrated that TNFR agonists limit T_{reg} suppressor function. Although TNF is widely accepted as a mediator of T_{reg} function, the underlying molecular mechanisms still have not been revealed. It is known that T_{reg} cells require TCR and IL-2R stimulation in order to function adequately (18, 29). Correspondingly, various lines of evidence have demonstrated that IL-2-dependent activation of Stat5 is important for T_{reg} development and maintenance (76, 83, 84, 237). Thus, TNF might influence the signalling cascade downstream of the IL-2R and thereby change the suppressor function of T_{reg}. While conventional T cells activate various signalling pathways upon ligation to the IL-2R, T_{reg} predominantly activate the JAK-Stat5 pathway (74, 77). The inhibition of Stat5 activation, could therefore pose a

potential mechanism used by TNF to abrogate T_{reg} function. Thus, the ability of TNFR agonists to influence the activation of Stat5, i.e. the expression of phosphorylated Stat5 (pStat5), in activated T_{reg} was examined in this work.

3.5.1 Variations of staining protocol for pStat5 with BAF3 cells

Traditionally, western blot is used for studying cell-signalling events. Yet, large cell numbers are required to perform those immunoblots, which is a great limitation for the applicability of this technique in small cell populations such as T_{reg} (238). Additionally, western blot only allows the measurement of the average expression of a phospho-protein in non-separated cell subsets. By using flow cytometry, however, signalling events can be evaluated on a single-cell basis while cell subsets can be identified simultaneously based on their surface maker expression (239). Therefore flow cytometry was used to examine the phosphorylation status of Stat5 in response to TNF in this study. However, the pStat5 protocol supplied by the manufacturer was only available for western blotting. Therefore, a variety of experiments were required in order to find the most adequate variation for flow cytometry. Ba/F3 cells were used for the establishment of this staining protocol as they expand easily and express high levels of pStat5 under specific circumstances. The Ba/F3 cell line is generated from pre-B cells and depends on IL-3 for growth. Removing IL-3 for six up to twelve hours leads to a strong induction of pStat5 once the cultures are re-stimulated with IL-3 (240). Thus, for the following experiments, starved BA/F3 cells were incubated with IL-3 for 30 minutes at 37 °C and then stained with the rabbit monoclonal antibody (mAB) against pStat5/Tyr694.

3.5.1.1 Alterations in fixation, permeabilization and incubation with the primary antibody

In all of the following results, the protocol for intracellular staining of pStat5 as described in 2.4.2 is referred to as the standard protocol. In order to establish the most adequate procedure, variations of this final protocol had to be performed beforehand.

First of all, it was tested if the time of incubation was relevant for the quality of flow cytometry data. As shown in Figure 24, it was possible to stain pStat5 after incubation with the primary antibody overnight and for 30 minutes. However, if the incubation was performed overnight, the separation of the $pStat5^+$ and $pStat5^-$ populations was more defined (Figure 24A). Then, it was of interest if the fixation with 4 % formaldehyde or the permeabilization with 100 % methanol was necessary for an adequate staining of pStat5. Previous data has demonstrated that a strong permeabilization agent, such as methanol, is required for pStat5 detection due its nuclear location (241). Further, it has been shown that

rapid fixation, which can be achieved by applying formaldehyde together with methanol, is necessary in order to inhibit the activity of intracellular phosphatases and, thereby, capture the original phosphorylation status (242). It was seen here that it was possible to stain pStat5 if cells were not fixed in formaldehyde but permeabilized with methanol, both after incubation overnight (Figure 24C) and for 30 minutes (Figure 24D). Nevertheless, without fixation the $pStat5^{+}$ cells were generally not as distinguishable from the $pStat5^{-}$ cells. The separation of both subsets was less precise if the unfixed cells were incubated with the primary antibody for only 30 minutes (Figure 24D). On the other hand, it was not possible to detect pStat5 if cells were not permeabilized with methanol but fixed with formaldehyde (Figure 25). Therefore, in every future experiments cells were fixed with 4 % formaldehyde, permeabilized with 100 % methanol and incubated with the primary antibody overnight.

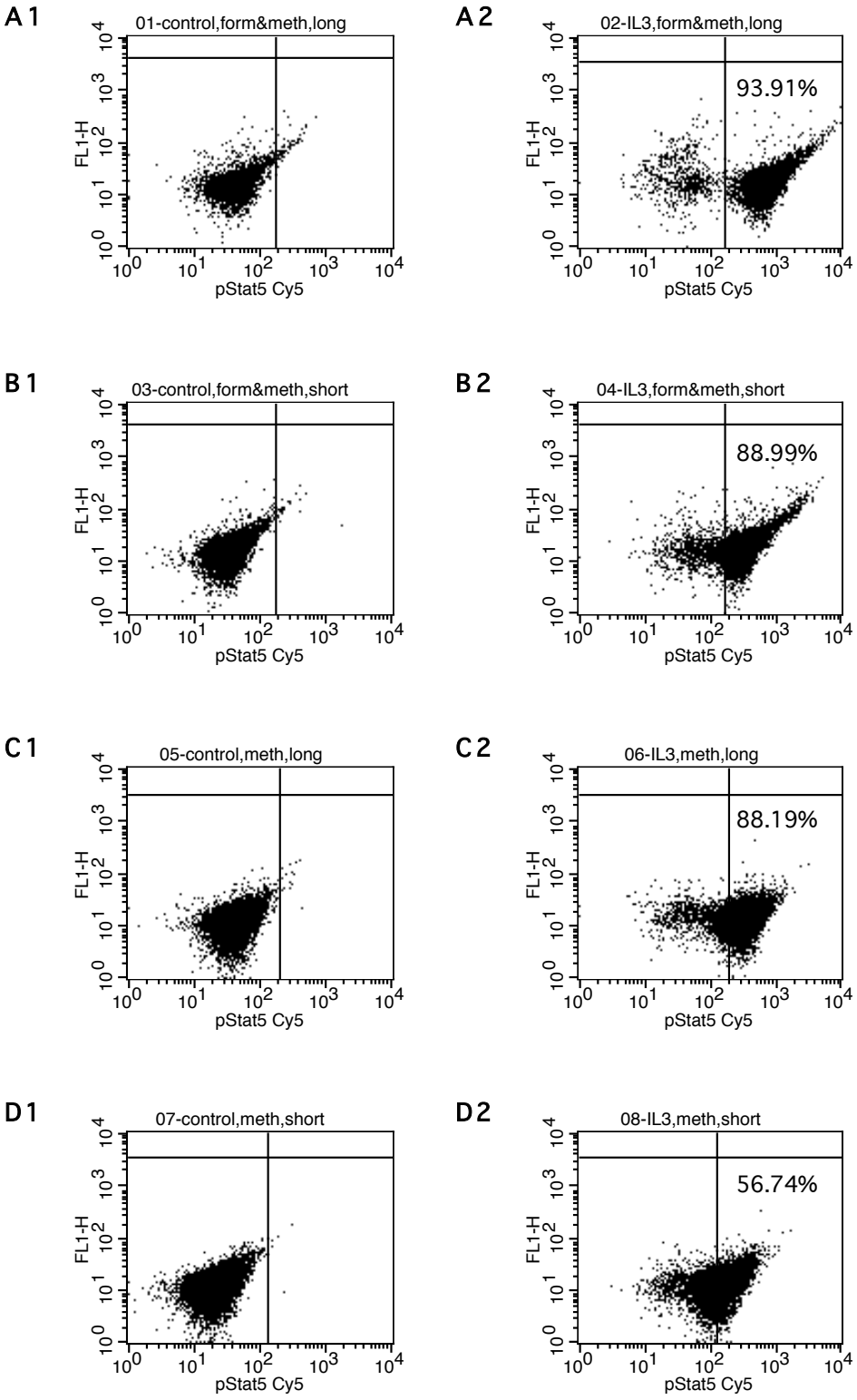

Figure 24: Variations of staining protocol: Non-stimulated (A1-D1) vs. activated with 1 µg IL-3 (A2-D2). Ba/F3 cells were cultivated without IL-3 for 12 hours before restimulation with IL-3 (right panels, "2") or left without IL-3 (left panels, "1"). 30 min later, cells were harvested and assayed for pStat5 by various protocols. Standard protocol, antibody incubation overnight (A); Standard protocol, antibody incubation for 30 min (B); without formaldehyde, incubation overnight (C); without formaldehyde, incubation for 30 min (D).

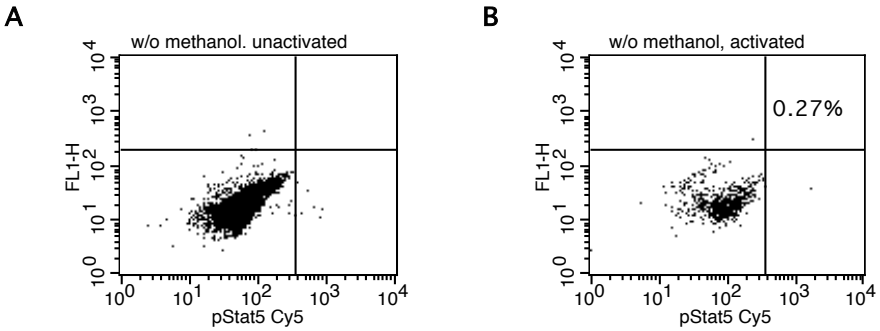

Figure 25: Staining of pStat5 without permeabilization with methanol: Non-stimulated (A) vs. stimulated with 1 µg IL-3 (B). Ba/F3 cells were cultivated without IL-3 for 12 hours before restimulation with IL-3 (B) or left without IL-3 (A). 30 min later, cells were harvested and assayed for pStat5 according to the standard protocol without permeabilization with methanol.

It was also assessed if pStat5 was detectable when the protocol recommended by eBioscience for Foxp3 staining was performed, which includes using the fixation and permeabilization solutions supplied in their Foxp3 staining kit. However, this was not successful, as, unlike expected, none of the IL-3 stimulated Ba/F3 cells showed pStat5 expression after the staining procedure (Figure 26).

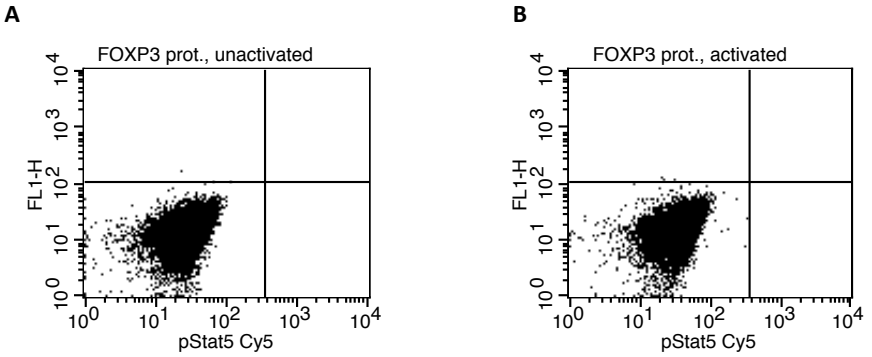

Figure 26: Staining of intracellular pStat5 with Foxp3 staining protocol in non-stimulated (A) and IL-3 activated cells (B). Ba/F3 cells were cultivated without IL-3 for 12 hours before restimulation with IL-3 (B) or left without IL-3 (A). 30 min later, cells were harvested and assayed for pStat5 using the Foxp3 staining kit from eBioscience.

3.5.1.2 Concentration of Triton X-100 in the required buffers

The staining protocol states that 0.3 % of Triton X-100 should be used in all buffers. In order to identify if the addition of Triton X-100 was necessary, cells were stained using buffers that contained 0 %, 0.1 % and 0.3 % Triton X-100. After performing the staining procedure with the different concentrations of Triton X-100, no relevant differences of the percentages of $pStat5^+$ cells or of the separation of $pStat5^+$ and $pStat5^-$ populations were

noticed (data not shown). Thus, Triton X-100 was used in the buffers at the suggested concentration of 0.3 %.

3.5.1.3 Titration of Rabbit mAB pStat5 Tyr694 (clones: D47E7 and C71E5)

Titrations of two antibody clones against pStat5 were conducted in order to find an appropriate dilution of the mAB, with which good quality flow cytometry data could be achieved. A two-fold serial dilution of the D47E7 clone was performed, starting with 1:200. The C71E5 clone was utilized in dilutions of 1:100, 1:200 and 1:400. If the cells were stained with the C71E5 clone the staining was most effective if a 100- or 400-fold dilution had been used (data not shown). Yet, the D47E7 clone generally produced better results. The highest proportions of $pStat5^+$ populations in response to IL-3 re-stimulation were obtained using a 400- and 800-fold dilution of the D47E7 clone (Figure 27). Since there was no relevant difference between the 1:400 and a 1:800 dilution, a 600-fold dilution was used standardly in the following experiments.

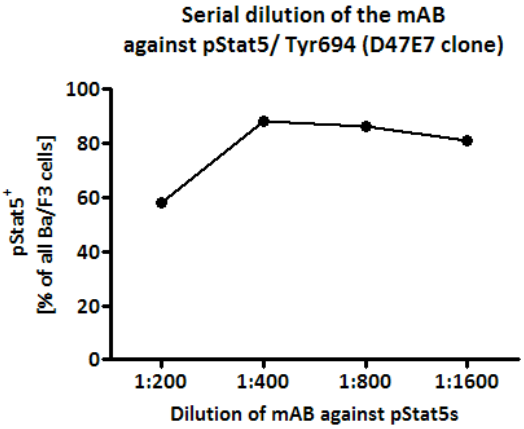

Figure 27: Proportion of $pStat5^+$ cells depending on the dilution of the rabbit anti-mouse mAB pStat5/Tyr694 (D47E7 clone). Ba/F3 cells were cultivated without IL-3 for 12 hours before restimulation with IL-3. 30 min later cells were harvested and stained with a monoclonal antibody against pStat5/Tyr694 (D47E7 clone) at various dilutions starting from 1:200.

3.5.1.4 Comparison of starved BA/F3 cells with continuously cultured BA/F3 cells

Starved and IL-3-stimulated Ba/F3 cells had been used throughout all of the previous experiments in order to determine the most effective alteration of the western blot protocol for staining against intracellular pStat5. A positive control would also be needed in future experiments on the expression of pStat5 in lymphocytes. As starved Ba/F3 cells are not as rapidly available as regular growing Ba/F3 cells, it was of question if the continuously cultured cells could be used as a substitute. The quality of flow cytometry data following the staining against intracellular pStat5 was, therefore, compared between continuously cultured

and starved Ba/F3 cells. As demonstrated in Figure 28, the pStat5$^+$ population was smaller and less separated from the pStat5$^-$ subset in the continuously cultured Ba/F3 cells when compared to the starved Ba/F3 cells. Yet, 88.87 % of growing cells were pStat5$^+$ in response to stimulation with IL-3. This was accepted to be sufficient and staining of pStat5 in continuously cultured Ba/F3 was used as a positive control in future experiments.

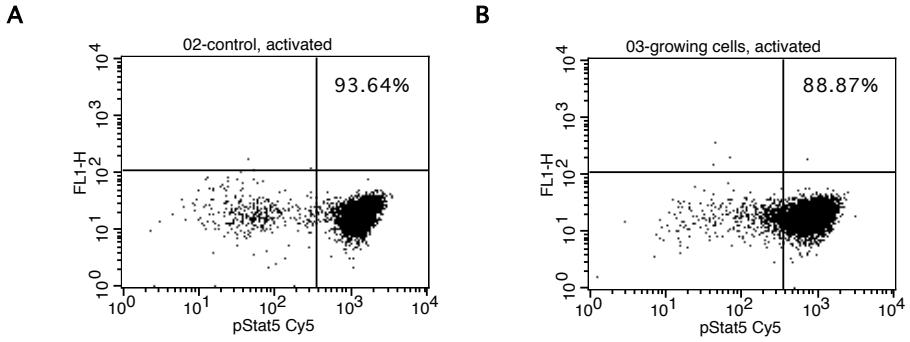

Figure 28: Positive control for staining against intracellular pStat5 using starved BAF3 after re-stimulation (A) and continuously cultured (B) cells. (A) Ba/F3 cells were cultivated without IL-3 for 12 hours, and after restimulation with IL-3 for 30 min they were harvested and assayed for pStat5. (B) Ba/F3 cells that had continuously been cultured with 1 µg IL-3 were harvested in the pro-B cell stadium and assayed for pStat5.

3.5.2 Variations of staining protocol with lymphocytes

3.5.2.1 Altering the order of the staining protocol

As it was of interest how TNFR agonists change pStat5 expression in different sub-populations of lymphocytes, it had to be determined if staining against pStat5 was also possible in T cells using the method that was established for the Ba/F3 cell line. Furthermore, naïve splenocytes also needed to be stained with antibodies against extracellular markers in order to identify different cell populations, such as CD4$^+$CD25$^+$ T_{reg}. Yet, the staining protocol for pStat5 includes methanol, which could potentially interfere with the staining process of surface molecules for flow cytometric analysis. Thus, it was required to find a method that produced high quality data for flow cytometric analysis of both extracellular and intracellular molecules. Therefore, the comparability of staining against intracellular pStat5, which includes the fixation and permeabilization processes, and staining against extracellular markers was assessed. The standard FACS staining, which is described in 2.4.1, was used as a control for the extracellular staining. The following variations of the standard protocol were applied: [1] staining against extracellular markers followed by fixation and permeabilization; [2] fixation and permeabilization followed by staining against extracellular markers; [3] staining against extracellular markers followed by permeabilization; [4] permeabilization

followed by staining against extracellular markers. The fixation process was always performed, because it had been shown that pStat5 was only detectable if cells were fixed with formaldehyde (Figure 25). A method was found to be effective if an adequate number of cells were positive for the surface marker in question and if the discrimination between the positive and negative populations could be made based on the surface marker. If the surface staining was performed after the cells were either fixed and permeabilized [2] or only permeabilized [4], the staining against CD4 and B220 was effective with every antibody. However, the cell populations could not be discriminated if surface staining was conducted prior to the permeabilization process, regardless of fixation [1] or not [3]. Yet, staining against CD25 did not show constant results. Thus, the emphasis was put on finding a method, which could readily detect CD25. The percentages of $CD25^+$ cells within the $CD4^+$ cell subset were assessed. If approximately 10 % were $CD25^+$, which is the expected proportion of T_{reg} cells in mice, the particular staining method was considered acceptable. For the results refer to Table 1. Similar to CD4 and B220, detection of CD25 was not possible if staining was performed before the permeabilization with methanol [1], [3]. The most reliable method was the method that had been proposed by Cell Signalling, i.e. treating cells with formaldehyde and methanol for fixation and permeabilization before the immunostaining [2].

	Standard FACS staining	[1] 1.FACS staining 2.Fixation and permeabilization	[2] 1.Fixation and permeabilization 2.FACS staining	[3] 1.FACS staining 2.Permeabilization	[4] 1.Permeabilization 2.FACS staining
CD4-AF488	✓	✓	✓	✓	✓
CD4-PE	✓	X	✓	X	✓
CD4-AF647	✓	✓	✓	✓	✓
B220-PerCP	✓	X	✓	X	✓
B220-PE	✓	X	✓	X	✓
CD25-APC	✓	(✓)	X	X	X
CD25-B/Strep-FITC	✓	X	(✓)	X	(✓)
CD25-B/Strep-PerCP	✓	X	X	X	X
CD25-PE	✓	NA	✓	NA	✓
CD25-B/Strep-PE	✓	NA	✓	NA	NA
CD25-FITC (7D4 clone)	✓	X	X	NA	NA

Table 1 Comparison of the results of staining against extracellular markers while altering the standard protocol for staining against pStat5. ✓= staining possible; X = staining not possible; NA = not available

3.5.2.2 Alteration of fluorochromes conjugated to streptavidin

In the former experiments, the staining of CD25 was more precise if a biotin-labelled primary antibody had been utilized, but the quality of the acquired data was also dependent on the fluorochromes conjugated to streptavidin. Thus, different types of streptavidin-coupled fluorochromes were compared. The staining process was performed in the following orders: [1] fixation, permeabilization, incubation with the biotin-labelled primary antibody, incubation with streptavidin and other antibodies or [2] incubation with the biotin-labelled primary antibody, fixation, permeabilization, incubation with streptavidin and other antibodies. No relevant differences in the data quality could be identified between those two variations (data not shown). Therefore, for reasons of convenience, staining with both the biotin-labelled primary antibody and streptavidin was performed after fixation and permeabilization [1]. In contrast to preliminary experiments, it was observed here that the quality of data and the percentages of $CD25^+$ within the $CD4^+$ population were equally satisfying for all of the fluorochromes tested (Figure 29).

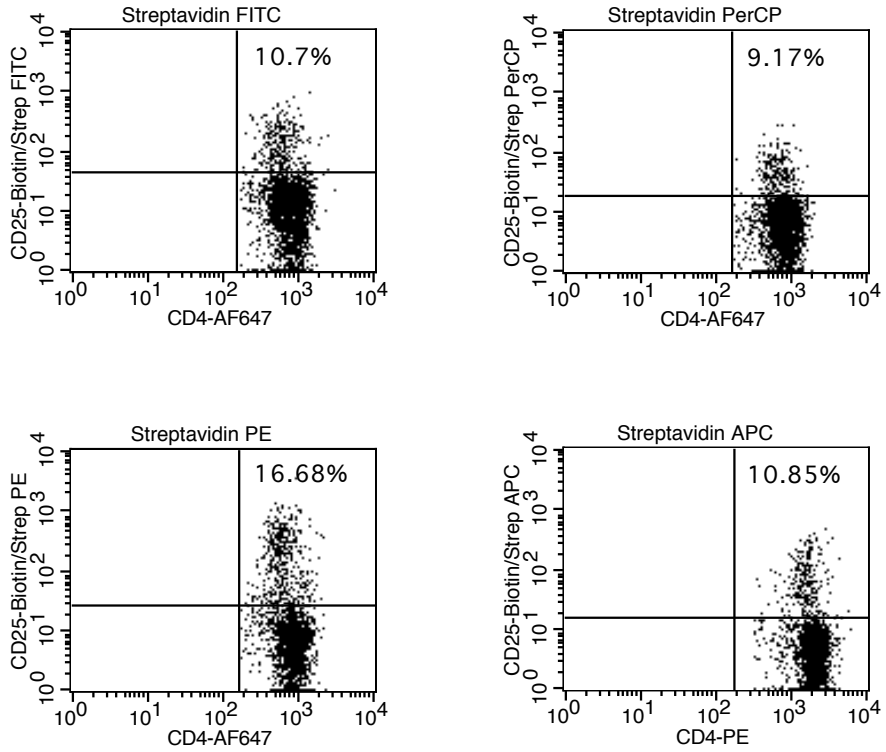

Figure 29: Staining against CD25 depending on the streptavidin-conjugated fluorochrome. Naïve Splenocytes were stained against CD4 and CD25, for the latter biotin-labelled primary antibodies and a variation of fluorochromes conjugated to streptavidin were used. The percentages of $CD4^+CD25^+$ cells (upper right, UR) were calculated.

3.5.2.3 Alteration of fixation solution and buffers

In order to analyse the importance of Triton X-100 and the concentration of formaldehyde, which are used in the fixation solutions, splenocytes were stained against

extracellular markers following the standard FACS staining protocol. Cells were then fixed using 2 % formaldehyde, 4 % formaldehyde or 4 % formaldehyde with 0.3 % Triton X-100. The highest proportions of $CD25^+$ cells out of all $CD4^+$ cells were found after fixation with 4 % formaldehyde without any additional Triton X-100 in the fixation solution (data not shown). Fixation with formaldehyde and Triton X-100 led to a complete loss of the $CD25^+$ cell population when using APC-labeled anti-CD25-mAB (data not shown).

Further, the impact of different concentrations of Triton X-100 in the required buffers for the intracellular staining protocol on the efficiency of surface staining was assessed. The highest proportion of $CD4^+CD25^+$ cells and the best staining results were seen if 0.3 % Triton X-100 was used. Therefore, 0.3 % was the standard concentration of Triton X-100 in the buffers in future applications of this protocol, while the fixation was performed with 4 % formaldehyde without any additional Triton X-100.

3.5.2.4 Substitution of methanol by acetone

Acetone could be less aggressive for the cells during the permeabilization step, and, therefore, it was assessed if the substitution of methanol by acetone was applicable. Extracellular markers could not be detected by flow cytometry when the staining process was performed before the permeabilization with acetone. However, the extracellular makers were detectable if cells first were permeabilized with acetone and then incubated with the antibodies against those markers. This is consistent with the previous results for the permeabilization with methanol, which had shown that surface molecules were not detectable when staining was performed before the permeabilization process (Table 1). Moreover, staining of CD25-Biotin/Streptavidin-PE was performed both with methanol and acetone. In direct comparison, the separation of $CD4^+CD25^+$ from $CD4^+CD25^-$ was more defined after the permeabilization with methanol (data not shown). Thus, acetone was not used as a substitute for permeabilization in future experiments.

3.5.2.5 Staining against CD45.1, CD45.2 and Foxp3 for further identification of T_{reg}

Additional reliable markers for the identification of T_{reg} other than CD25 were required. For this purpose, it was tested if staining with antibodies against CD45.1, CD45.2 and Foxp3 were compatible with the staining against intracellular pStat5. For both CD45.1 and CD45.2 (data not shown) the data was satisfactory. Thus, mouse lines, which are specific for CD45.2, could be used as source for $CD4^+CD25^+$ T_{reg}. By staining against CD45.2 and CD45.1 it was, therefore, possible to differentiate between regulatory and effector T cells. Assaying against Foxp3 with the pStat5 staining protocol was also tested. The proportion of

$CD4^+Foxp3^+$ out of all $CD4^+$ cells was 7.36 % (Figure 30), which is close to the estimated 10 % T_{reg} cells in the $CD4^+$ population. Thus, Foxp3 could be used as an additional T_{reg} marker in the experiments regarding pStat5 expression in T_{reg} and T_{eff}.

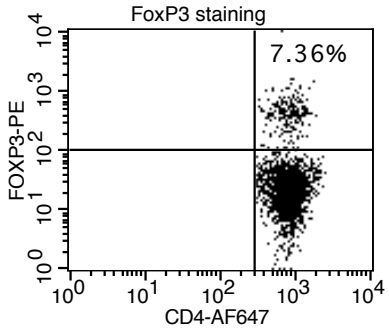

Figure 30: Staining against Foxp3 with pStat5 protocol. Naïve splenocytes were stained with Foxp3-PE and CD4-AF647 using the standard staining protocol for pStat5. Dot plot was gated on $CD4^+$ cells and the percentage of $CD4^+Foxp3^+$ cells (UR) was calculated.

3.6 Expression of phosphorylated Stat5 in regulatory and effector T cells

T_{reg} are highly dependent on IL-2 for their activation and function (13) and induce Stat5 as the main mediator of signal transduction downstream of the IL-2R (74). As shown in the previous experiments, TNCscTNF80 has a negative effect on T_{reg} function (refer to 3.3). In T_{eff}, TNFR2 signalling serves as a co-stimulator for IL-2 induction during early T cell activation through the activation of the Akt/NF-κB pathway (181, 243). Yet, the effect of TNFR agonists during activation of T_{reg} remains unknown. It was hypothesized that TNCscTNF80 attenuates signal transduction in response to TCR/IL-2R stimulation and thereby inhibits T_{reg} suppressor capacity.

3.6.1 Change in expression of phosphorylated Stat5 after short-term incubation with TNFR agonists

Following IL-2-driven activation, T cells are expected to express pStat5 rapidly (244). However, specific data on the kinetics of Stat5 upregulation in $CD4^+Foxp3^+$ T_{reg} cells is rare. Yet, it is likely that Stat5 is also activated quickly in T_{reg} cells upon stimulation. TNFR2 signalling might therefore affect T_{reg} function through interaction with intracellular processes during early cell activation. Thus, the influence of different TNFR agonists on the expression of pStat5 during short-term incubation for up to three hours was examined. Regulatory and non-regulatory T cells were identified using the extracellular markers $CD4^+$ and $CD25^+$. Subsequently, expression of pStat5 was analysed in the $CD4^+CD25^-$ and $CD4^+CD25^+$ subsets.

3.6.1.1 Expression of pStat5 after 30 minutes

Lymphocytes were stimulated with anti-CD3ε antibody, IL-2 and a TNFR agonist for 30 minutes and the expression of pStat5 was compared between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. As seen in Figure 31A, $CD4^+CD25^+$ cells showed a higher expression of pStat5 after incubation with IL-2 and anti-CD3ε together in comparison to the non-stimulated cells and cells that had only received anti-CD3ε. If TNFR agonists were present in the culture, the expression of pStat5 in $CD4^+CD25^+$ cells was further enhanced. There were minor differences between the different TNFR agonists. The TNFR1 agonist hTNF had the smallest effect on pStat5 enhancement, while the TNFR2 agonist TNCscTNF80 enhanced pStat5 expression the most and the combined TNFR1 and TNFR2 agonist TNCwtTNF showed intermediate effects. In $CD4^+CD25^-$ cells, however, the level of pStat5 did not change at all, neither following the stimulation with anti-CD3ε alone nor after incubation with anti-CD3ε and IL-2 together (Figure 31B).

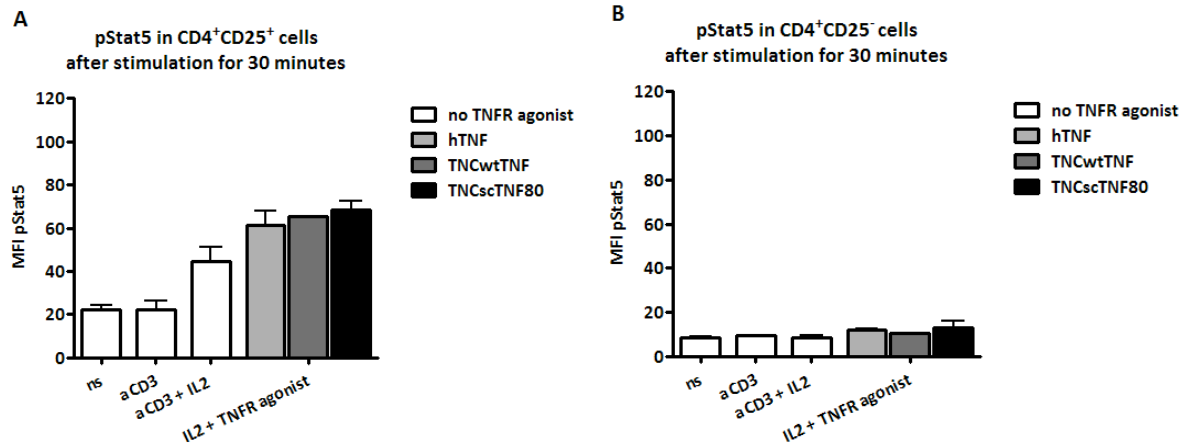

Figure 31: Expression of pStat5 in $CD4^+CD25^+$ (A) and $CD4^+CD25^-$ (B) cells after activation with different TNFR agonists for 30 minutes. Naïve splenocytes were cultured alone, with hTNF (light grey bars), TNCwtTNF (dark grey bars) or TNCscTNF80 (black bars) in the presence of 10 ng/ml IL-2 and 0.5 µg/ml anti-CD3ε antibody. Non-stimulated (ns) cells served as a negative control; incubation with anti-CD3ε antibody alone served as a positive control. Shown is the mean fluorescence intensity (MFI) of pStat5 in $CD4^+CD25^+$ (A) cells and $CD4^+CD25^-$ cells (B) as the mean + SD of two technical replicates.

3.6.1.2 Expression of pStat5 after 60 minutes

A culture of lymphocytes was stimulated with anti-CD3ε antibody, IL-2 and various TNFR agonists for 60 minutes. Some cells were incubated with IL-2 for 30 minutes before the TNFR agonists were added for another 30 minutes, others were incubated with the TNFR agonist first and IL-2 was added after 30 minutes.

Figure 32A demonstrates that the $CD4^+CD25^+$ cells showed a stronger expression of pStat5 after incubation with anti-CD3ε and IL-2 for 60 minutes in comparison to the $CD4^+CD25^+$ cells that had received anti-CD3ε alone. If the cells were incubated with IL-2 before the TNFR agonist was added, the expression of pStat5 only marginally increased compared to the incubation with IL-2 and anti-CD3ε alone. On the other hand, pre-incubation with a TNFR agonist for 30 minutes followed by the addition of IL-2 for another 30 minutes enhanced the expression of pStat5 in $CD4^+CD25^+$ cells. The greatest effect was seen after pre-incubation with TNCscTNF80, whereas the effect of TNCwtTNF was weaker and hTNF again only induced minor changes. Similar to the results upon stimulation for 30 minutes, the expression of pStat5 in the $CD4^+CD25^-$ cells did not change at all following the different stimulation processes for 60 minutes (Figure 32B).

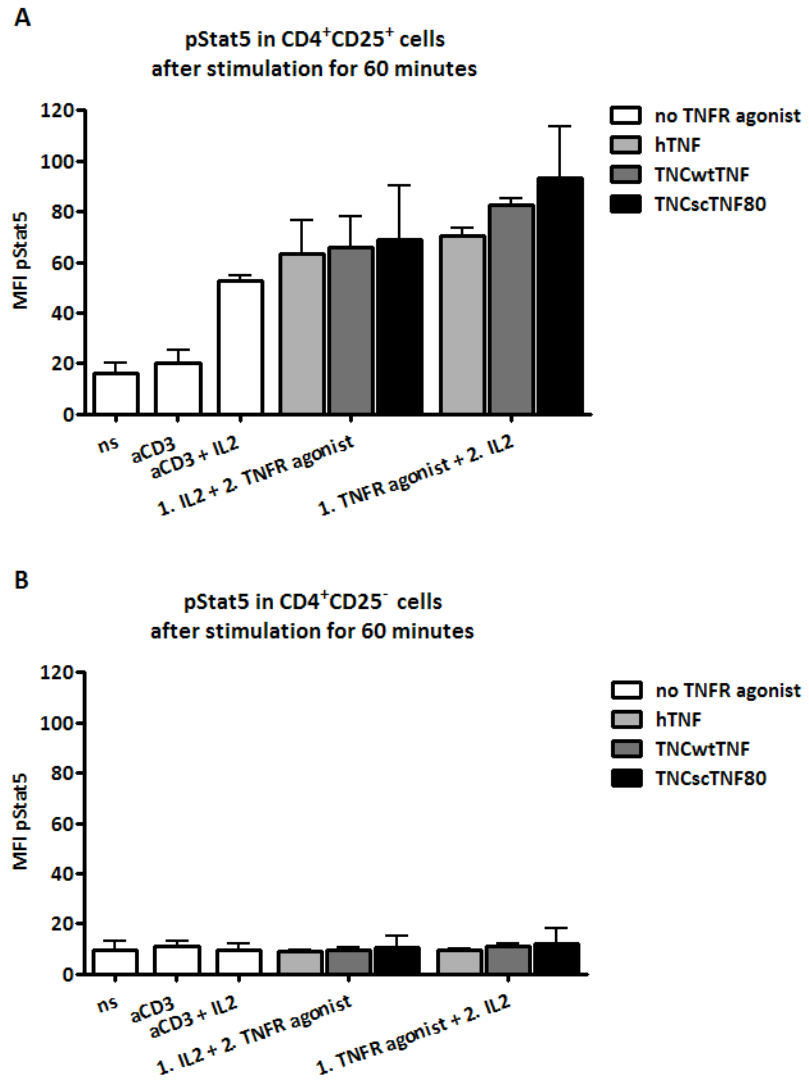

Figure 32: Expression of pStat5 in $CD4^+CD25^+$ (A) and $CD4^+CD25^-$ (B) cells after activation with different TNFR agonists for 60 minutes. All cells but the non-stimulated (ns) negative control were activated with anti-CD3ε antibody. Cells were additionally treated with different combination of 10 ng/ml IL-2 and 10 ng/ml TNFR agonists. In one approach cells were incubated with IL-2 for 60 min and a TNF agonist was added for the last 30 min of incubation; in another approach the cells were incubated with a TNFR agonist for 60 min and IL-2 was added for the last 30 min. Incubation with anti-CD3ε antibody alone or anti-CD3ε antibody and IL-2 served as positive controls. Shown is the MFI of pStat5 of $CD4^+CD25^+$ (A) and $CD4^+CD25^+$ (B) cells as the mean + SD of two technical replicates.

3.6.1.3 Expression of pStat5 after three hours

Next, the behaviour of pStat5 in $CD4^+CD25^-$ and $CD4^+CD25^+$ cells following incubation for three hours was analysed. The expression was generally low in non-stimulated $CD4^+CD25^-$ and $CD4^+CD25^+$ cells. Yet, $CD4^+CD25^+$ cells showed an enhanced expression of pStat5 following stimulation with anti-CD3ε alone or in combination with IL-2 for three hours (Figure 33A). However, there was only a slight increase in the pStat5 expression in

$CD4^+CD25^-$ cells after stimulation with anti-CD3ε for three hours. The level of pStat5 in $CD4^+CD25^-$ cells, which had been stimulated with both anti-CD3ε and IL-2, was even lower when compared to cells that only received anti-CD3ε stimulation (Figure 33B).

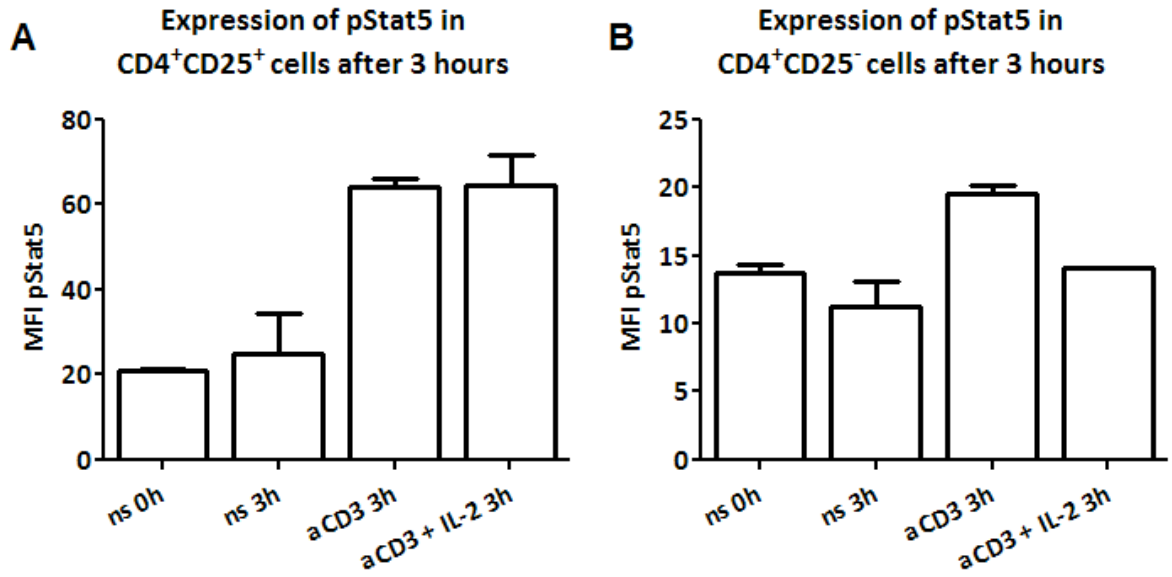

Figure 33: Expression of pStat5 in $CD4^+CD25^-$ (A) and $CD4^+CD25^+$ (B) cells after stimulation for three hours. Naïve splenocytes were activated with 0.5 µg/ml anti-CD3ε antibody alone or together with 10 ng/ml IL-2 for 3 hrs. Non-stimulated (ns) cells that were stained either directly or after 3 hrs in culture served as a negative control. Demonstrated is the mean + SD of two technical replicates. The values show the MFI for pStat5 of $CD4^+CD25^+$ (A) or $CD4^+CD25^-$ (B) cells.

3.6.1.4 Expression of CD25 in $CD4^+Foxp3^-$ cells after stimulation with anti-CD3ε antibody for three hours

In order to examine the expression of pStat5 in T_{reg}, a reliable marker was needed for the identification of T_{reg}. In the standardisation of the protocol for staining against intracellular pStat5, which is described in 3.5, the focus was put on CD25, the IL-2 receptor α-chain. However, the $CD4^+CD25^-$ cells, presumably T_{eff}, did not show any upregulation of pStat5 upon short-term stimulation, while the $CD4^+CD25^+$ strongly activated Stat5. CD25 is known to be upregulated on T cells upon stimulation of their TCR or IL-2R (72, 249). Thus, gating on $CD4^+CD25^+$ cells might identify activated effector cells rather than T_{reg}, even after short-term stimulation. Therefore, it was of interest if the $CD4^+CD25^+$ cells actually represent the T_{reg} population in those short-term studies. In the non-stimulated cells the proportion of $CD4^+CD25^+Foxp3^+$ T_{reg} out of all $CD4^+$ cells was 7.48 %, which was similar to the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} after stimulation with anti-CD3ε antibody for three hours (8.09 %). Yet, a high proportion of $CD4^+$ expressed CD25 but not Foxp3 following activation with anti-CD3ε for only three hours; 18.58 % of the stimulated $CD4^+$ cells were

$CD4^+CD25^+FoxP3^-$ vs. 2.87 % of the non-stimulated $CD4^+$ cells (Figure 34). Therefore, it could be shown that CD25 is upregulated on non-regulatory $CD4^+Foxp3^-$ cells even upon TCR stimulation for as short as three hours. Thus, CD25 could not be used as an exclusive marker to identify T_{reg} during short-term studies.

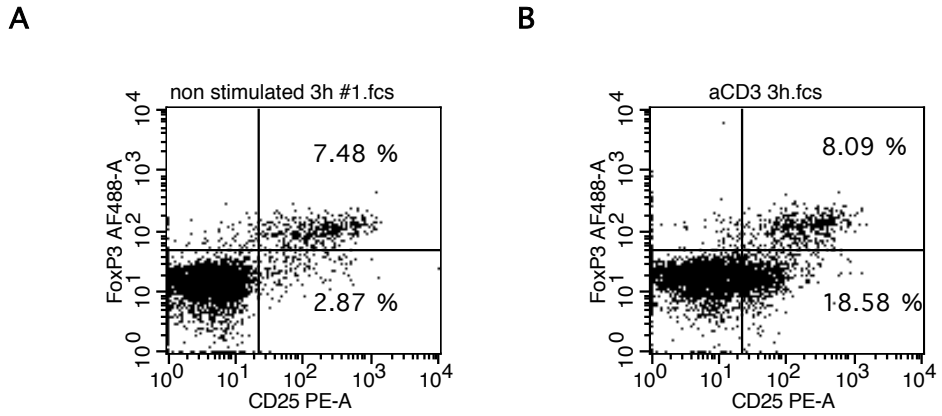

Figure 34: Expression of CD25 and Foxp3 in $CD4^+$ cells after stimulation for 3 hours. Splenocytes were cultivated either alone, which served as the non-stimulated control (A), or in the presence of 0.5 µg/ml anti-CD3ε antibody (B) for 3 hrs. The data is representative for a total of four repeats. The dot plots were gated on $CD4^+$ cells and the percentages of $CD25^+Foxp3^+$ (UR) and $CD25^+Foxp3^-$ (lower right, LR) cells were calculated.

3.6.2 Expression of pStat5 during long-term incubation

3.6.2.1 Expression of pStat5 in the $CD4^+$ cell population

In addition to the expression of pStat5 during short-term incubation, it was also planned to observe the development of pStat5 expression in T_{reg} over long-term incubation, as the inhibitory effect of TNCscTNF80 on the suppressive capacity of T_{reg} was demonstrated in suppression assays over 72 hours. Thus, TNFR2 signalling might abrogate T_{reg} function in the later phases of cell activation. Initially, the development of pStat5 expression in $CD4^+$ cells during incubation for 72 hours was assessed in order to determine the effect of long-term stimulation with anti-CD3ε on the activation of Stat5 in T cells. Figure 35 shows that there was a slight increase of pStat5 expression in $CD4^+$ cells after stimulation with the anti-CD3ε antibody three hours. Following stimulation for 24 hours, the expression of intracellular pStat5 reached its maximum and then started to decline after 48 hours. After 72 hours of stimulation, the expression in the activated cells was nearly as low as in the non-stimulated cells. Thus, pStat5 is only transiently upregulated in $CD4^+$ cells upon TCR stimulation.

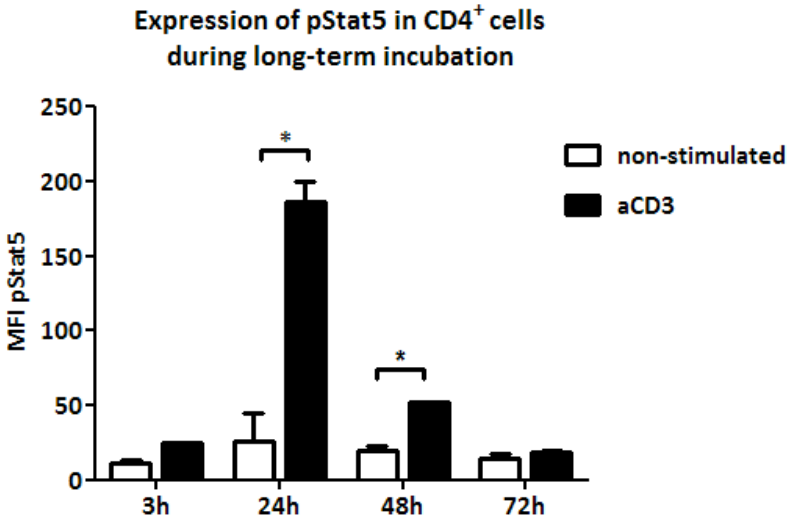

Figure 35: Expression of pStat5 in $CD4^+$ cells over 72 hours. Naïve splenocytes were cultivated without (white bars) or with 0.5 µg/ml anti-CD3ε antibody (black bars) for 72 hrs. The data demonstrates the mean fluorescence intensity of pStat5 of $CD4^+$ cells. Shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.6.2.2 Upregulation of CD25 on $CD4^+Foxp3^-$ cells after stimulation with anti-CD3ε antibody

$CD4^+$ lymphocytes showed maximal pStat5 expression after 24 hours of stimulation. Yet, the expression of pStat5 during long-term incubation might show different kinetics in T_{reg}. However, a reliable marker for T_{reg} was required in order to study cell-signalling events in T_{reg} cells during long-term incubation. As explained in 3.6.1.4, the expression of CD25 on $CD4^+Foxp3^-$ T_{eff} cells is already enhanced after short-term activation with anti-CD3ε antibody. Additionally, the expression of CD25 on non-stimulated and activated $CD4^+Foxp3^-$ T_{eff} cells was assessed during incubation for up to 72 hours. While CD25 expression remained at the same level on non-stimulated $CD4^+Foxp3^-$ cells during the whole course of incubation, it was increased on cells that had been activated with anti-CD3ε antibody. The majority of $CD4^+Foxp3^-$ cells were $CD25^+$ after stimulation for 24 and 48 hours (74.16 % and 85.05 % respectively). The increase of CD25 expression, however, was not as pronounced after stimulation for three and 72 hours (Figure 36). Therefore, it was shown that CD25 on its own does not a serve as a reliable marker for T_{reg} cells during long-term incubation.

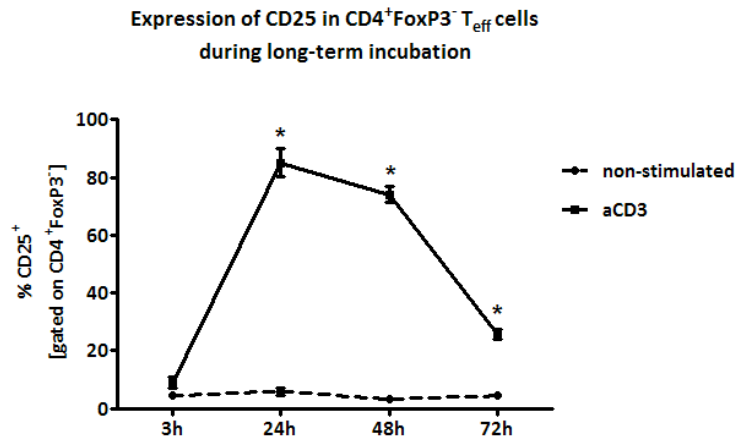

Figure 36: Expression of CD25 in $CD4^+Foxp3^-$ cells during long-term incubation. Naïve splenocytes were cultivated either alone (dotted line, circles) or in the presence of 0.5 µg/ml anti-CD3ε antibody (line, squares) for up to 72 hrs. The percentages of $CD25^+$ cells in the $CD4^+Foxp3^-$ population were determined. The data is a representative of three individual experiments, shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.6.2.3 Expression of pStat5 in $CD4^+Foxp3^+$ cells

As CD25 could not be used as a reliable marker for T_{reg}, Foxp3 was utilized to identify T_{regs} in the experiments regarding the long-term expression of pStat5. The level of pStat5 in $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells was compared after activation for up to 72 hours in the presence of TNCscTNF80 in order to determine if TNFR2 signalling has different effects on the activation of Stat5 in T_{reg} and T_{eff}. As demonstrated in Figure 37A, the expression of pStat5 was increased in the $CD4^+Foxp3^+$ cells after activation for three hours and was {Romio 2011 #402}at its maximum upon activation for 24 hours, both after stimulation with anti-CD3ε antibody alone or with anti-CD3ε antibody and TNCscTNF80 together. Then, following stimulation for 48 hours or more, the level of pStat5 declined rapidly. Interestingly, after incubation for 48 and 72 hours the expression of pStat5 in $CD4^+Foxp3^+$ cells was highest in the non-stimulated controls. Moreover, stimulation with anti-CD3ε and TNCscTNF80 for 48 and 72 hours resulted in a slightly lower expression of pStat5 when compared to stimulation with anti-CD3ε alone. Stimulation with hTNF for 24 hours reduced pStat5 expression in the $CD4^+Foxp3^+$ cells to a small extend, but did not alter pStat5 levels after 48 or 72 hours (data not shown). In the $CD4^+Foxp3^-$ cell subset expression of pStat5 was only observed after activation with anti-CD3ε antibody for 24 hours, both alone and in the presence of TNCscTNF80; TNCscTNF80 slightly enhanced pStat5 expression at this time point (Figure 37B). Taken together, it was shown that TNFR agonists did not considerably alter the expression of pStat5, neither in T_{reg} or T_{eff} cells.

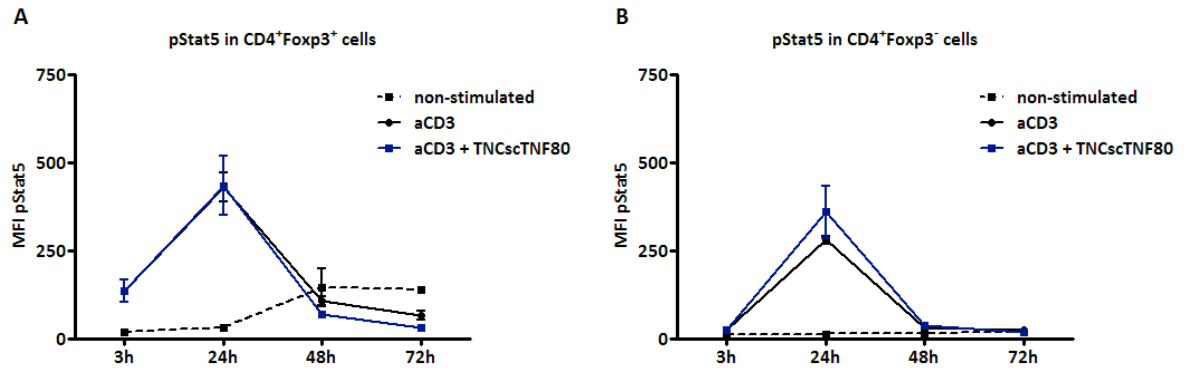

Figure 37: Expression of pStat5 in $CD4^+Foxp3^+$ (A) and $CD4^+Foxp3^-$ (B) cells during long-term incubation. Naïve splenocytes were cultivated for up to 72 hrs either in the absence of stimuli (ns; dotted line), with 0.5 µg/ml anti-CD3ε antibody (black line) or with 0.5 µg/ml anti-CD3ε antibody and 10 ng/ml TNCscTNF80 (blue line). The data originates from an experiment representing a total of three repetitions. The data is shown as the mean + SD of two technical replicates.

3.6.2.4 Expression of pStat5 in $CD4^+CD45.2^+$ T_{reg} cells

In a different approach the CD45.1/CD45.2 model was used to identify T_{reg} during the experiments on the effect of TNFR agonists on pStat5 expression. Therefore, splenocytes from mice with different isoforms of CD45, i.e. CD45.1 and CD45.2, were used for the suppression assays. $CD4^+CD25^+$ cells were purified from naïve splenocytes from CD45.2 mice by magnetic separation; subsequently staining against CD45.2 could identify T_{reg}. This method allowed studying the different effects of TNCscTNF80 on pStat5 expression in T_{eff} that were stimulated alone and in T_{reg} or T_{eff} that were co-cultured in suppression assays.

First, the percentages of $pStat5^+$ T_{eff} cells in cultures of $CD4^+CD45.2^-$ T_{eff} alone or in co-cultures with purified $CD4^+CD25^+$ T_{reg} cells from CD45.2 mice at ratios of 3:1 were analysed during long-term incubation. When cultured alone, the stimulation with anti-CD3ε alone and with anti-CD3ε and TNCscTNF80 together similarly enhanced the percentages of $pStat5^+$ in the T_{eff} population. The only difference was that treatment with TNCscTNF80 for 24 hours slightly increased the proportion of $pStat5^+$ cells when compared to stimulation with anti-CD3ε alone (Figure 38A). However, if $CD4^+CD25^+$ cells were present in the culture, the percentage of $pStat5^+$ cells in the $CD4^+CD45.2^-$ subset was enhanced by the stimulation with TNCscTNF80 for 24 hours, but reduced by TNCscTNF80 stimulation for 72 hours (Figure 38B).

Furthermore, the percentages of $pStat5^+$ cells in the $CD4^+CD45.2^+$ cell population, representing the purified $CD4^+CD25^+$ T_{reg}, were analysed. Figure 38C shows that the maximum of $pStat5^+$ expressing T_{reg} was seen following stimulation for 24 hours. Stimulation with additional TNCscTNF80 slightly increased the proportion of $pStat5^+$ cells after three and

24 hours. Yet, the proportion of pStat5$^+$ cells was reduced following TNCscTNF80 treatment for 48 and 72 hours. In addition, the MFI of pStat5 was used to analyse the activation of Stat5 in CD4$^+$CD45.2$^+$ T$_{reg}$ cells that had been in co-cultures with CD4$^+$CD45.2$^-$ cells in the presence of different TNFR agonists for 24 and 48 hours. As seen in Figure 38D, TNCwtTNF and TNCscTNF80 increased the level of pStat5 after 24 hours. Interestingly, the expression of pStat5 was significantly ($p<0.05$) reduced by TNCscTNF80 treatment for 48 hours, whereas the other TNFR agonist did not notably change the expression of pStat5 at that time point.

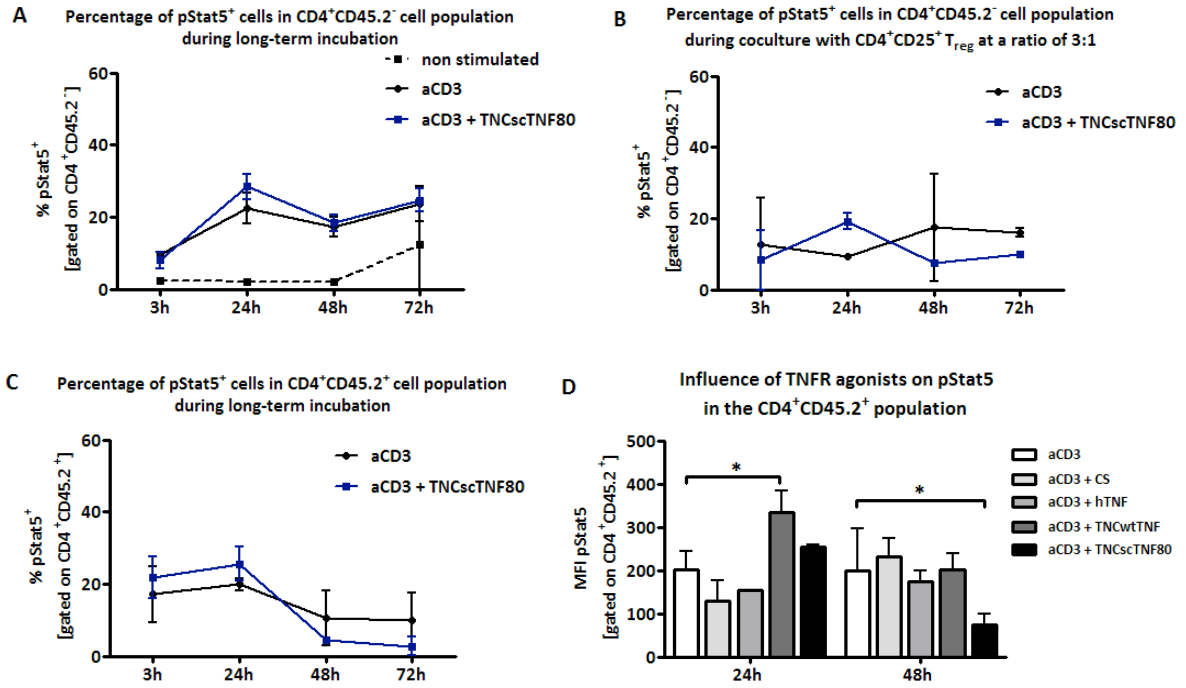

Figure 38: Expression of pStat5 in CD4$^+$CD45.2$^-$ T cells and CD4$^+$CD45.2$^+$ T$_{reg}$ cells. (A), (B) CFSE-labelled CD45.1 splenocytes were cultured alone (A) or with purified CD4$^+$CD25$^+$ cells from CD45.2 mice at ratios of 3:1 (B) for up to 72 hrs and stimulated with 0.5 µg/ml anti-CD3ε alone (black lines) or with 0.5 µg/ml anti-CD3ε and 10 ng/ml TNCscTNF80 (blue lines). Non-stimulated cells (dotted lines) served as a negative control. Shown are the percentages of pStat5$^+$ cells in the CD4$^+$CD45.2$^-$ subset as the mean + SD of two technical replicates. (C) The percentages of pStat5$^+$ were analysed in CD4$^+$CD45.2$^+$ cells that had been co-cultured with CD4$^+$CD45.2$^-$ at ratios of 1:3 in the presence of anti-CD3ε alone (black lines) or anti-CD3ε and TNCscTNF80 (blue lines) for up to 72 hrs. Shown is the mean + SD of two technical replicates. (D) The MFI of pStat5 was analysed in CD4$^+$CD45.2$^+$ cells that had been co-cultured with CD4$^+$CD45.2$^-$ cells at ratios of 1:3 for 24 and 48 hrs in the presence of anti-CD3ε alone or anti-CD3ε together with different TNFR2 agonists. Shown is the mean + SD of two technical replicates. * indicates a statistically significant difference of the indicated groups as determined by two-way ANOVA with Bonferroni post-test.

3.7 Expression of phosphorylated ZAP70

T_{reg} need to be stimulated via their TCR in order to gain suppressor function. However, once activated they suppress responder cells in an antigen non-specific manner (18, 55). It was shown in previous experiments of this work that TNFR agonists attenuate T_{reg} suppressor function. Because signalling events downstream of the IL-2R were only marginally changed in T_{reg} after TNCscTNF80 treatment, it was hypothesized that TNFR2 signalling might alter TCR-dependent signal induction. The phosphorylation status of the tyrosine kinase ZAP70 was chosen for the studies on the effects of TNCscTNF80 on TCR signalling, because it is one of the first mediators to be activated and it is required for activation of most downstream signalling cascades (49, 50).

3.7.1 Establishment of a method for stimulation of T cells leading to the expression of pZAP70

In order to study the effects of TNFR agonists on the rapid upregulation of phosphorylated ZAP70 in T_{reg}, it was necessary to establish a method, which allowed staining against both surface markers and intracellular molecules shortly after the activation of lymphocytes. First of all, an adequate activation method needed to be found. Therefore, splenocytes were stimulated with anti-CD3ε antibody for up to 60 minutes and stained against pZAP70 and various T_{reg} markers. Then, the expression of pZAP70 was analysed in both effector and regulatory T cells. Yet, although the anti-CD3ε antibody has been proven to be an appropriate stimulus for T cell (245) and T cells had been activated with the anti-CD3ε antibody in the former experiments, no relevant change of pZAP70 expression was detectable in stimulated cells. The expression of pZAP70 in $CD4^+CD25^+Foxp3^+$ cells was only slightly increased by stimulation with anti-CD3ε for 30 or 60 minutes, whereas stimulation for 10 minutes actually decreased the level of pZAP70 expression (Figure 39A). The $CD4^+CD25^-Foxp3^-$ T_{eff} did not show any change of the expression of pZAP70 at all (data not shown). Furthermore, stimulation with anti-CD3ε antibody and IL-2 together also failed to enhance the expression of pZAP70, both in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells (Figure 39B and C).

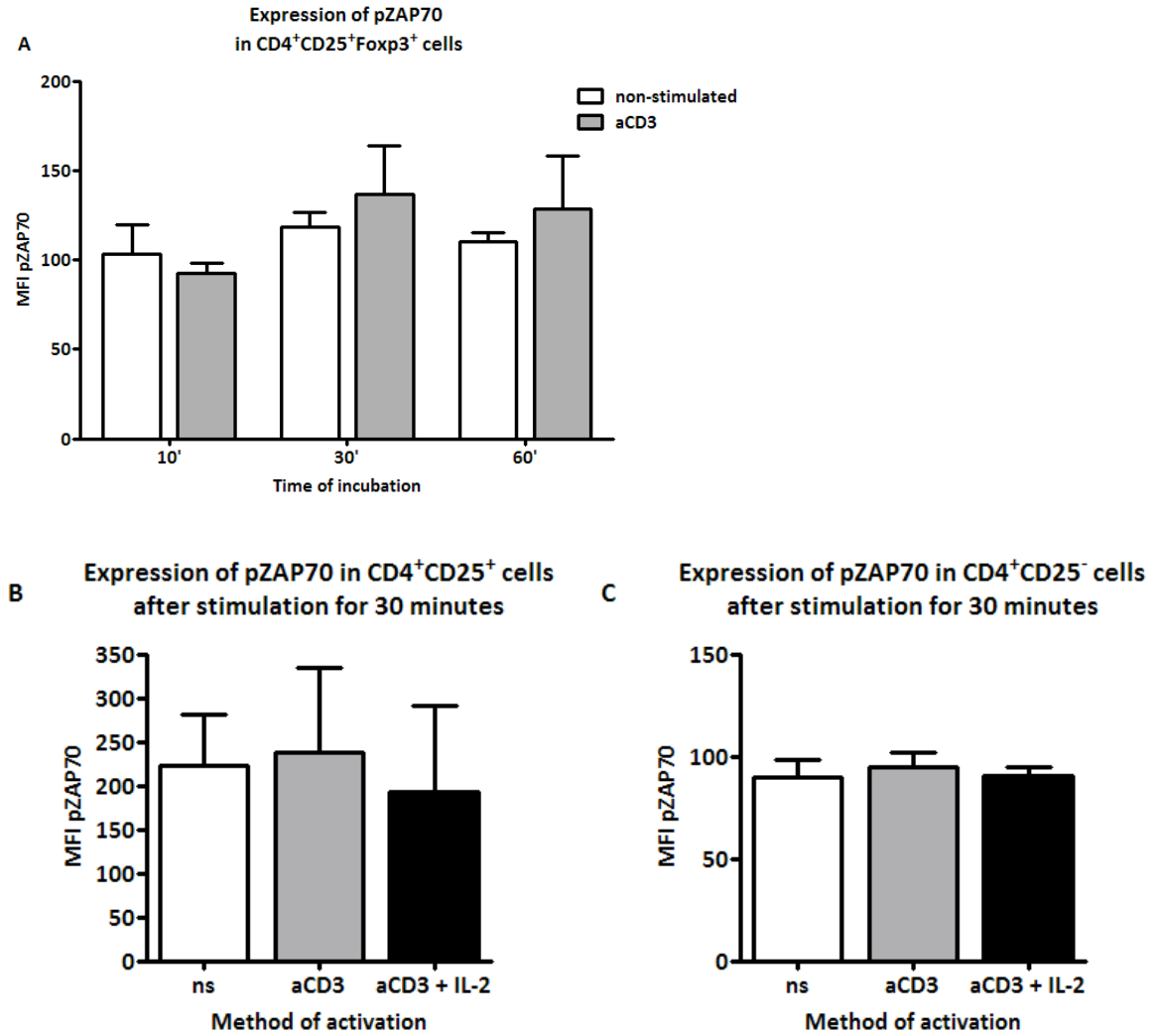

Figure 39: Expression of pZAP70 in T_{reg} and T_{eff} after short-time stimulation. (A) Naïve splenocytes were cultivated either in cell culture medium alone (white bars) or in the presence of 0.5 µg/ml anti-CD3ε antibody (grey bars) for 10, 30 and 60 min. Cells were gated on $CD4^+CD25^+Foxp3^+$ and the MFI of pZAP70 was determined. Shown is the mean + SD of two technical replicates. (B), (C) Naïve splenocytes were cultivated for 30 min either in the presence of 0.5 µg/ml anti-CD3ε alone (grey bars) or 0.5 µg/ml anti-CD3ε and 10 ng/ml IL-2 (black bars). Non-stimulated (ns) cells, which were cultivated only in cell culture medium (white bars), served as a negative control. The MFI of pZAP70 in $CD4^+CD25^+$ (B) and $CD4^+CD25^-$ (C) was determined. Shown is the mean + SD of two technical replicates.

As it was shown that incubation with anti-CD3ε antibody did not result in any activation of ZAP70 in $CD4^+$ cells, different stimulation techniques were performed: cross-linking of the anti-CD3ε antibody, activation with concanavalin A (Con A) and stimulation with H_2O_2. Cross-linking of the receptor or stimulation with the mitogen Con A are well-known methods to induce T cell activation and proliferation (246-249). For the anti-CD3ε cross-link, the lymphocytes were incubated with anti-CD3ε antibody, biotin-

labelled IgG and streptavidin. Stimulation with H_2O_2 was performed because in a previous report it had been demonstrated that H_2O_2 is a more potent inducer of ZAP70 phosphorylation than the anti-CD3ε antibody (250). Both cross-linking of the anti-CD3ε antibody (Figure 40B) and stimulation with Con A (Figure 40C) elevated the expression of pZAP70 in $CD4^+$ cells to a certain degree. H_2O_2, however, induced an obvious shift of pZAP70-positive cells in the $CD4^+$ cell population (Figure 40D). Interestingly, a relatively large fraction of $CD4^-$ cells also showed pZAP70 expression (Figure 40D). This is unexpected since ZAP70 is only expressed in T cells and NK cells, but not in B cells (251). Nevertheless the mAB used for staining against pZAP70 is not specific and also cross-reacts with phosphorylated Syk, which is widely expressed in B cells (252). Thus, a high proportion of $B220^+$ cells, 26.56 %, were also stained positive with this antibody (Figure 40E). Taken together, the best results for pZAP70 induction in T cells were achieved by applying the stimulation technique with H_2O_2. Thus, H_2O_2 was used for activation of lymphocytes in experiments on the expression of phosphorylated ZAP70 in regulatory and effector $CD4^+$ T cells throughout this work.

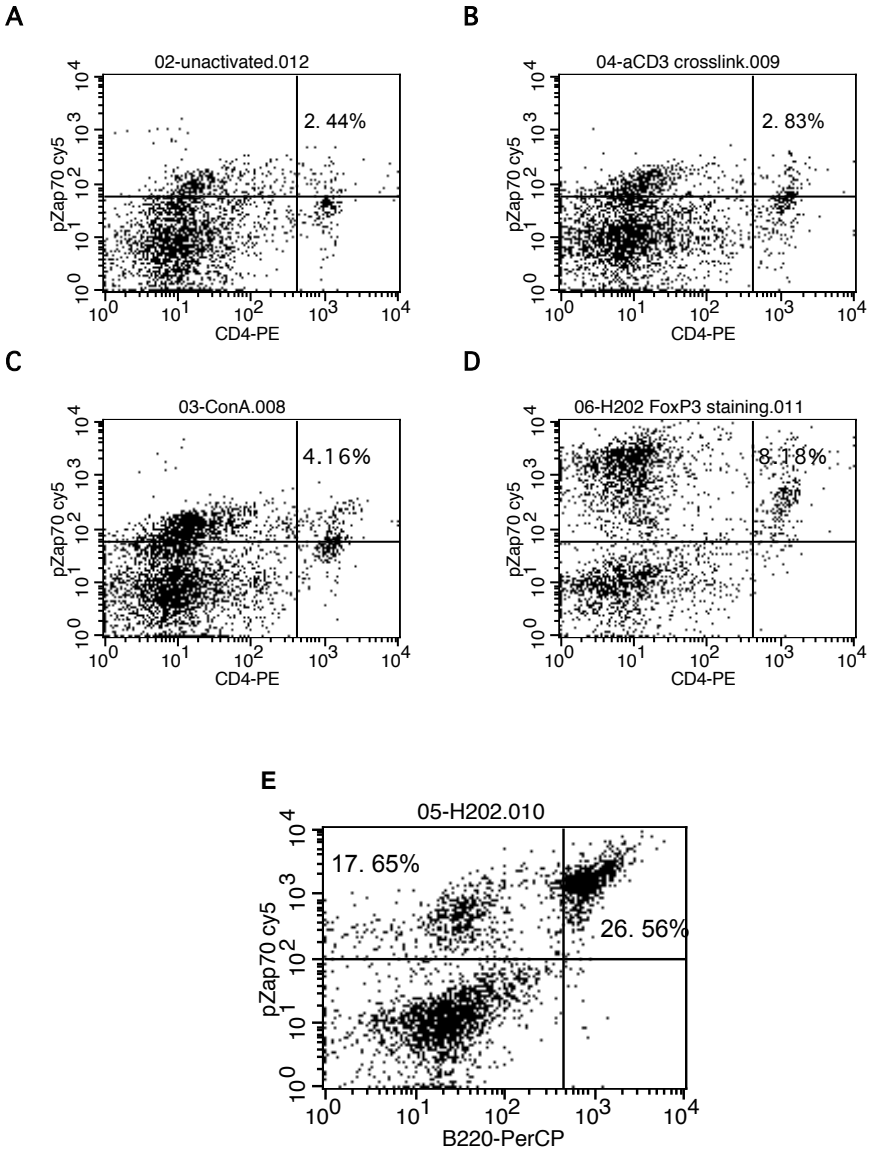

Figure 40: Comparison of the effect of different activation methods on pZAP70 expression. (A)-(D) pZAP70 expression in $CD4^+$ cells. Naïve splenocytes were incubated for 5 min with 1 µg/ml anti-CD3ε, 1 µg/ml biotin-labeled IgG and a 1:300 dilution of streptavidin (B), with 5 µg/ml Con A (C) or with 0.01 % H_2O_2 (D). Non-stimulated naïve splenocytes (A) served as a negative control. The percentages of $CD4^+pZap70^+$ cells (UR) were determined. (E) pZAP70 expression in $B220^+$ cells. Naïve splenocytes were incubated with 0.01 % H_2O_2 for 5 min. The percentages of $B220^+pZAP70^+$ cells (UR) and $B220^-pZAP70^+$ cells (upper left, UL) were determined.

Further experiments were conducted in order to optimize the activation process with H_2O_2. In a study by Haas *et al.*, 11 mM (0.035 %) H_2O_2 were used for stimulation (250). It was of interest, if the reducing or enhancing the concentration of H_2O_2 had an influence on the data quality and detectability of pZAP70. Thus, the pZAP70 expression was assessed following incubation with either 0.01 % or 0.05 % H_2O_2. Incubation for 15 minutes was chosen, because it had been shown that H_2O_2-induced pZAP70 expression reached its

maximum after 15 minutes (250). Additionally, the impact of diluting the primary antibody against pZAP70 was examined. To this end, the cells were stained with anti-pZAP70 in a 100-fold or 50-fold dilution. As seen in Figure 41, H_2O_2 enhanced the expression of pZAP70 in $CD4^+$ cells in comparison to the non-stimulated controls. If 0.01 % H_2O_2 were used, the induction of pZAP70 was stronger. This was noticeable for both antibody dilutions. However, if the primary antibody was used in 50-fold dilution (Figure 41B), phosphorylated ZAP70 was detectable to a stronger extend than by applying a 100-fold dilution (Figure 41A). Taken together, optimal detection of pZAP70 in $CD4^+$ lymphocytes was achieved by stimulating the cells with 0.01 % H_2O_2 and subsequently staining them with a 50-fold dilution of the primary antibody against pZAP70.

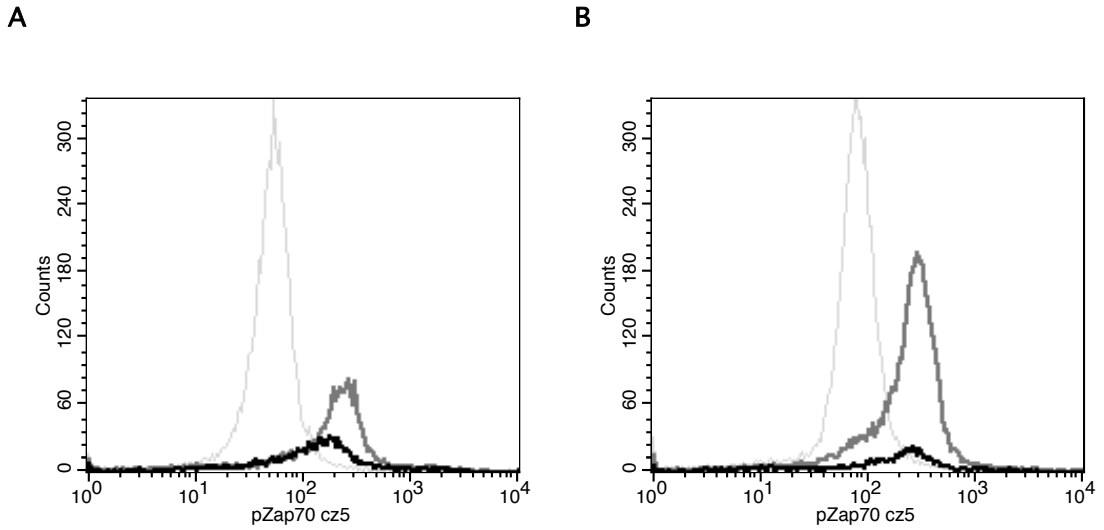

Figure 41: Effect of H_2O_2 on the expression of pZAP70 in $CD4^+$ cells. Naïve splenocytes were activated with 0.01 % H_2O_2 (grey lines) and 0.05 % H_2O_2 (black lines) for 15 minutes. Non-stimulated cells (light grey lines) served as a negative control. Cells were then stained with a 1:100 (A) or a 1:50 (B) dilution of the mAB against pZAP70. The plots are gated on $CD4^+$ cells and are representative for one of two technical replicates.

Haas *et al.* have also shown that the maximal pZAP70 expression following anti-CD3ε stimulation was detected as early as 30 seconds after activation when using the phospho-flow technique (250). As demonstrated above, no pZAP70 expression was detectable after stimulation with 0.5 µg/ml anti-CD3ε antibody for 10, 30 or 60 minutes. Thus, it was of interest if pZAP70 is upregulated at earlier time points following the anti-CD3ε-induced cell activation. Moreover, it was examined if higher anti-CD3ε antibody concentrations were able to induce pZAP70 upregulation. For this purpose, the level of pZAP70 was compared between lymphocytes that were activated with 5 µg/ml anti-CD3ε antibody or 0.01 % H_2O_2 for either 30 seconds or five minutes. As shown in Figure 42, stimulation with H_2O_2 enhanced

the expression of pZAP70 in $CD4^+$ cells, both after incubation for 30 seconds (Figure 42A) and for five minutes (Figure 42B). Yet, pZAP70 induction was slightly higher following stimulation for 30 seconds. Stimulation with anti-CD3ε antibody for 30 seconds or five minutes actually reduced pZAP70 expression when compared to the non-stimulated controls.

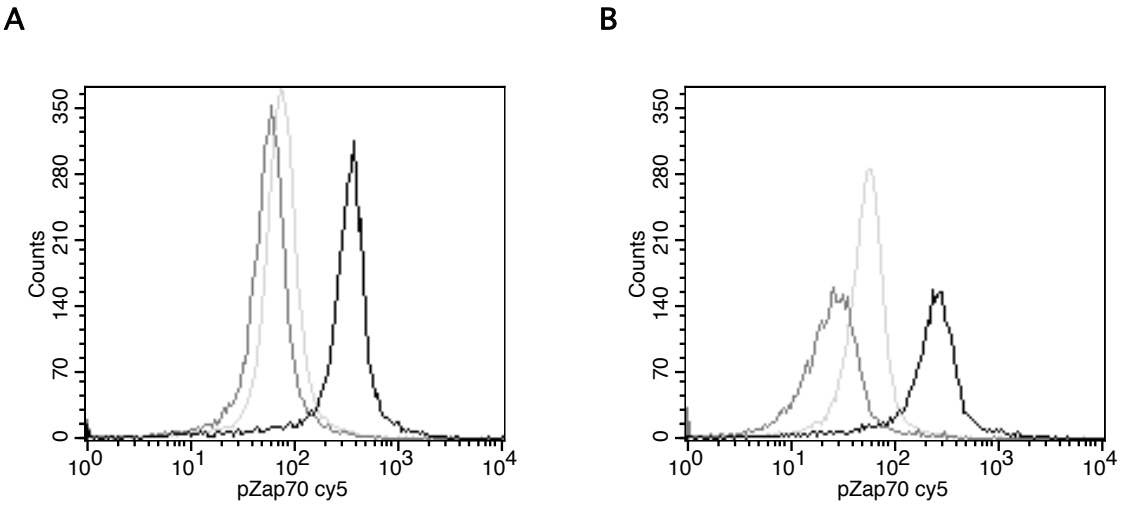

Figure 42: Difference in the expression of pZAP70 in $CD4^+$ cells after activation with anti-CD3ε antibody or H_2O_2. Naïve splenocytes were incubated with anti-CD3ε antibody (grey lines) or H_2O_2 (black lines) for 30 s (A) and 5 min (B); non-stimulated cells (light grey lines) served as a negative control. The plots are gated on $CD4^+$ cells.

3.7.2 Analysis of the effect of TNCscTNF80 on the expression of pZAP70

The activation process and staining protocol for pZAP70 in T cells could be optimized in the preliminary experiments. Thus, it was possible to study the effect of TNCscTNF80 on the phosphorylation of ZAP70 in both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells. In the following experiments, H_2O_2-stimulated splenocytes were treated with TNCscTNF80, fixed and permeabilized as described before for the detection of pStat5 (refer to 3.5.2) and then stained against pZAP70, CD4 and Foxp3. As demonstrated in Figure 43, the expression of pZAP70 was enhanced if cells were stimulated with H_2O_2 and TNCscTNF80 simultaneously when compared to the stimulation with H_2O_2 alone. This effect could be observed in $CD4^+Foxp3^-$ (Figure 43A) and $CD4^+Foxp3^+$ (Figure 43B) cells. However, in the following experiments those results could not be repeated (Figure 44). Moreover, if cells were incubated with TNCscTNF80 before the stimulation with H_2O_2 was started, no strong alteration of pZAP70 expression was detected (Figure 43A and Figure 43B). Taken together, the experiments on the effect of TNCscTNF80 on pZAP70 induction in T cells showed conflicting results. Thus, it remained unclear if TNCscTNF80 has an influence on pZAP70 expression in T_{reg} and T_{eff}.

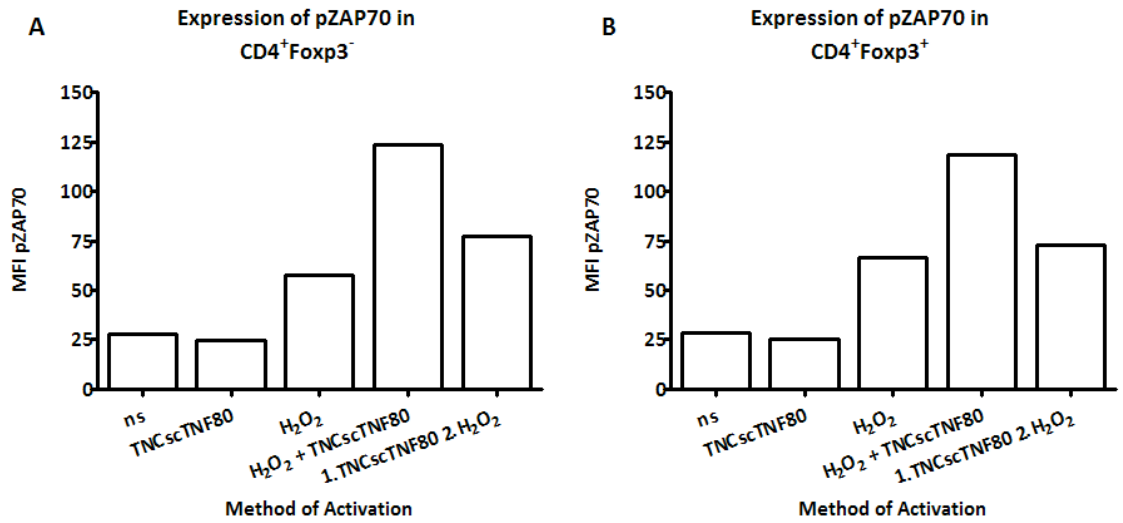

Figure 43: Impact of TNCscTNF80 on the expression of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells. Naïve splenocytes were activated with 0.01 % H_2O_2 alone or in the presence of 10 ng/ml TNCscTNF80. Part of the cells received H_2O_2 at the same time as TNCscTNF80, whereas the other part was incubated with TNCscTNF80 for 15 min prior to the stimulation with H_2O_2. Non-stimulated (ns) cells and cells that only received TNCscTNF80 were used as controls. Shown is the MFI of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells.

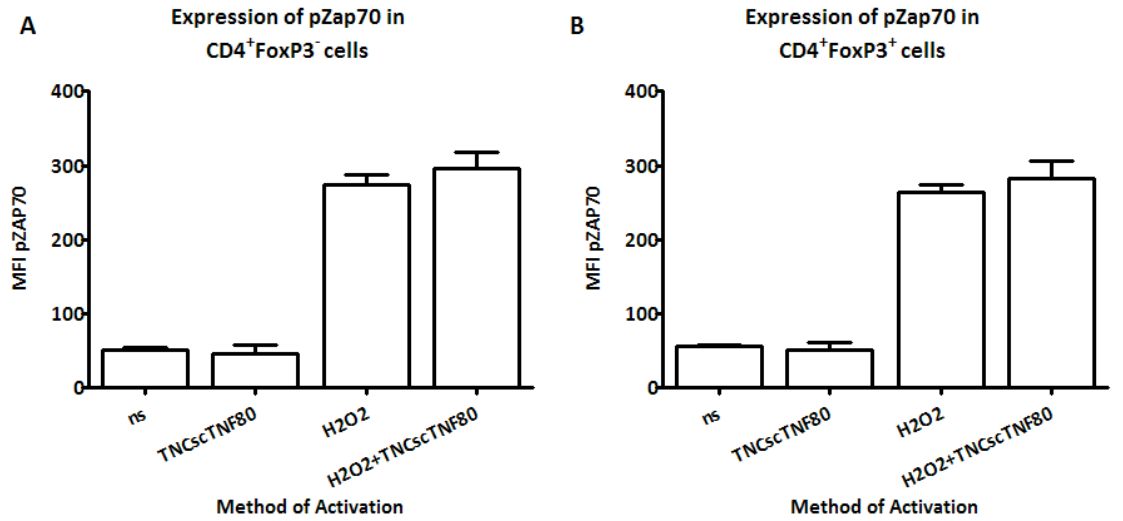

Figure 44: Impact of TNCscTNF80 on the expression of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells. Naïve splenocytes were activated with 0.01 % H_2O_2 alone or in the presence of 10 ng/ml TNCscTNF80. Non-stimulated (ns) cells and cells that only received TNCscTNF80 were used as controls. The MFI of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells was determined. Shown is the mean + SD of three technical replicates.

3.7.3 Serial dilution of TNCscTNF80

As diverging results were found for the impact of TNCscTNF80 on pZAP70 expression in $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells, it was of interest if changing the

concentration of TNCscTNF80 had an effect on pZAP70 expression. Thus, splenocytes were treated with increasing concentration of TNCscTNF80, ranging from 0.1 ng/ml to 10 ng/ml. However, no increased pZAP70 expression in $CD4^+Foxp3^-$ (Figure 45A) or $CD4^+Foxp3^+$ (Figure 45B) could be shown following TNCscTNF80 treatment. Even if 10 ng/ml TNCscTNF80, the concentration used in the suppression assays, were added to the cell cultures, no obvious change of pZAP70 expression was noticeable. Treatment with 0.1 ng/ml and especially 1 ng/ml TNCscTNF80 actually decreased pZAP70 levels in both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells. Nevertheless, the MFI values showed a relatively high standard deviation for those concentrations. If the percentage of $CD4^+Foxp3^-$ or $CD4^+Foxp3^+$ being positive for pZAP70 were compared, no differences could be shown between stimulation with H_2O_2 alone or with H_2O_2 and any given concentration of TNCscTNF80 (data not shown).

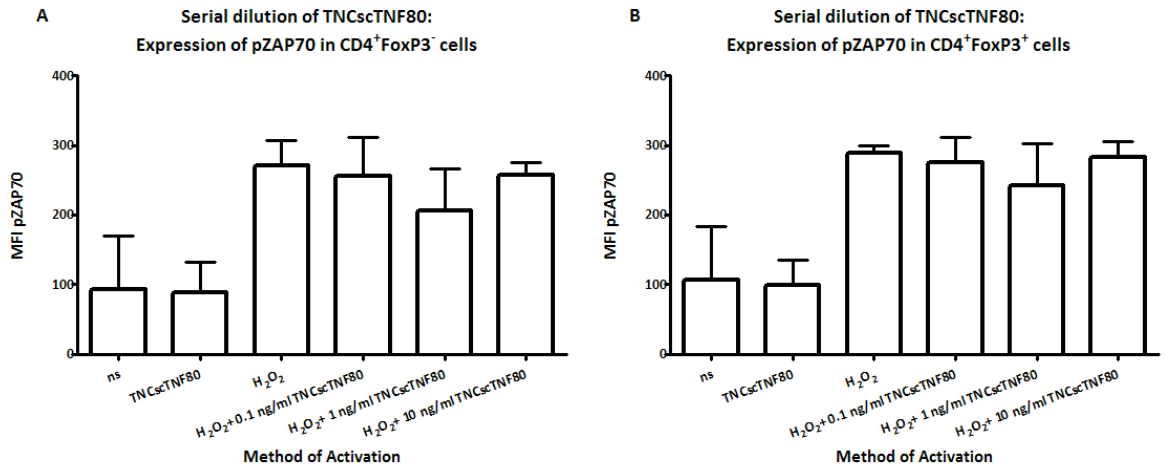

Figure 45: Impact of the concentration of TNCscTNF80 on the expression of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells. Naïve splenocytes were activated with 0.01 % H_2O_2 alone or in the presence of increasing concentrations of TNCscTNF80 for 5 min. Non-stimulated (ns) cells and cells that only received 10 ng/ml TNCscTNF80 were used as controls. The MFI of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells was determined. The data is representative for two individual experiments. Shown is the mean + SD of two technical replicates.

3.7.4 Time series of pZAP70 expression

Further, the expression of pZAP70 in T_{eff} and T_{reg} cells following stimulation for up to 60 minutes was analysed. Over the time there was a reduction in the expression of pZAP70 in $CD4^+Foxp3^-$ (Figure 46A) and $CD4^+Foxp3^+$ (Figure 46B) cells. After activation for five minutes, both cell populations showed the highest level of pZAP70. The expression steadily decreased until, after 60 minutes, it was nearly as low as in the non-stimulated control. In both cell populations there was no considerable difference in between stimulation with or without TNCscTNF80. Taken together, the present data shows that TNCscTNF80 does not influence

the expression of pZAP70 in $CD4^+Foxp3^-$ T_{eff} or $CD4^+Foxp3^+$ T_{reg} cells during the early H_2O_2-induced T cell activation.

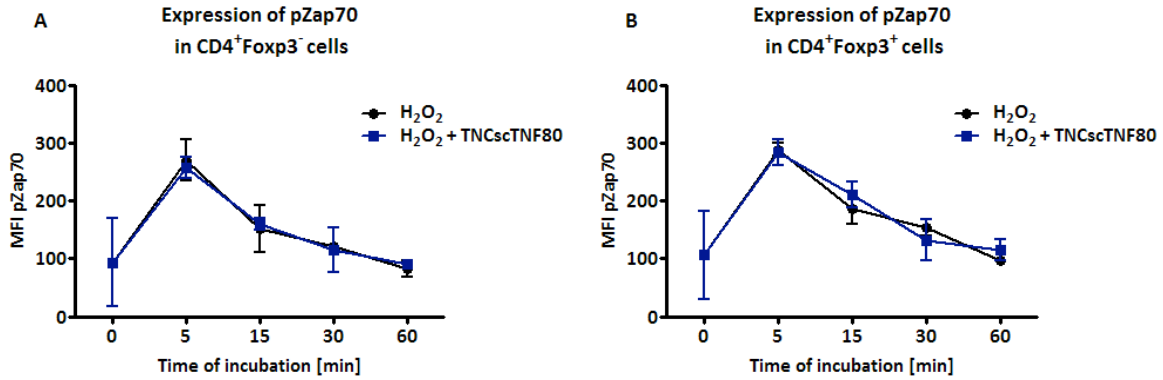

Figure 46: Kinetics of the pZAP70 expression in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells following stimulation with H_2O_2 or H_2O_2 and TNCscTNF80. Naïve splenocytes were incubated with 0.01 % H_2O_2 (circles, black lines) or with 0.01 % H_2O_2 and 10 ng/ml TNCscTNF80 (squares, blue lines) for 5 up to 60 min. Staining against pZAP70 in non-stimulated cells served as a control for the initial pZAP70 expression. The MFI of pZAP70 in $CD4^+Foxp3^-$ (A) and $CD4^+Foxp3^+$ (B) cells was determined. The data is representative for two independent experiments, shown is the mean + SD of two technical replicates.

3.8 Suppression of lymphocytes by myeloid derived suppressor cells (MDSC)

Myeloid-derived suppressor cells are a heterogeneous subset of immature myeloid cells that expand under pathological conditions, especially cancer (37). Previous studies had shown that myeloid cells have the capacity to suppress T cell proliferation in response to CD3/CD28 co-signalling (253). This MDSC-mediated suppression of immune responses is enhanced in tumor-bearing organisms and appears to play an essential part in the immune escape of tumor cells (40). It has been observed that TNF has enhancing effects on the survival and accumulation of MDSC in tumor-bearing mice (224) and that suppressive function of MDSC is attenuated when signalling via TNF receptor, and more specifically via TNFR2, on MDSC is abrogated (225, 226, 254). Thus, TNF appears to promote MDSC suppressor function. In the following experiments, it was studied how MDSC affect T cell proliferation and to what extend this is changed through signalling via TNFR2.

3.8.1 Alteration of ratios of splenocytes and MDCS

While MDSC mainly evolve under pathologic conditions *in vivo* (37), *in vitro* generation of MDSC is achieved by cultivation in the presence of GM-CSF. It has been observed that bone marrow derived cells acquired suppressive function after cultivation for four days with high doses of GM-CSF or cultivation for eight to ten days with low-dose

GM-CSF (42). In this work, it was of interest if bone marrow derived cells, which were cultured with GM-CSF for four days, acquired the ability to suppress the anti-CD3-induced proliferation of T cells. Furthermore, it was assessed at what ratio of MDSC to splenocytes the suppressor capacity was the strongest. As shown in Figure 47, the proliferation of $CD4^+$ and $CD8^+$ cells was readily suppressed when MDSC at ratios of 1:4 and higher were present in the culture. While 75.06 % of $CD4^+$ and 95.73 % of $CD8^+$ proliferated after stimulation with anti-CD3 for 72 hours, the fraction of proliferating cells was reduced to 1.36 % of $CD4^+$ (Figure 47A) and 12.92 % of $CD8^+$ (Figure 47B) cells respectively in the presence of MDSC at ratios of 1:1. Taken together, it could be shown that GM-CSF-generated bone marrow cells are capable of suppressing the anti-CD3-induced proliferation of T cells.

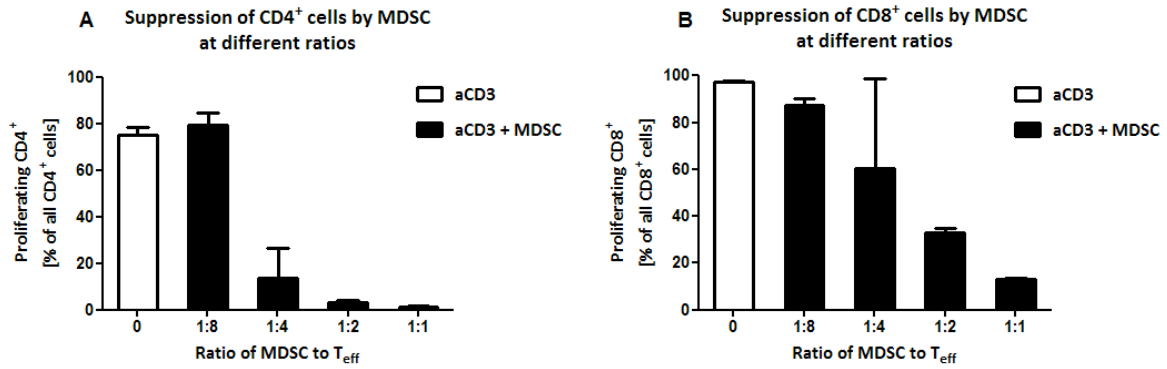

Figure 47: Suppression of $CD4^+$ (A) and $CD8^+$ (B) cells by MDSC at different ratios. 2×10^5 CFSE-labeled splenocytes were stimulated for 72 hrs with 1 µg/ml anti-CD3ε antibody in the presence of MDSC. The MDSC were added at ratios of 1:8, 1:4, 1:2 or 1:1 (black bars). Lymphocytes only stimulated with anti-CD3ε antibody (white bars) served as a positive control. Shown is the mean + SD of two technical replicates.

3.8.2 Suppression of T cells by MDSC generated with TNCscTNF80

The aim of the following studies was to examine to what extend MDSC are able to suppress T cell proliferation if they had been generated with the TNFR2 agonist TNCscTNF80. As shown in Figure 48, the proliferation of $CD4^+$ and $CD8^+$ cells after 72 hours in culture was decreased by the MDSC in a concentration-dependent manner. Interestingly, low ratios of TNCscTNF80-treated MDSC (1:8 and 1:4) enhanced the T cell proliferation when compared to the proliferation in the absence of MDSC. Yet, the proliferation of both $CD4^+$ (Figure 48A) and $CD8^+$ (Figure 48B) cells was reduced in the presence of the pre-treated MDSC at ratios of 1:2 and 1:1. Thus, it could be demonstrated that MDSC are able to exert their suppressive function if they have been generated under the influence of the TNFR2 agonist TNCscTNF80.

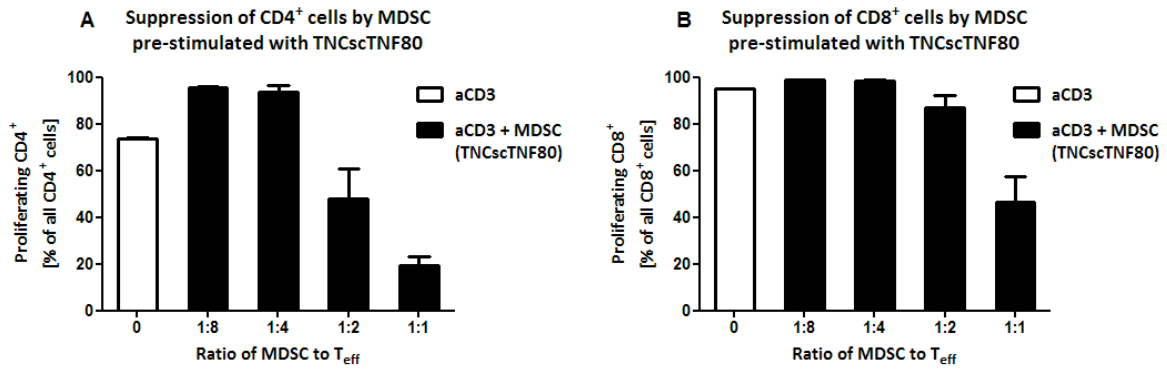

Figure 48: Suppression of $CD4^+$ (A) and $CD8^+$ (B) cells by MDSC after pre-incubation with TNCscTNF80. 2 x 10^5 CFSE-labeled splenocytes were stimulated for 72 hrs with 1 µg/ml anti-CD3ε antibody in the presence of MDSC, which had been in culture with GM-CSF and TNCscTNF80 for four days. The MDSC were added at ratios of 1:8, 1:4, 1:2 or 1:1 (black bars). Lymphocytes stimulated with anti-CD3ε antibody alone (white bars) served as a positive control. Shown is the mean + SD of two technical replicates.

3.8.3 Alteration of the suppressive capacity of MDSCs by TNCscTNF80

After having shown that BMDC acquire suppressive function following cultivation for four days with GM-CSF alone or with GM-CSF and the TNFR2-specific agonist TNCscTNF80, the influence of TNCscTNF80 on the suppressor capacity was examined. Therefore, the MDSC-mediated suppression following generation with or without TNCscTNF80 was compared. In addition, the suppression mediated by WT and $TNFR2^{-/-}$ MDSC was compared. Moreover, it was also assessed if the length of cultivation under GM-CSF had an impact on the suppressor capacity of MDSC. Since MDSC can differentiate into mature cells without suppressive activity (42), prolonged periods of cultivation with GM-CSF might cause a reduction of total suppressor cell numbers and thus of T cell suppression.

The suppression of both $CD4^+$ and $CD8^+$ cells by MDSC was most effective when WT MDSC had been cultivated with GM-CSF for four days prior to the suppression assay. WT MDSC, which had been stimulated for six or more days, were less suppressive. After generation with both GM-CSF and TNCscTNF80 for four days, the suppression of $CD4^+$ cells (Figure 49A) and $CD8^+$ cells (Figure 49B) was slightly alleviated when compared to generation with GM-CSF alone. 6.16% of $CD4^+$ cells proliferated when co-cultured with WT MDSC that had been generated only with GM-CSF vs. 8.09 % of the $CD4^+$ cells in co-cultures with WT MDSC that had been generated with additional TNCscTNF80. The proliferation of $CD8^+$ cells was 46.85 % vs. 52.84 % respectively. This TNCscTNF80-induced attenuation of the suppression mediated by the WT MDSC was more pronounced after cultivation for six or more days. In the presence of $TNFR2^{-/-}$ MDSC, the

proliferation of $CD4^+$ and $CD8^+$ cells was higher when compared to the co-cultures with WT MDSC (Figure 49), indicating that TNFR2-deficient MDSC are less suppressive than their WT counterparts. TNCscTNF80 did not noticeably affect the suppressive capacity of $TNFR2^{-/-}$ MDSC (data not shown). Thus, it was shown that bone marrow derived cells from WT mice were able to suppress T cell proliferation following stimulation with GM-CSF for four days, while the suppressor capacity was attenuated by prolonged periods of cultivation. Generation in the presence of the TNFR2-specific agonist TNCscTNF80 inhibited MDSC-induced suppression. This indicates that signalling via TNFR2 on MDSC limits their suppressive function. On the other hand, TNFR2-deficient MDSC showed reduced suppressor capacity, suggesting that TNFR2 signalling is important for MDSC-mediated suppression.

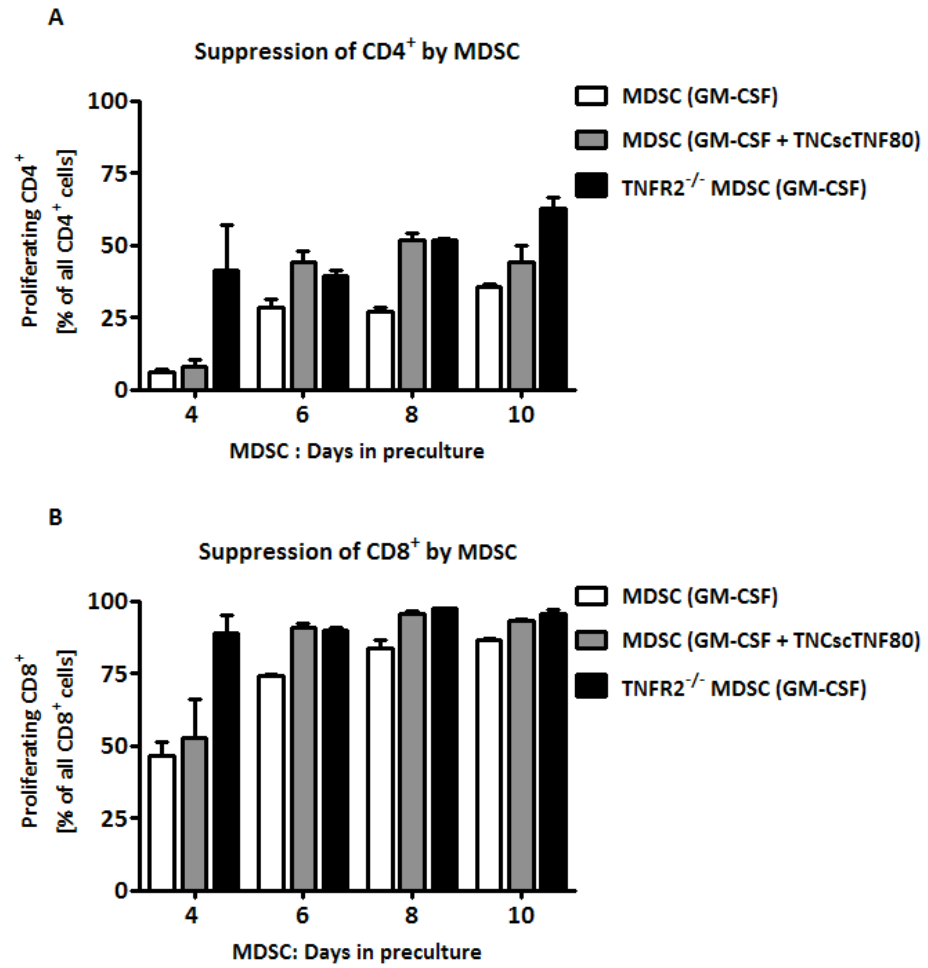

Figure 49: TNCscTNF80 alters the suppression of $CD4^+$ (A) and $CD8^+$ (B) cells by MDSCs. CFSE-labeled splenocytes were cultivated with 1 µg/ml anti-CD3ε antibody and 0.5 µg/ml anti-CD28 antibody for 72 hrs in the presence of MDSC from WT mice at a ratio of 1:1, which were generated with GM-CSF alone (white bars) or with additional TNCscTNF80 (grey bars) for four up to ten days, or in the presence of MDSC from $TNFR2^{-/-}$ mice that were generated with GM-CSF alone for four up to ten days (black bars). Shown is the mean + SD of two technical replicates.

4 Discussion

4.1 Effect of TNCscTNF80 on T cell proliferation and cytokine production

Former studies have revealed that TNF, through signalling via the TNFR2, acts as a co-stimulatory factor for lymphocytes, promoting their survival and proliferation during the TCR-induced T cell activation (181, 183). Correspondingly, it was observed in this work that the TNFR2-specific agonist TNCscTNF80 increased the proliferation of $CD4^+$ and $CD8^+$ cells in response to stimulation with an anti-CD3ε antibody. Further, it was shown that this enhancing effect was exclusively mediated through the TNFR2, since TNFR2-deficient T cells failed to respond to TNCscTNF80 treatment.

When lymphocytes were stimulated with high amounts of anti-CD3ε antibody and TNCscTNF80, the proliferation rate reached a plateau; further enhancing the concentration of anti-CD3ε did not increase the proliferation any more. It had been reported that co-stimulation via TNFR2 decreases the threshold for antigen-driven induction of cell activation (180, 255). Thus, cells might easily receive the required signals for full activation in the presence of high amounts of both TCR and TNFR2 stimulating agents; increasing the amount of anti-CD3ε will not further enhance the proliferative response in that case. Nevertheless, it could be shown in this work that the addition of increasing amounts of TNCscTNF80 still enhanced proliferation in a dose-dependent manner, even when cells were stimulated with high doses of anti-CD3ε. Thus, TNFR2 appears to mediate co-stimulatory signals even if T cells are optimally activated through their TCR. Moreover, it could be shown that the proliferation of TNFR2-deficient T cells in response to TCR stimulation was lower when compared to their WT counterparts. This provides further proof for the importance of TNFR2 as a co-stimulatory receptor during TCR-driven immune responses.

Treatment with TNCscTNF80 did not only promote T cell proliferation, but also enhanced secretion of the proinflammatory cytokine IFN-γ in a dose-dependent manner. However, while the co-stimulatory effects of TNFR2 on cell proliferation were observable at all levels of anti-CD3ε stimulation, effects on IFN-γ secretion were only detectable when higher amounts of anti-CD3ε antibody were used for TCR stimulation. Conversely, increasing the amount of anti-CD3ε antibody in the presence of TNCscTNF80 promoted further production of IFN-γ. In line with the TNCscTNF80-induced effects on T cell proliferation, TNCscTNF80 also increased IFN-γ production in a dose-dependent manner in the presence of high concentrations of anti-CD3ε. This indicates that TNFR2 mediates strong co-stimulatory

signals for IFN-γ production. This is consistent with previous observations showing that T cells deficient for TNFR2 release considerably less IFN-γ (255) and that stimulation of TNFR2 enhanced IFN-γ production (181). Nevertheless, the combined TNFR1 and TNFR2 agonist TNCwtTNF enhanced the secretion of IFN-γ to a higher extend than TNCscTNF80. This effect was observed after T cell stimulation for 48 and 72 hours. This suggests that TNFR1 signalling additionally promotes IFN-γ production. However, it has been shown that the production of IFN-γ upon CD3/CD28 stimulation *in vitro* is increased in TNFR1-deficient mice, indicating that TNFR1 signalling actually leads to a reduction of IFN-γ secretion (256). *In vivo* studies, on the other hand, have demonstrated that TNFR1-deficient mice show an impaired $CD8^+$ expansion and IFN-γ production upon allogeneic stimulation, which suggests that TNFR1 might have an important role in T cell expansion and production of proinflammatory cytokines (256). Taken together, it could be shown that TNFR2 promotes proliferation and cytokine production of lymphocytes. However, the role TNFR1 plays as a co-stimulatory receptor for T cell proliferation and cytokine production remains to be elucidated.

4.2 Change of $CD4^+CD25^+$ suppressor capacity by TNFR2-specific agonist TNCscTNF80

Regulatory T cells are crucial for the maintenance of self-tolerance (18). Conversely, T_{reg} dysfunction has been associated with different immunological disorders (205, 206, 218, 257). A variety of those autoimmune diseases, such as rheumatoid arthritis, psoriatic arthritis, Crohn's disease and ulcerative colitis are nowadays treated with anti-TNF agents (193, 195). It could be demonstrated that, in addition to various other effects, neutralization of TNF restores T_{reg} numbers and function in rheumatic patients and thus promotes control over activated self-reactive immune cells (205). Similar results were shown for IBD (207–209) and autoinflammatory diabetes mellitus type 1 (236, 257). Nevertheless, some patients do not respond to anti-TNF therapy or even show an onset of new inflammatory processes (194, 199, 209, 243, 258). Thus, there is great interest in how exactly TNF influences T_{reg} function.

In this study, it was found that the TNFR2 agonist TNCscTNF80 has an inhibitory effect on T_{reg}-mediated suppression of $CD4^+$ and $CD8^+$ cell proliferation. TNCscTNF80 enhanced the proliferation of anti-CD3ε-activated T cells under T_{reg}-mediated suppression to levels that were comparable to the proliferation levels found in the absence of T_{reg}. Moreover, TNCscTNF80 failed to attenuate the suppressor capacity of TNFR2-deficient T_{reg}. This

indicates that the inhibition of T_{reg}-induced suppression by TNCscTNF80 is exclusively mediated via TNFR2.

Previous studies have also shown that TNF impairs T_{reg}-mediated suppression and that neutralizing TNF can restore their suppressor capacity (206, 211). Further, it has been reported that TNF induces inhibition of T_{reg} suppressor function through signalling via TNFR2, which consecutively leads to an enhancement of NF-κB activation (210). However, Chen *et al.* reported that, although the T_{reg}-driven suppression of T_{eff} was partially abrogated after incubation with TNF for 48 hours, it was restored after exposure to TNF for 72 hours (216). Differences in the experimental set-up might account for the contradictory effects of TNF on T_{reg} suppression seen in studies by Chen *et al.* and in this work. While TNF was used in a concentration 10 ng/ml in their suppression assays, 10 ng/ml of TNCscTNF80 were utilized in this study. TNF is supposed to influence T_{reg} function only via TNFR2 (210, 217, 218, 222). Yet, soluble TNF has only limited ability to stimulate TNFR2 (175), whereas the TNFR2-selective TNCscTNF80 mimics the membrane-bound form of TNF and thereby activates TNFR2 more efficiently (228). Therefore, TNCscTNF80 will mediate stronger effects than TNF when used in the same concentrations. Conversely, it has been reported in other studies that sTNF concentrations as high as 50 ng/ml were required to achieve modulation of human T_{reg} function (210, 211). In line with this, it could be shown in the present study that the inhibition of T_{reg}-induced suppression is reduced if the T_{eff}-T_{reg} co-cultures are treated with lower concentrations of TNCscTNF80.

In addition to TNF-mediated effects on T_{reg}, Chen *et al.* also observed a TNF-mediated reduction of the susceptibility of T_{eff} towards T_{reg}-induced suppression. They reported that a fraction of T_{eff} cells upregulate TNFR2 upon TCR stimulation, which renders them more resistant towards suppression by conventional $TNFR2^{-}$ T_{reg}. Highly activated $TNFR2^{+}$ T_{reg}, however, maintain the capacity to suppress those $TNFR2^{+}$ T_{eff} (218). Thus, signalling via TNFR2 might stimulate T_{eff} to a greater extend than T_{reg} under certain circumstances and, thereby, cause an impaired T_{reg}-mediated suppression of T cell responses. Arguing against this theory, however, is the observation that TNF preferentially upregulates the TNFR2 on T_{reg} and not on T_{eff} (259). Moreover, it was demonstrated in the present work that TNCscTNF80 is capable of inhibiting T_{reg}-mediated suppression of $TNFR2^{-/-}$ T_{eff} cells, while it does not change the suppression induced by $TNFR2^{-/-}$ T_{reg}, indicating that TNCscTNF80 influences T_{reg} function rather than the responsiveness of T_{eff} towards suppression.

While TNCscTNF80 did not affect the suppression by $TNFR2^{-/-}$ T_{reg}, it completely reversed the suppression induced by $TNFR1^{-/-}$ T_{reg}. This indicates that TNFR1-deficient T_{reg}

are more affected by TNFR2 signalling. A possible explanation for this is that signalling pathways downstream of TNFR2 are activated to a stronger extend in the absence of TNFR1 signalling. Normally, the adaptor protein TRAF2, which is important for the signalling cascade downstream of the TNFR2, is subject to degradation when both TNF receptors are stimulated (187, 188). Thus, in case of TNFR1-deficiency, cytosolic TRAF2 concentrations might be higher, leading to an increased activation of NF-κB and c-Jun. Since enhanced activity of the transcription factors NF-κB and AP-1 stimulate IL-2 production by T_{reg} (60), higher IL-2 concentrations might be found in the cell cultures. Il-2, then in turn, could promote higher proliferation levels of T_{eff}.

4.3 $CD4^+Foxp3^+$ T_{reg} surface makers: Under the influence of TNCscTNF80

Various surface markers are preferentially expressed by $CD4^+Foxp3^+$ T_{reg}, part of which also appear to contribute to their regulatory function, such as CTLA-4, CD25, CD39 and CD73 (109, 126, 155). It was demonstrated in this study that the TNFR2 agonist TNCscTNF80 attenuates T_{reg}-mediated suppression of T cell responses. Yet, although TNF is known to influence T_{reg} function (211), it is not clear what the underlying mechanisms are. Thus, the effect of TNCscTNF80 on the expression of surface molecules was studied over long-term anti-CD3ε-induced stimulation.

4.3.1 CD25: A T_{reg} and activation marker under the influence of TNCscTNF80

The IL-2 receptor α-chain, CD25, has long been used as a T_{reg} marker (17), yet effector T cells without suppressive activity also upregulate CD25 upon stimulation (260). In the present study, it was shown that the majority of $Foxp3^+$ cells expressed CD25 independently of their activation status. However, at the early time point of three hours approximately 25 % of the $Foxp3^+$ cells were $CD25^-$, indicating that Foxp3-expressing T_{reg} do not necessarily need to express CD25. This is consistent with previous data, which showed that 20-30 % of $CD4^+Foxp3^+$ cells are $CD25^-$ (261) and that those $CD4^+CD25^-Foxp3^+$ cells retain a T_{reg} phenotype and suppressive activity (262). In this work, it was shown that $Foxp3^+$ T_{reg} upregulated CD25 upon activation via their TCR complex, which is also consistent with previous findings (260, 263–265). While additional stimulation with the TNFR2 agonist TNCscTNF80 for three or 24 hours did not alter CD25 expression on T_{reg} cells, stimulation for 48 and 72 hours slightly reduced the anti-CD3-induced upregulation of CD25. In contrast to these results, it has been reported before that the expression of CD25 remains at the same level when T_{reg} are treated with anti-CD3ε and activated monocytes, which release cytokines

such as TNF, IL-1β and IL-6 (266). Controversially, Chen *et al.* observed an enhancing effect of TNF on the expression of CD25 on T_{reg} (216). Yet, differences in the experimental set-up might account for this discrepancy. First of all, the cells were stimulated with IL-2 and TNF in that study, while in the experiments for this work anti-CD3ε and TNCscTNF80 were used. Moreover, the effect of TNF on CD25 expression was examined in purified $CD4^+CD25^+$ cells that were later identified by gating on $CD25^+$ cells. In the present study, however, T_{reg} were identified by Foxp3 expression. By gating on CD25, not only T_{reg} but also activated T cells might be included in the analysis, as enhanced expression of CD25 is expected upon stimulation with IL-2 (72). Nevertheless, a more recent report showed that *in vivo* stimulation with TNF results in higher expression of CD25 on $CD4^+Foxp3^+$ cells when compared to IL-2 stimulation alone (267). Yet, in both studies the effects of unspecific TNF were examined. Ligation to the TNFR2 by a TNFR2-specific agonist, however, appears to downregulate the CD25 expression on T_{reg} in later stages of the anti-CD3-induced cell activation.

4.3.2 TNCscTNF80 influences the expression of adenosine-generating ectonucleotidases on $Foxp3^+$ cells

Several lines of evidence have shown that T_{reg} express high amounts of CD39 and CD73, two ectonucleotidases that hydrolyse ATP into the inhibitory molecule adenosine, which interferes with T cell activation and function (155, 157, 158, 268, 269). In this work it was observed that almost all non-stimulated $Foxp3^+$ T_{reg} were $CD39^+$, which is consistent with previous reports (157, 158). A slight reduction of CD39 surface expression on T_{reg} upon TCR stimulation was seen here. Yet, this decrease might not be sufficient for a reduction of the overall ectonucleotidase activity. Conversely, it was shown that T_{reg} maintain adenosine production upon activation (155). Noteworthy, CD39 expression was further reduced in the presence of the TNFR2 agonist TNCscTNF80. In contrast to these results, however, it has been previously demonstrated that T_{reg} express the same level of CD39 when co-cultured with activated monocytes from synovial fluid of rheumatic patients that secret TNF, IL-1β and IL-6 (266). On the other hand, it was observed that T_{reg} from the synovial fluid of patients with rheumatic arthritis, which is known to harbour high levels of TNF, exhibit higher amounts of CD39 (270). The divergent results are most likely due to differences in the experimental methods. In addition to TNF other factors might account for the alteration of CD39 expression on T_{reg} in those studies, since a great variety of proinflammatory cells and mediators are present in the synovial fluid of rheumatic patients.

Moreover, it could be demonstrated that nearly all non-stimulated Foxp3$^+$ T_{reg} showed a constant high expression of CD73. Stimulation with anti-CD3ε did not alter the level of CD73, which is consistent with a previous study that showed that, upon stimulation, Foxp3$^+$CD4$^+$ cells maintain high levels of CD73, while Foxp3$^-$CD4$^+$ cells down-modulate CD73 expression (269). Furthermore, treatment with TNCscTNF80 did not influence the expression of CD73. Yet, a downregulation of CD73 was observed on human Foxp3$^+$ T_{reg} in the synovial fluid of patients suffering from rheumatoid arthritis (270). It remains to be clarified if and how TNF alters the expression of CD73 on T_{reg}. The present results, however, do not indicate that TNCscTNF80 inhibits T_{reg} function by reducing CD73 activity. Interestingly, TNFR2 signalling decreased the expression of CD39. CD39 catalyses that rate-limiting step in the nucleotide metabolism cascade, i.e. the generation of adenosine monophosphate (AMP) from ATP (155, 157). Thus, limiting the amount of CD39 on T_{reg} could pose a mechanism, by which TNF attenuates the T_{reg}-driven suppression even when CD73 expression is maintained on T_{reg} cells. As the downregulation of CD39 was observed at later stages of the cell activation, this abrogation of T_{reg} function might require prolonged TCR and TNFR2 stimulation.

4.3.3 Influence of TNCscTNF80 on the expression of the TNFR superfamily member OX40 on Foxp3$^+$ cells

OX40 is another surface marker that is predominately expressed on T_{reg} cells, but also upregulated on effector T cells upon activation (271). It was observed in this work that the majority of naive Foxp3$^+$ T_{reg} were OX40$^+$. In addition, OX40 was further upregulated on T_{reg} after TCR stimulation, which is consistent with a previous report (272). The TNFR superfamily member OX40 has costimulatory effects on T_{conv}, promoting their proliferation and survival (273). Yet, the effects of OX40 engagement on T_{reg} function are controversially discussed. Whereas some data suggests that OX40 expression is necessary for T_{reg} suppressor capacity (274–276), others propose that OX40 stimulation actually attenuates T_{reg}-induced suppression (271, 272, 277, 278). More recent data showed that OX40 stimulation together with exogenous IL-2 induced profound T_{reg} expansion, whereas OX40 engagement in absence of additional IL-2 resulted in the abrogation of T_{reg} function (279).

It could be speculated that TNCscTNF80 enhances OX40 expression on T_{reg} cells and thereby contributes to their reduced suppressor function. Yet, the presence of TNCscTNF80 changed OX40 expression only marginally. Correspondingly, TNFR2-deficient T_{reg} were shown to harbour equal amounts of OX40 when compared to their WT counterparts (280),

indicating that TNFR2 signalling does not influence OX40 expression. Other reports, however, suggested that TNF increases OX40 expression by T_{reg} (210, 259). Methodical differences could account for the conflicting results. Preferential upregulation of OX40 on $CD4^+Foxp3^+$ was seen after three days of stimulation with IL-2 and TNF (259) or following stimulation with TNF alone, which, however, was used in unusually high concentration of 50 ng/ml (210), while anti-CD3ε and lower concentrations of TNFR2-specific TNCscTNF80 were used for stimulation in this work. In addition, OX40 upregulation was seen in a study that utilized isolated $CD25^{++}$ and $CD25^-$ cells, which were classified as not being highly pure, and then used CD25 as a T_{reg} marker for the analysis of OX40 expression (210). Yet, contaminating T_{eff} cells might have outgrown the $CD25^{++}$ $T_{reg.}$ Those T_{eff} might have upregulated CD25 and OX40 expression in response to activation since both CD25 and OX40 are markers for T cell activation (260, 273). Thus, it is feasible that not T_{reg} but stimulated T_{eff} upregulated OX40 in that study.

4.3.4 TNCscTNF80 does not change expression of CTLA-4 on $Foxp3^+$ cells

CTLA-4 is an inhibitory receptor on T cells, which like CD28 binds to CD80/CD86 on APC, but instead of mediating costimulatory effects it actually reduces T cell responses (281). While conventional T cells only show CTLA-4 on their cell surface upon activation, T_{reg} constantly express CTLA-4 (105, 122). It was also found in this study that non-stimulated T_{reg} expressed relatively high amounts of CTLA-4. Strikingly, stimulated T_{reg} did not enhance the expression of CTLA-4 on their cell surface. Expression of CTLA-4 was even attenuated after stimulation for 48 or 72 hours. In contrast to the present results, previous studies have reported that CTLA-4 is upregulated upon stimulation (105, 122, 282, 283), mainly through re-cycling to the cell surface (122). Yet, ConA (105), PMA (282, 283) or anti-CD3 together with anti-CD28 (122) were used for stimulation of T cells in those reports, while T cells were stimulated with anti-CD3ε antibody alone in the present study. However, it has been shown that CD28 co-stimulation is necessary for maximal CTLA-4 expression (284, 285). Furthermore, while surface expression of CTLA-4 is upregulated following T cell stimulation, it is also subject to rapid endocytosis. Therefore, only a small amount of CTLA-4 is steadily expressed on the cell surface upon stimulation despite a constant movement of CTLA-4 molecules to the plasma membrane (285).

Because CTLA-4 is essential for T_{reg}-mediated suppression (109, 140), changing the surface expression of CTLA could be a possible inhibitory mechanism by which TNCscTNF80 limits T_{reg} suppressor capacity. Yet, no change of CTLA-4 expression on

Foxp3$^+$ cells could be observed if TNCscTNF80 was added to the cell cultures. Thus, TNCscTNF80 does not seem to modulate T_{reg} suppression by alteration of the immunosuppressive receptor CTLA-4.

4.3.5 TNSscTNF80 limits GITR expression on Foxp3$^+$ T_{reg}

Engagement to GITR on T_{conv} has costimulatory effects resulting in T cell proliferation and increased cytokine production (265, 286, 287). Yet, its function on T_{reg} has been the discussed controversially over the last years. Whereas some studies suggest that ligation to GITR attenuates T_{reg} suppression (34) or that signalling via GITR does not influence T_{reg} function but rather renders T_{eff} resistant to T_{reg} function (286), others found enhancing effects on T_{reg} numbers and suppressor capacity (258, 287). However, the effects of GITR on T_{reg} seem more complex, as a more recent report showed that GITR exerts contradictory functions in T_{reg} depending on the circumstances. According to that study, GITR engagement results in the loss of Foxp3$^+$ T_{reg} under pathogenic situation, while it promotes T_{reg} expansion in healthy mice (288).

In the experiments for this thesis it was found that nearly all Foxp3$^+$ cells expressed GITR over the whole incubation time of up to 72 hours regardless of stimulation or not. This is consistent with previous reports showing that naive *ex vivo* CD4$^+$CD25$^+$ T_{reg} strongly express GITR (34, 261, 265). In addition, it was observed that T_{reg} downmodulate GITR expression following treatment with the TNFR2 agonist TNCscTNF80 for 48 and 72 hours. Since GITR appears to promote T_{reg} proliferation and function under physiological conditions (258, 265, 288), the TNFR2-driven downregulation of GITR on T_{reg} might contribute to the TNCscTNF80-mediated abrogation of T_{reg} suppressor capacity. Moreover, downregulation of GITR, like CD25 and CD39, was seen after prolonged stimulation with anti-CD3ε and TNCscTNF80 for 48 and 72 hours. Thus, TNCscTNF80 appears to inhibit those factors, which are important for T_{reg} function, only at later stages of anti-CD3-driven activation. This is consistent with the results from the suppression assays that showed that TNCscTNF80 induces abrogation of T_{reg} suppression after stimulation for 72 hours.

4.4 Effect of TNCscTNF80 on pStat5 expression in T_{eff} and T_{reg}

It was observed that the TNFR2 agonist TNCscTNF80 inhibits the T_{reg}-induced suppression of T cells, but the underlying molecular mechanism of TNF-driven alteration of T_{reg} function is unknown. Prolonged stimulation with TNCscTNF80 reduced the levels of the surface expression of CD25, CD39 and GITR on T_{reg} cells, which at least in part might

account for their defective suppressor function. Constant IL-2 signals are required in order for T_{reg} to maintain high levels of CD25 (75, 289). Thus, impaired IL-2 signalling might be the reason for the TNCscTNF80-induced reduction of CD25. Moreover, disturbing the signalling cascade downstream of the IL-2R could explain why TNCscTNF80 abrogates T_{reg} function, since IL-2 is essential for T_{reg} function and homeostasis (13, 68). In T_{reg}, ligation to the IL-2R predominately induces the phosphorylation and thereby activation of the transcription factor Stat5. However, the MAPK and PI3K pathways, which are induced in IL-2-stimulated T_{eff}, are not activated (78). Thus, it was hypothesized that TNCscTNF80 could act via Stat5 and inhibit T_{reg} function by reducing its activation.

After the staining process against pStat5 had been optimized, the effects of TNFR agonists on Stat5 activation in T_{reg} and T_{eff} were examined during short- and long-term stimulation. First, it was demonstrated that pStat5 levels are enhanced in $CD4^+CD25^+$ cells after short-term stimulation with IL-2 and anti-CD3ε, while pStat5 expression did not increase in $CD4^+CD25^-$ cells. Nevertheless, both $CD4^+CD25^+$ regulatory and $CD4^+CD25^-$ effector T cells are supposed to respond to IL-2 stimulation with an increased Stat5 activation (13). However, the observation that pStat5 is only induced in $CD4^+CD25^+$ but not $CD4^+CD25^-$ cells does not necessarily mean that T_{reg} predominantly activate pStat5. T cells only express the high-affinity IL-2 receptor complex upon TCR-driven activation. Once upregulated, this receptor complex is more efficiently bound by IL-2, which in turn further enhances CD25 expression in a Stat5-dependent manner (75, 290). Thus, gating on $CD4^+CD25^+$ might select activated rather than regulatory T cells. Conversely, it was shown that a high proportion of $CD4^+Foxp3^-$ cells express CD25 in response to anti-CD3ε stimulation for as short as three hours. The differences of pStat5 expression in $CD4^+CD25^-$ and $CD4^+CD25^+$ cells observed in these experiments were, therefore, due to the separation of non-activated and activated cells. During the short-term stimulation, addition of TNFR agonists enhanced pStat5 expression in those activated $CD4^+CD25^+$ cells. The increase of pStat5 was always highest in the presence of the TNFR2 agonist TNCscTNF80, while the TNFR1 agonist hTNF showed only minor changes of pStat5 expression and the combined TNFR1/TNFR2 agonist TNCwtTNF had intermediate effects. It has been reported that TNFR2 signalling enhances Akt and NF-κB expression during TCR stimulation and thereby promotes IL-2 production (183). Thus, TNFR2 signalling appears to increases IL-2 levels in the cell cultures, which in turn further promotes the IL-2 driven activation of Stat5 in the stimulated T_{eff} cells.

It is known that Stat5 is activated in response to IL-2 signalling (244). Yet, in the suppression assays in this thesis, which demonstrated the negative effect of TNCscTNF80 on

T_{reg} suppressor function, T cells were stimulated only with anti-CD3ε antibody without any exogenous IL-2. However, it could be shown in this work that anti-CD3ε stimulated $CD4^+$ cells express pStat5 during long-term incubation even in the absence of exogenous IL-2. Thus, it was demonstrated that no additional IL-2 is needed for anti-CD3-induced activation of Stat5. This can be explained by the upregulation of IL-2 production and CD25 expression in response to TCR stimulation (291). IL-2R signalling in T cells is, thereby, increased and subsequently induces Stat5 activation. In the present study, however, strong Stat5 activation was only observed upon stimulation for 24 hours, indicating that aCD3-induced IL-2 production is only transiently upregulated. Conversely, it has been shown that IL-2 production is at its maximum 12-18 hours after TCR-mediated stimulation and then starts to decline (291).

Upon long-term stimulation, the $CD4^+Foxp3^+$ cells showed enhanced pStat5 levels as early as three hours after stimulation, whereas pStat5 was not detectable in $CD4^+Foxp3^-$ responder cells until 24 hours of stimulation. This is in line with a previous study, in which it was shown that T_{reg} were the first to respond to IL-2 during the initiation of immune responses, while effector T cells required repetitive antigenic stimulation (292). Yet, in $CD4^+Foxp3^-$ cells expression of pStat5 was only observed following activation with anti-CD3ε for 24 hours. A cardinal factor of T_{reg} cells is their ability to supress IL-2 production by T_{eff} cells (56). Therefore, less IL-2 might be available in the cell culture for both T_{eff} and T_{reg} cells during the course of suppression. Those reduced IL-2 level might not be sufficient to induce pStat5 expression in T_{eff}. T_{reg}, however, are stimulated efficiently even by sub-optimal IL2-R signalling (289). Conversely, the present results showed that pStat5 was expressed in $CD4^+Foxp3^+$ T_{reg} after prolonged stimulation as well, when IL-2 levels were presumably low. Furthermore, this observation is in line with the theory that T_{reg} mediate suppression through consumption of IL-2 and, thereby, deprive T_{eff} of the essential IL-2R signals (126). Regarding the TNFR agonists, neither the TNFR2-speficic TNCscTNF80 nor the TNFR1-speficic agonist hTNF considerably changed pStat5 expression in $CD4^+Foxp3^+$ cells. Only a minor reduction of pStat5 expression was observed after stimulation with TNCscTNF80 for 48 or 72 hours. This argues against the hypothesis that TNF affects T_{reg} suppressor function by modulating the cell-signalling cascade downstream of the IL-2 receptor.

In a different approach, CD45.1 and CD45.2 were used to separate T_{eff} and T_{reg} cells. When the effector cells were cultured alone, TNCscTNF80 only enhanced the pStat5 levels after 24 hours, while no difference was noticeable at other time-points. TNCwtTNF also

increased pStat5 levels in T_{eff} (data not shown). As they both activate TNFR2, while only TNCwtTNF binds to TNFR1 as well, this effect is likely modulated through TNFR2. Conversely, neither control supernatant nor the TNFR1-specific hTNF could increase the level of pStat5 expression (data not shown). This is consistent with previous reports showing that TNFR2, and not TNFR1, acts as a co-stimulator during TCR-driven immune responses (181, 183). When T_{eff} and T_{reg} were co-cultured, the proportion of $pStat5^+$ T_{eff} and T_{reg} cells was slightly enhanced after short-term stimulation with TNCscTNF80, while it was moderately decreased upon long-term stimulation with TNCscTNF80. Thus, TNFR2 signalling seems to act as co-stimulator in both T_{eff} and T_{reg} after shorter periods of stimulation. Accordingly, TNCwtTNF enhanced the pStat5 expression in T_{reg} cells after 24 hours, while the TNFR1-specific agonist hTNF did not influence the pStat5 levels. However, TNFR2 signalling appears to limit Stat5 activation both in effector and regulatory T cells at later stages of the T_{reg}-mediated suppression. This trend towards reduction of pStat5 expression after prolonged treatment with TNCscTNF80 was also seen when Foxp3 was used to identify T_{reg}.

Contrarily, Chen *et al.* reported that TNF enhanced both CD25 expression and IL-2-induced activation of Stat5 in $CD4^+CD25^+$ T_{reg} (216). Yet, in that study pStat5 expression was examined separately in T_{reg} and T_{eff} monocultures. Thus, in contrast to the present work, the effect of TNF on pStat5 during the course of T_{reg}-mediated suppression was not determined. Further, cell cultures were activated with IL-2 and TNF and subsequently the expression of pStat5 in $CD25^-$ and $CD25^+$ cells was measured. As described above (4.3.1), this might not separate regulatory and effector but rather unactivated and activated cells. Conversely, more recent data, which demonstrated that TNF enhanced CD25 on $CD4^+Foxp3^+$ T_{reg}, did not find any effect on the activation of Stat5 (267). Yet in that study, the pStat5 levels were analysed four hours after *in vivo* stimulation, while the effects of long-term treatment with TNF on pStat5 expression were not examined. Whereas in the present work, no alteration of pStat5 in $CD4^+Foxp3^+$ cells could be shown after short-term stimulation with TNCscTNF80 for three hours, long-term stimulation with this TNFR2 agonist led to a slight reduction of pStat5. Thus, only prolonged exposure to TNF might reduce IL2-R signalling in T_{reg} and consequently inhibit their suppressor capacity. Interestingly, it was also shown in this thesis that TNCscTNF80 enhanced CD25 expression on $Foxp3^+$ T_{reg} cells after 24 hours, but decreased its surface expression after 48 and 72 hours of stimulation. Thus, TNFR2-dependent downregulation of CD25 might cause inhibition of IL-2R signalling and thereby account for the reduced Stat5 activation. Moreover, TNCscTNF80 also induced

downregulation of the CD39 and GITR expression by T_{reg} after stimulation for 48 and 72 hours. Thus, it seems that especially prolonged TNFR2 stimulation inhibits T_{reg} function.

However, the results obtained in the experiments using Foxp3 to differentiate between effector and regulatory cells demonstrated only very mild effects of TNCscTNF80 on pStat5 expression in $CD4^+Foxp3^-$ T_{eff} and $CD4^+Foxp3^+$ T_{reg}. The conflicting results might be due to the methodical limitations that occurred when using the CD45.1/CD45.2 model for identification of T_{reg} and T_{eff} cells. Effector cells were defined as $CD4^+CD45.2^-$. Yet, naïve splenocytes from CD45.1 mice also comprise approximately 10 % $CD4^+CD25^+$ T_{reg}. Thus, T_{reg} cells have to be expected in the $CD4^+CD45.2^-$ population, which therefore does not entirely represent the T_{eff} subset. In contrast, gating on $CD4^+Foxp3^-$ will identify only true effector cells. Moreover, the recovery of $CD4^+CD25^+$ cells after magnetic separation is relatively low, which can contribute to the reduced data quality. Thus, further studies are required to clarify the effect of TNFR2 signalling on the activation of IL-2-depended transcription factors in T_{reg} and its possible implications on T_{reg} suppressor capacity.

4.5 Effect of TNCscTNF80 on pZAP70 expression in T_{eff} and T_{reg}

It was demonstrated that the TNFR2 agonist TNCscTNF80 did not have major effects on IL-2 receptor signalling. Yet, it is also possible that TNFR2 stimulation changes T_{reg} suppression by influencing the signalling cascade downstream of the TCR. In order to assess the effect of TNCscTNF80 on TCR signalling, the expression of pZAP70, a downstream molecule of this receptor complex, was studied. Similar to the experiments with pStat5 staining, a series of preliminary studies had to be performed in order to optimize the phospho-flow staining protocol for the detection of pZAP70 in $Foxp3^+$ T_{reg} and $Foxp3^-$ T_{eff}. Although it was shown in a previous report that ZAP70, both in its non-phosphorylated and phosphorylated form, was upregulated in T_{reg} and non-T_{reg} $CD4^+$ cells upon activation with anti-CD3ε antibody for at least 30 minutes (238), in the present study no pZAP70 was detectable after stimulation with anti-CD3ε antibody. Moreover, no enhanced levels of pZAP70 were detectable in T_{reg} and T_{eff} upon combined aCD3/IL-2 stimulation. Therefore, other methods of stimulation needed to be found.

Previous studies on the detection of signalling events with western blots have demonstrated that H_2O_2 can be used as a stimulator for tyrosine phosphorylation of signalling molecules downstream of the TCR (293, 294). It is not completely clear, how H_2O_2 can simulate antigenic stimulation of T cells. H_2O_2, being a generator of free radicals, might promote the formation of ROS that inhibit protein tyrosine phosphatases, resulting in the

relatively higher activity of protein tyrosine kinases. Those kinases in turn can facilitate the phosphorylation of mediators like ZAP70 (293). When signalling events are studied in limited cell population, techniques like western blot are not practical because they require large cell numbers. The phospho-flow method, on the other hand, allows simultaneous analysis of surface markers for the identification of small cellular subsets and phosphorylated intracellular signalling molecules. Regarding the compatibility of H_2O_2 stimulation and flow cytometric analysis, it has been shown that pZAP70 is detectable by both western blot and flow cytometry upon H_2O_2 stimulation (250). In the present study, it could also be demonstrated that stimulation with H_2O_2 resulted in a pronounced expression of pZAP70 by $CD4^+$ cells. Thus, H_2O_2 stimulation could be used as an efficient method for the activation of the proximal TCR signalling cascade.

After establishing a suitable activation method, it was possible to analyse the impact of TNFR2 signalling on pZAP70 expression in both $CD4^+Foxp3^-$ T_{eff} and $CD4^+Foxp3^+$ T_{reg} cells. Nevertheless, the effects of TNCscTNF80 on H_2O_2-stimulated cells were not consistent. While TNCscTNF80 enhanced pZAP70 expression in T_{reg} and T_{eff} in one experiment, those results could not be replicated in future experiments. Furthermore, comparing the pZAP70 expression in $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ cells showed that both T_{reg} and T_{eff} activate ZAP70 to the same extend regardless of their activation status. Thus, the present results suggest that proximal TCR signalling is not different in T_{reg} or T_{eff} cells and that signalling via the TNFR2 does not influence the activation of ZAP70 in neither of those cells. On the contrary, Hanschen *et al.* have demonstrated that T_{reg} upregulate pZAP70 more rapidly and to a higher extend than non-T_{reg} cells (238). Differences in the experimental set-up might account for the contrasting results, as T cells were activated with anti-CD3ε in that study, whereas in the present work the more unphysiological H_2O_2 was used. In a different study it was demonstrated that $CD4^+CD25^+$ T_{reg} show reduction of proximal TCR signalling following CD3 cross-linking, including an impaired recruitment of ZAP70 to the CD3ζ chain and diminished ZAP70 activity (58). In that report western blotting was performed to show coimmunoprecipitation of CD3ζ and ZAP70, which might produce different results than the measurement of the ZAP70 phosphorylation level by flow cytometry.

In order to exclude that the concentration of TNCscTNF80 has potential effects on pZAP70 expression, serial dilutions of TNCscTNF80 were performed. Once again, stimulation with 10 ng/ml TNCscTNF80 did not increase pZAP70 expression in H_2O_2-activated T_{reg} and T_{eff} cells. Reducing the concentration of TNCscTNF80 did not have considerable effects on pZAP70 levels in T_{eff} or T_{reg} either. The expression of pZAP70 was

also examined depending on the incubation time. The maximal expression of pZAP70 in both $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ was noticed after stimulation with H_2O_2 for five minutes, while it declined constantly until the 60-minute end point. This is in contrast to the observation made by Haas *et al*. who found that stimulation with H_2O_2 for 15 minutes led to the maximal expression of pZAP70 (250). Yet, in the present study staining was performed with a mAB against phosphorylated tyrosine 319 (Tyr319) of pZAP70, whereas Haas *et al.* studied the phosphorylation levels of pZAP70 at Tyr493 and Tyr292 (250). Following TCR stimulation, ZAP70 is recruited to the receptor complex (49). ZAP70 then in turn is phosphorylated at Tyr319, resulting in the release from its autoinhibited conformation. Subsequently, phosphorylation of Tyr493 is induced, which is required for full catalytic activity of ZAP70 (49, 295). Thus, phosphorylation at Tyr319 precedes phosphorylation of Tyr493. This might explain the different kinetics of pZAP70 upregulation upon H_2O_2 stimulation that were found in both studies. Regarding TNFR2 signalling, however, no considerable difference of the pZAP70 level could be shown in the presence of TNCscTNF80 at any time point. Thus, it was concluded that TNCscTNF80 and, therefore, signalling via TNFR2 does not change the activation of ZAP70. As ZAP70 is one the earliest mediators of the TCR signalling cascade, this indicated that TNCscTNF80 does not influence T_{reg}-induced suppression by changing proximal TCR signalling events.

4.6 Change of MDSC-induced suppression by TNFR2-agonist TNCscTNF80

Not only T_{reg} exhibit immunoregulatory functions, myeloid-derived suppressor cells are also enabled to limit T cell numbers and functions. It has been shown formerly that MDSC efficiently suppress T cell proliferation *in vitro* (253), which could be reproduced in this thesis. In the present study a marked suppression of $CD4^+$ and $CD8^+$ proliferation in response to anti-CD3 stimulation could be observed, especially when higher ratios of MDSC to splenocytes were utilized. Furthermore, it was shown in this work that MDSC, which had been cultured for six up to ten days prior to the suppression assays, showed a reduced ability to suppress T cell proliferation when compared to MDSC that had been cultured for only four days. In a previous report it was reported that suppressive MDSC develop from bone marrow cells after stimulation *in vitro* with GM-CSF for four days, while they differentiated into mature cells without suppressor capacity following longer periods of stimulation (42). Thus, the reduction of suppressor capacity of MDSC that were cultured for more than four days might be due to advanced cell maturation towards non-suppressive cells.

MDSC, which had been generated with GM-CSF and the TNFR2-specific agonist TNCscTNF80, were also able to suppress T cell proliferation. However, cultivation in the presence of TNCscTNF80 resulted in the generation of MDSC that showed reduced suppression of T cell proliferation, indicating that TNFR2 signalling inhibits the generation of suppressive MDSC. Contrarily, Zhao *et al.* suggested that TNF does not change the suppressor capacity of MDSC, but rather mediates anti-apoptotic effects in those cells. This is thought to contribute to the survival and peripheral accumulation of MDSC in tumor-bearing mice, thereby facilitating MDSC-induced suppression of antitumor immune response (224). Moreover, there is also data available that suggests that TNF enhances suppressor capacity of MDSC (225, 226).

Interestingly, it was observed in the present study that TNFR2-deficient MDSC were less suppressive when compared to their WT counterparts, indicating that TNFR2 signalling promotes the suppressive capacity of MDSC. This stands in stark contrast to the observation that the TNFR2 agonist TNCscTNF80 attenuated MDSC-induced T cell suppression. A possible explanation for this phenomenon is that MDSC appear to undergo more rapid maturation in the absence of TNFR2 signalling (225, 254). Cultivation for more than fours days did not considerably reduce the suppressive activity of MDSC from $TNFR2^{-/-}$ mice, while WT MDSC gradually lost their suppressor capacity. A great proportion of the $TNFR2^{-/-}$ bone marrow cells might have already developed into mature non-suppressive DC after four days of stimulation. WT MDSC, on the other hand, do not differentiate as quickly and thereby retain their suppressor capacity even after longer periods of cultivation. Thus, TNFR2 signalling seems to play a role in the disruption of myeloid cell maturation, which results in the accumulation of immature myeloid cells with suppressive functions. However, if TNFR2 signalling was blocking MDSC maturation, GM-CSF-induced generation with additional TNCscTNF80 should have slowed down the maturation of MDSC and resulted in an enhancement of MDSC suppressor function. Nevertheless, the present results showed that generation with TNCscTNF80 abrogated MDSC-induced T cell suppression. This suggests that TNFR2 signalling inhibits rather than enhances MDSC-mediated suppression. More in depth studies are required to clarify the reasons for the discrepancy of the existing data.

5 Conclusion

In this work, it could be shown that TNFR2 signalling has a co-stimulatory effect on the proliferation and cytokine production of effector T cells and inhibits T_{reg}- and MDSC-mediated suppression of these effector lymphocytes. It could also be demonstrate that signalling via the TNFR2 has certain implications on T_{reg} phenotype and intracellular signalling events. It was shown that T_{reg} down-regulate the surface molecules CD25, GITR and CD39 after prolonged exposure to TNFR2 agonists. It is possible that the reduced activity of those surface molecules leads to a reduction of IL-2R signalling, adenosine production and stimulatory signals for T_{reg} expansion and, thereby, eventually results in an abrogation of T_{reg} suppressor capacity. Regarding intracellular signalling events, it was found that stimulation of TNFR2 has marginal effects on the IL-2-induced activation of Stat5, while it does not affect proximal TCR signalling pathways. This reduction of pStat5 up-regulation was only seen after long-term stimulation. Since the TNFR2-mediated down-regulation of surface molecules was only seen at later time-points as well, this indicates that mainly prolonged TNFR2 signalling negatively affects T_{reg} suppressor function. Likewise, the MDSC-induced suppression was also more pronounced when those cells were exposed to TNFR2 agonists for longer periods of time. Since TNF is mainly produced during the course of inflammation, the defective function of regulatory immune cells in response to prolonged exposure towards TNF could contribute to an impaired clearing of proinflammatory cells, especially after the acute phase of an immune response. Eventually, overshooting inflammatory processes and autoimmune disorders may develop or be worsened. Thus, anti-TNF reagents might have the potential to promote both T_{reg} and MDSC function and thereby contain dysregulated immune responses.

6 References

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