

Novel aspects of TGF β in human CD4⁺ T cell biology

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1 Summary

During this thesis, several novel aspects of TGF β in human CD4⁺ T cell biology were investigated. First, we were interested, which factors control the resting state of CD4⁺ T cells. Genome-wide transcriptional analysis of serum-deprived CD4⁺ T cells revealed TGF β to be the most prominent factor controlling the homeostasis of CD4⁺ T cells. Furthermore, we could show that TGF β is especially important for the control of CD4⁺ T cell homeostasis in the presence of low-level T cell stimulating signals.

The finding that TGF β is constantly signaling in human CD4⁺ T cells formed the basis for the investigation of the transcriptional changes in CD4⁺ T cells in response to clinical drug-mediated blockade of TGF β signaling. This analysis in combination with validation experiments resulted in the identification of three novel biomarker genes, which can be used to monitor the effectiveness of clinical drugs targeting TGF β signaling.

Furthermore, we investigated the suppressive mechanisms of TGF β and Treg cells. We were able to demonstrate that the inhibitory potential of TGF β on human CD4⁺ T cells is independent of Treg cells. In contrast, Treg cell-mediated inhibition of CD4⁺ T cells is partially dependent on TGF β .

In addition, we investigated the influence of TGF β on the induction of 'induced' human Treg cells derived from naïve T cells. In contrast to the murine system, we could induce suppressive FOXP3⁺ Treg cells via T cell activation ('activation-induced' Treg cells), independent of the presence of TGF β . The transcriptional profile of these 'activation-induced' Treg cells was related to natural Treg cells, when addressing the Treg-specific genes.

In contrast to mere T cell activation, the addition of TGF β in the process of Treg cell induction ('TGF β -induced' Treg cells) led to changes in the expression of several transcription factors in comparison to activation-induced Treg cells. One gene downregulated in natural as well as TGF β -induced Treg cells but not in activation-induced Treg cells was the chromatin organizer SATB1. Therefore, regulation of this gene was investigated in more detail. T cell activation as well as TGF β were identified to be implicated in the regulation of SATB1.

Taken together, during this thesis several new aspects of TGF β in the control of human CD4⁺ T cells homeostasis were demonstrated. This data formed the basis to develop biomarkers for clinical therapies targeting the TGF β signaling pathway. Moreover, some implications of TGF β on the biology of Treg cells were studied.

2 Table of contents

1	Summary.....	I
2	Table of contents	III
2.1	Figure legend.....	VI
2.2	Table legend.....	VII
3	Abbreviations	VIII
4	Introduction	1
4.1	The Immune system	1
4.2	T cells	1
4.2.1	Development of CD4 ⁺ T cells.....	1
4.2.2	Differentiation of CD4 ⁺ T cells.....	2
4.2.3	Memory T cells	3
4.2.4	T cell homeostasis	4
4.3	Regulatory T cells	5
4.4	Transforming Growth Factor β	7
4.4.1	The TGF β signaling pathway.....	8
4.4.2	TGF β effects on immune cells.....	9
4.4.3	Role of TGF β in cancer.....	12
4.5	TGF β and Treg interaction	13
4.6	SATB1	14
4.7	Objectives.....	16
5	Material and Methods	17
5.1	Materials	17
5.1.1	Reagents	17
5.1.2	Plastic ware	18
5.1.3	Equipment.....	19
5.1.4	TGF β - Inhibitors.....	19
5.1.5	Software.....	19
5.2	Methods	20
5.2.1	Peripheral blood samples	20
5.2.2	Isolation of CD4 ⁺ T cells, CD8 ⁺ T cells, B cells and monocytes	20
5.2.3	Isolation of CD4 ⁺ T cell subpopulations.....	20
5.2.4	Cell counting.....	21
5.2.5	CD4 ⁺ T cell culture assays.....	21

5.2.6	Serum deprivation and TGF β add-back experiments.....	21
5.2.7	CD4 ⁺ T cell treatments.....	21
5.2.8	Induction of Treg cells.....	22
5.2.9	Flow cytometry.....	22
5.2.10	FACS sorting	23
5.2.11	Proliferation assay	23
5.2.12	Suppression assay	24
5.2.13	Th1/Th2 cytokine measurement.....	24
5.2.14	Immunofluorescence for SMAD2 and SMAD3	24
5.2.15	Cell lysis and Western Blot.....	25
5.2.16	EMSA.....	25
5.2.17	ChIP	26
5.2.18	RNA Isolation.....	26
5.2.19	Real-time-PCR.....	27
5.2.20	Microarray procedure.....	27
5.2.21	Data analysis and software.....	28
5.2.22	Statistics	29
6	Results.....	31
6.1	TGF β controls T cell homeostasis.....	31
6.1.1	Transcriptional changes in human CD4 ⁺ T cells after serum deprivation.....	31
6.1.2	The TGF β signaling pathway is a major target after serum deprivation.....	32
6.1.3	Serum-deprived human CD4 ⁺ T cells present a 'TGF β loss signature'	34
6.1.4	Identification of novel TGF β target genes in resting CD4 ⁺ T cells.....	36
6.1.5	CD4 ⁺ T cell display an activated TGF β signaling pathway	39
6.1.6	Ablation of TGF β signaling is associated with reduced SMAD phosphorylation..	42
6.1.7	Presence of constitutive TGF β signaling leads to decreased T cell proliferation .	44
6.2	Identification of biomarkers for a new clinical TGF β R kinase inhibitor.....	45
6.2.1	The TGF β R kinase inhibitor restores TGF β -mediated T cell inhibition	45
6.2.2	The inhibitor counter-regulates most TGF β -mediated transcriptional changes....	47
6.2.3	Biomarker candidates for clinical TGF β RI kinase inhibitor therapy.....	50
6.2.4	Further development of biomarkers for clinical TGF β RI kinase inhibitor therapy.	52
6.3	Interaction of TGF β and Treg cells	54
6.3.1	TGF β mediates T cell inhibition in the absence of Treg cells	55
6.3.2	The TGF β RI kinase inhibitor reduces the suppressive effect of Treg cells	56

6.4	Implication of TGF β on 'induced' human Treg cells	57
6.4.1	FOXP3 induction is a late activation response	58
6.4.2	Human induced Treg cells show suppressive capacity	61
6.4.3	The transcriptional profile of induced Treg cells is related to natural Treg cells... ..	63
6.4.4	SATB1 levels are reduced in natural and TGF β -induced Treg cells	67
6.5	TGF β is a key player of SATB1 regulation in activated CD4 ⁺ T cells.....	69
6.5.1	T cell activation leads to SATB1 upregulation	69
6.5.2	SATB1 is downregulated by TGF β in activated CD4 ⁺ T cells.....	69
6.5.3	SATB1 levels of expression are reduced in memory CD4 ⁺ T cells	70
6.5.4	TGF β downregulates SATB1 under Th1 and Th2 promoting conditions.....	72
6.6	Interplay of TGF β , SATB1 and Treg cells.....	75
6.6.1	Is TGF β -mediated SATB1 downregulation FOXP3 dependent?	76
7	Discussion.....	81
7.1	TGF β controls T cell homeostasis	81
7.2	Identification of biomarkers for clinical therapies targeting TGF β	84
7.3	Interaction of TGF β and Treg cells	87
7.4	The role of TGF β in the induction of human Treg cells	89
7.5	SATB1 regulation in CD4 ⁺ T cells.....	92
7.6	Interplay of TGF β , FOXP3 and SATB1 in Treg cells	95
8	Zusammenfassung	99
9	Acknowledgements	101
10	References.....	103
11	List of publication	115

2.1 Figure legend

FIGURE 1: T HELPER CELL DIFFERENTIATION AND REGULATION	2
FIGURE 2: THE TGF β SIGNALING PATHWAY	9
FIGURE 3: TGF β EFFECTS ON CELLS OF THE INNATE AND THE ADAPTIVE IMMUNITY	10
FIGURE 4: PROLIFERATION OF CFSE- LABELED CELLS	24
FIGURE 5: EXPERIMENTAL SETUP TO ADDRESS THE ROLE OF EXOGENOUS FACTORS	31
FIGURE 6: TIME OF SERUM WITHDRAWAL SEPARATES THE SAMPLE GROUPS	32
FIGURE 7: THE TGF β SIGNALING PATHWAY IS A MAJOR TARGET AFTER SHORT-TERM SERUM DEPRIVATION	34
FIGURE 8: EXPRESSION CHANGES IN THE TGF β SIGNALING PATHWAY IN RESPONSE TO SERUM DEPRIVATION	35
FIGURE 9: FOLD CHANGES OF KNOWN TGF β TARGET GENES IN RESPONSE TO SERUM DEPRIVATION	36
FIGURE 10: EXPERIMENTAL SETUP TO IDENTIFY TGF β TARGET GENES	36
FIGURE 11: IDENTIFICATION OF NEW TGF β TARGET GENES	37
FIGURE 12: RT-PCR OF EXEMPLARY TGF β TARGET GENES	39
FIGURE 13: SMAD3 PHOSPHORYLATION IN HUMAN LYMPHOCYTES	40
FIGURE 14: SMAD2 AND SMAD3 ARE PHOSPHORYLATED IN PRIMARY HUMAN CD4 ⁺ T CELLS	41
FIGURE 15: SMAD-PHOSPHORYLATION IN CD4 ⁺ T CELL SUBPOPULATIONS	41
FIGURE 16: SMAD-PHOSPHORYLATION IN REGULATORY T CELLS	42
FIGURE 17: KINETICS OF DE-PHOSPHORYLATION OF SMAD2 AND SMAD3	42
FIGURE 18: DOSE DEPENDENCY OF SMAD2 AND SMAD3 RE-PHOSPHORYLATION	43
FIGURE 19: CD4 ⁺ T CELL PROLIFERATION IS DECREASED IN THE PRESENCE OF TGF β 1	44
FIGURE 20: TGF β -MEDIATED T CELL INHIBITION IS RESTORED BY LY2109761	46
FIGURE 21: LY2109761 RESTORES THE TGF β -MEDIATED BLOCK OF PROLIFERATION IN NAÏVE AND MEMORY T CELLS	47
FIGURE 22: SETUP TO STUDY LY2109761 INDUCED EXPRESSION CHANGES IN RESTING CD4 ⁺ T CELLS	48
FIGURE 23: TGF β 1 REGULATED GENES ARE COUNTER-REGULATED BY LY2109761 IN RESTING CD4 ⁺ T CELLS	48
FIGURE 24: SETUP TO STUDY LY2109761 INDUCED EXPRESSION CHANGES IN ACTIVATED CD4 ⁺ T CELLS	49
FIGURE 25: TGF β 1 REGULATED GENES ARE COUNTER-REGULATED BY LY2109761 IN ACTIVATED CD4 ⁺ T CELLS ..	50
FIGURE 26: HEATMAP DISPLAYING EXPRESSION VALUES OF DIFFERENTIALLY EXPRESSED GENES	51
FIGURE 27: SCHEMATIC WORKFLOW FOR <i>IN VITRO</i> BIOMARKER VALIDATION EXPERIMENTS	52
FIGURE 28: SCHEMATIC WORKFLOW FOR <i>IN VIVO</i> BIOMARKER VALIDATION EXPERIMENTS	53
FIGURE 29: TGF β -TREG INTERACTION SCHEME	54
FIGURE 30: TGF β MEDIATES T CELL INHIBITION IN THE ABSENCE OF TREG CELLS	55
FIGURE 31: THE TGF β R KINASE INHIBITOR PARTIALLY REDUCES TREG CELL-MEDIATED SUPPRESSION	57
FIGURE 32: FOXP3 EXPRESSION IS INDUCED UPON T CELL STIMULATION IN NAÏVE T CELLS	59
FIGURE 33: FOXP3 PROTEIN LEVELS ARE UPREGULATED IN RESPONSE TO T CELL STIMULATION	59
FIGURE 34: INDUCED TREG CELLS PROLIFERATE IN RESPONSE TO T CELL ACTIVATION	60
FIGURE 35: EXPERIMENTAL SETUP TO TEST THE SUPPRESSIVE CAPACITY OF INDUCED TREG CELLS	61
FIGURE 36: INDUCED TREG CELLS SHOW CELL-CONTACT DEPENDENT SUPPRESSIVE CAPACITY	62
FIGURE 37: HIERARCHICAL CLUSTERING REVEALED INDUCED TREG CELLS TO CLUSTER WITH NATURAL TREG CELLS ..	64
FIGURE 38: HEATMAP DISPLAYING EXPRESSION VALUES OF DIFFERENTIALLY EXPRESSED GENES	65
FIGURE 39: SATB1 EXPRESSION IS DECREASED IN NATURAL AND TGF β -INDUCED TREG CELLS	67
FIGURE 40: SATB1 PROTEIN LEVELS ARE REDUCED IN NATURAL TREG CELLS	68
FIGURE 41: SATB1 EXPRESSION IS INCREASED IN RESPONSE TO T CELL ACTIVATION	69
FIGURE 42: TGF β DOWNREGULATES SATB1 IN ACTIVATED CD4 ⁺ T CELLS	70
FIGURE 43: SATB1 EXPRESSION IS NOT ALTERED BY TGF β IN RESTING CD4 ⁺ T CELLS	70
FIGURE 44: SATB1 EXPRESSION IS REDUCED IN MEMORY CD4 ⁺ T CELLS	71
FIGURE 45: SATB1 RNA REGULATION IN ACTIVATED CD4 ⁺ T CELL SUBPOPULATIONS	72
FIGURE 46: SATB1 IS DOWNREGULATED BY TGF β UNDER TH1/TH2 PROMOTING CONDITIONS	73
FIGURE 47: THE PROTEIN LEVEL OF SATB1 IS REDUCED BY TGF β UNDER TH1/TH2 PROMOTING CONDITIONS	74
FIGURE 48: TGF β BLOCKS TH1/TH2 CYTOKINE SECRETION	75
FIGURE 49: TGF β -SATB1 INTERACTION SCHEME	76
FIGURE 50: FOXP3 BINDS TO THE SATB1 GENE	77
FIGURE 51: FOXP3 BINDS TO THE SATB1 GENE	77
FIGURE 52: KINETIC OF SATB1 AND FOXP3 EXPRESSION IN RESPONSE TO CD4 ⁺ T CELL STIMULATION.	78
FIGURE 53: SATB1 EXPRESSION IS UNALTERED IN RESTING T CELLS UPON FOXP3 TRANSFECTION	79

2.2 Table legend

TABLE 1: FACS ANTIBODIES	23
TABLE 2: EMSA OLIGONUCLEOTIDES	26
TABLE 3: CHIP PRIMER	26
TABLE 4: RT-PCR PRIMER	27
TABLE 5: MICROARRAY EXPERIMENTS FOR TGF β IN T CELL HOMOEOSTASIS	27
TABLE 6: MICROARRAY EXPERIMENTS FOR TGF β RI KINASE INHIBITOR ANALYSIS	28
TABLE 7: MICROARRAY EXPERIMENTS FOR INDUCED TREG CELL ANALYSIS	28
TABLE 8: DIFFERENTIALLY EXPRESSED GENES IN NATURAL AND TGF β -INDUCED TREG CELLS	66

3 Abbreviations

μl	micro liter
aAPC	artificial antigen-presenting cell
ab	antibody
bp	base pair
CCR	chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
ChIP	chromatin immune precipitation
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
ddH ₂ O	double distilled water
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
EDTA	ethylenediamine tetraacetate
EMSA	electrophoretic mobility shift assay
EtOH	ethanol
FACS	fluorescence activated cell sorter
FC	fold change
FCS	fetal calf serum
FSC	forward scatter
GO	gene ontology
hrs	hours
H ₂ O	milliQ-filtered, autoclaved water
IFN	interferon
IL	interleukin
Kb	kilo base pairs
KDa	kilo Dalton
MHC	major histocompatibility complex
mM	millimolar
mRNA	messenger RNA
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGE ₂	prostaglandin E ₂
RNA	ribonucleic acid
RT	room temperature
RT-PCR	real time polymerase chain reaction
SATB1	special AT-rich binding protein 1
SBE	SMAD binding element
TCR	T cell receptor
TE	tris/EDTA
TGFβ	transforming growth factor beta
Th	T helper
Treg cell	regulatory T cell

4 Introduction

4.1 The Immune system

The immune system of vertebrates is a highly evolved system that protects the organism from invasion by foreign pathogens. The main tasks of the immune system are the recognition and elimination of invading bacteria, fungi and parasites and the killing of virally infected cells. The immune system is composed of two major subdivisions: the innate and the adaptive immune system. The innate immune system provides an immediate defence mechanism mediated by macrophages, dendritic cells, granulocytes, mast cells, and natural killer cells. Innate immune responses are characterized by a non-specific recognition of classes of microorganisms.

When pathogens successfully evade the innate immune response, the adaptive immune system becomes activated. The adaptive immune response is mediated by B and T lymphocytes and is specified by the ability of clonal selection of highly antigen-specific lymphocytes and results in the development of immunological memory.

4.2 T cells

4.2.1 Development of CD4⁺ T cells

Mammalian T cells of diverse functional types share a complex developmental history. They originate from pluripotent precursors in the bone marrow or fetal liver from where they migrate to the thymus. T cell progenitors respond to the thymic environment by extensive proliferation and by initiating the transcriptional program of T cell differentiation. This differentiation process consists of an irreversible progression through distinct developmental stages characterized by gradual turn off of non-T cell lineage associated genes (Hayday and Pennington, 2007), (Petrie, 2003). During thymic development, T cells undergo T cell receptor (TCR) gene rearrangements, leading to a high structural diversity of the TCR. The survival of T cells depends on the successful assembly of a functional TCR. T cells expressing a functional TCR undergo positive and negative selection. During positive selection CD4⁺CD8⁺ double-positive T cells are selected for further survival and maturation. Selection is based on the appropriate degree of interaction between their TCR and peptide-MHC complexes on thymic epithelial cells. Depending on the class of MHC molecules that are recognized by T cells, they are positively selected either to helper CD4⁺ or to effector CD8⁺ single positive T cells. In the process of negative selection, double-positive or single-positive T cells that express TCRs with

high affinity for self-antigens are eliminated (central tolerance). After successful selection, T cells migrate from the thymus to the periphery.

4.2.2 Differentiation of CD4⁺ T cells

Peripheral helper CD4⁺ T cells recognize MHC class II molecules and generally cannot destroy infected cells or pathogens. Instead, activated helper CD4⁺ T cells lead to the activation of other immune cells, such as CD8⁺ T cells or B cells. Upon antigen stimulation, helper CD4⁺ T cells typically undergo distinct developmental pathways, attaining specialized properties and effector functions. T-helper (Th) cells were traditionally thought to differentiate into Th1 and Th2 cell subsets (Glimcher and Murphy, 2000) (Figure 1). This differentiation involves the coordinated expression of genes controlling tissue homing and effector functions. Signals from the TCR and the IL-12 (Th1) respectively the IL-4 (Th2) receptor act in synergy to induce specific transcription factors that mediate chromatin remodeling events at target genes (Agarwal and Rao, 1998), (Takemoto et al., 2000), (Lee et al., 2001), (Ansel et al., 2003). One chromatin-organizing factor implicated in Th cell differentiation is the special AT-rich-binding protein 1 (SATB1), which is predominantly expressed in T cells (see also chapter 4.6) (Alvarez et al., 2000), (Cai et al., 2006).

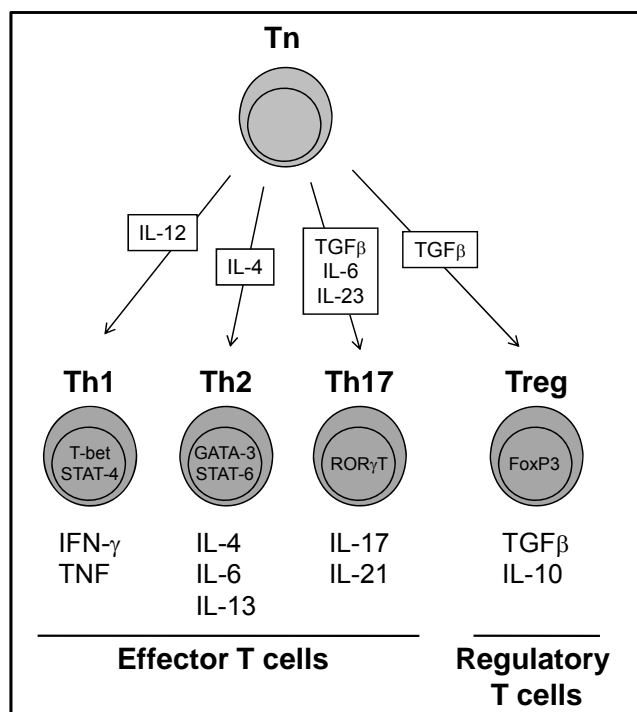


Figure 1: T helper cell differentiation and regulation

Predominant cytokines involved in the development of each lineage are associated with arrows. Cytokines noted below each lineage indicate key effector or regulatory cytokines secreted by differentiated cells of that lineage. Transcription factors for each lineage are placed in the nucleus. Tn: naïve postthymic CD4⁺ T cell precursor.

Th cell differentiation is mediated by various signaling proteins, including STAT4 and STAT6, and results in expression of the transcription factors GATA-3 and c-Maf (Th2 cells) or T-bet (Th1 cells) (Zheng and Flavell, 1997), (Szabo et al., 2000), (Mullen et al., 2001). Th1 cells

produce inflammatory cytokines, such as TNF, IFN- γ , and promote macrophage-mediated cellular immunity against intracellular infections and malignancy. Th2 cells secrete IL-4, IL-6 and IL-13, promoting antibody production by B cells to extracellular pathogens and allergic reactions.

Recently, a subset of IL-17-producing T (Th17) cells has been described and shown to play a crucial role in the induction of autoimmune tissue injury (Langrish et al., 2005), (Weaver et al., 2007). The key transcription factor for Th17 cell differentiation is the orphan nuclear receptor ROR γ t, which induces the transcription of genes encoding IL-17 (Ivanov et al., 2006). TGF β together with IL-6 and IL-23 appear to be required for the generation and maintenance of these cells (Bettelli et al., 2006, Veldhoen, 2006 #641).

In contrast to the before mentioned T helper cell subpopulations that exert immune responses, Treg (described in more detail in chapter 4.3) cells are important regulators of the immune system. Treg cells are able to inhibit the activation of other immune cells and thereby prevent autoimmunity and protect against tissue injury. Treg cells are characterized by the expression of the transcription factor FOXP3 and the production of TGF β and IL-10 (Fontenot et al., 2005), (Nakamura et al., 2001). The induction and maintenance of Treg cells seems to be dependent on TGF β (Chen et al., 2003), (Liu et al., 2008). Therefore, TGF β plays a dual role in T cell differentiation as it can induce anti-inflammatory Treg cells, but in the presence of IL-6, it promotes the development of inflammatory Th17 T cells.

4.2.3 Memory T cells

In the final phase of an immune response, most activated T cells (~90%) are deleted mainly by apoptosis, but a small fraction of cells survives and remains as a long-lived population of memory T cells (Sallusto et al., 1999), (Sallusto et al., 2004). The purpose of maintaining memory T cells is to provide a more efficient immune response to secondary infections. Memory T cells can be further divided into two subsets with distinct homing potentials and effector functions (Sallusto et al., 1999). Central memory T cells were identified to express the chemokine receptor CCR7 and the adhesion molecule CD62L (L-selectin) and were shown to home to T cell areas of secondary lymphoid organs (Iezzi et al., 2001). Upon TCR stimulation, central memory T cells first produce IL-2, but after proliferation they differentiate into effector cells and produce large amounts of effector cytokines, such as IL-4 or IFN- γ . On the contrary, effector memory T cells have lost CCR7 expression and are mainly negative for CD62L, migrate to inflamed peripheral tissues and are characterized by their ability to exert effector functions immediately upon activation (Masopust et al., 2001).

4.2.4 T cell homeostasis

While much is known about activation, differentiation, and expansion of lymphocytes involved in antigen responses, the maintenance of lymphocyte homeostasis before and after an immune response is less well understood (Chen, 2004; Greenwald et al., 2005; Grossman et al., 2004; Jameson, 2002; Seddon and Zamoyska, 2003). As suggested by Grossman and colleagues, T cell homeostasis requires temporary T cell activation followed by either self-renewal or differentiation (Grossman et al., 2004). T cell homeostasis was proposed to be regulated by several feedback loops most likely on the level of antigen presenting cells. The mechanisms involved are supposed to function in each differentiation compartment from the naïve to the memory T cell stage.

Using genetic approaches in murine model systems, it became apparent that internal stimuli, in particular self-peptide–MHC ligands for the T cell receptor and interleukin-7 receptor (IL-7R) interactions are essential for T cell survival and homeostasis (Brocker, 1997; Kirberg et al., 1997; Takeda et al., 1996) (Boise et al., 1995; Schluns et al., 2000; Tan et al., 2001; Vella et al., 1997; Vella et al., 1998). The necessity of MHC / TCR interaction for T cell maintenance was further supported by TCR α chain depletion in mature T cells resulting in a decay of T cells over time (Polic et al., 2001). In addition to IL-7 other common γ -chain cytokines such as IL-4 or IL-15 were shown to support survival of naïve T cells *in vitro* but IL-7 seems to be the dominant cytokine *in vivo* (Schluns et al., 2000; Tan et al., 2001). IL-15 can augment the homeostatic proliferation of naïve T cells, but important components of the IL-15 receptor are not expressed until naïve T cells begin homeostatic proliferation in response to IL-7 (Jameson, 2002). Nevertheless, genetic disruption of the common γ -chain receptor (present in the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors) or components of its signaling pathway results in impaired homeostasis of CD4⁺ T cells (Nakajima et al., 1997; Sohn et al., 1998), (Sadlack et al., 1995). Since the above mentioned genes are also necessary for T cell activation during immune responses, mechanisms must have evolved that allow T cells to choose between survival and homeostatic proliferation on the one hand and activation and immune response-associated proliferation on the other hand. Several explanations have been proposed so far, including inhibition by physical T cell-T cell interaction, competition for limiting resources, e.g. of IL-7 or involvement of inhibitory factors (Geiselhart et al., 2001; Kieper et al., 2002; Tan et al., 2001). In fact, genetic disruption of several genes encoding inhibitory factors, such as transforming growth factor- β (TGF β), IL-10 or CTLA-4, leads to significant disturbances of T cell biology resulting in severe lymphoproliferative diseases (Gorelik and Flavell, 2000; Kulkarni et al., 1993; Lucas et al., 2000; Shull et al., 1992; Takahashi et al., 1994; Tivol et al., 1995; Watanabe-Fukunaga et al., 1992). CTLA-4^{-/-} mice are characterized by a spontaneous lethal

lymphoproliferative disease with infiltrates of highly activated T cells in many tissues (Tivol et al., 1995). The role of CTLA-4 during thymic development and the requirement of T cell activation prior to cell surface expression suggests that CTLA-4 plays a role during T cell activation rather than being directly involved in regulation of peripheral T cell survival and homeostasis (Greenwald et al., 2005; Inobe and Schwartz, 2004). In comparison to CTLA-4, a different phenotype has been shown for IL-10-deficient mice. They show less acute inflammatory diseases, which are mostly manifested in the intestine (Kuhn et al., 1993). This bowel disease may be due to uncontrolled chronic inflammation induced by enteropathogens.

In mice there is clear evidence that TGF β is an important factor restraining the size of the T cell compartment (Christ et al., 1994; Gorelik and Flavell, 2000, 2002; Lucas et al., 2000; Yang et al., 1999). Mice defective in TGF β 1 develop symptoms of a lymphoproliferative disease 2-3 weeks after birth. Due to the pleiotropic effects of TGF β 1, studying the role of TGF β 1 on T cell homeostasis in TGF β 1^{-/-} mice is hampered by unrelated effects also responsible for the observed phenotype (Gorelik and Flavell, 2002; Lucas et al., 2000). To circumvent such limitations, transgenic mice with a dominant-negative TGF β -receptor II specifically expressed in T cells were previously introduced by Gorelik and colleagues (Gorelik and Flavell, 2000, 2002). Mice expressing such dominant-negative TGF β -RII within the T cell compartment show disruption of homeostasis, a strong inflammatory response and significant signs of autoimmunity (Gorelik and Flavell, 2000; Lucas et al., 2000). These mice are characterized by a reduced number of naïve T cells with a concomitant increase of memory T cells, of which a significant fraction demonstrates an activated phenotype. Furthermore, these cells show spontaneous differentiation *in vitro* in response to T cell activation.

Taken together, numerous inhibitory mechanisms have been revealed in murine models to be responsible for preventing T cells from being activated. However, even in the murine system their integration into homeostatic circuits is not fully understood.

In humans even less is known about the major signals involved in T cell homeostasis, which is mainly due to the inability of *in vivo* manipulation of single genes. It is tempting to speculate that the same factors involved in murine T cell homeostasis are also involved in human T cell homeostasis, especially since many of these factors have similar roles during induction of immunity. However, so far no experimental evidence exists supporting such postulate.

4.3 Regulatory T cells

Homeostatic control of the immune system is important to prevent excessive inflammation and/or autoimmunity. Autoimmune disorders are characterized by a breakdown in the mechanisms of tolerance to self-antigens. The negative selection of autoreactive T cells in the

thymus is the first self-tolerance mechanism (central tolerance), but even if it is highly efficient, a number of autoreactive cells overcome the selection barrier (Sakaguchi, 2000). As a result, autoreactive T cells are present in all individuals, and these T cells may be activated in the periphery leading to autoimmune responses (Shevach, 2000). However, as the percentage of individuals that develop autoimmunity is very low, mechanisms must exist that mediate peripheral tolerance to silence potentially pathogenic autoreactive T cells.

Besides the inhibitory cytokines described before, many cell types have been shown to possess the capacity to regulate immune responses, of which CD4⁺CD25⁺ Treg cells play the most important role. Treg cells are able to inhibit cells involved in both, innate and adaptive immune mechanisms. In humans, CD4⁺CD25⁺ Treg cells comprise approximately 5-10% of the circulating CD4⁺ T cells. Treg cells have been associated with multiple markers including CD25 (IL-2 receptor α chain), the T cell inhibitory receptor CTLA-4, the glucocorticoid-inducible tumor necrosis factor receptor GITR, CD69 and CD44. However, most of these molecules are also upregulated during T cell activation and thus are present on the cell surface of effector T cells. The transcription factor FOXP3 represents the most specific marker identified so far (Fontenot et al., 2005). More recently, downregulation of CD127 in combination with FOXP3 or CD25 staining has been described as the most specific approach to identify Treg cells (Liu et al., 2006), (Seddiki et al., 2006).

Natural Treg cells originate in the thymus during the process of negative selection in the thymus. Some highly self-reactive T cells avoid destruction and instead differentiate into natural Treg cells, which are able to survive in the periphery (Sakaguchi, 2004), (Kronenberg and Rudensky, 2005), (Lio and Hsieh, 2008). Furthermore, Treg cells are generated in the periphery by the conversion of conventional CD4⁺CD25⁻ T cells into Treg cells (induced Treg cells). Induced Treg cells develop as a consequence of activation of naïve CD4⁺CD25⁻ T cells under particular conditions of sub-optimal antigen and a certain cytokine milieu, such as TGF β , IL-10 or PGE₂ (Chen et al., 2003), (Tran et al., 2007), (Sharma et al., 2005), (Barrat et al., 2002), (Wakkach et al., 2003). A small proportion of these induced Treg cells is thought to remain in the periphery and to contribute to the circulating Treg cell pool. This would ensure that Treg cells have a very broad reactivity against self and foreign antigens (Pillai and Karandikar, 2007). This hypothesis is supported by data demonstrating that the TCR repertoire of Treg cells is similar to the one of naïve T cells (Akbar et al., 2007), (Pacholczyk et al., 2007). Until now, the exact mechanism for Treg cell-mediated suppression remains largely unknown. One suppressive mechanism of natural Treg cells is described to be cell-cell-contact-dependent. Upon activation, natural Treg cells express on their cell surface CTLA-4 and TGF β 1 (Nakamura et al., 2001), (Ostroukhova et al., 2006). Interactions of these two surface molecules with their cognate receptors on the target cell were demonstrated to suppress the

proliferation and cytokine production of the target cell (Nakamura et al., 2001), (Thornton and Shevach, 2000), (Piccirillo and Shevach, 2001), (Taylor et al., 2004). Further, Treg cells have been shown to secrete soluble inhibitory factors (IL-4, IL-10, IFN- γ , soluble TGF β), thereby inhibiting the activation of target cells (Wakkach et al., 2003), (Tang et al., 2004), (Asseman et al., 1999), (Birebent et al., 2004), (Chen and Wahl, 2003), (Fantini et al., 2004). The third suppressive mechanism of Treg cells is a passive mechanism. Treg cells constitutively express high levels of CD25 on their surface (IL-2 receptor α chain). Activated Treg cells compete with naïve T cells for IL-2, an essential T cell growth factor, leading to the inhibition of naïve T cell activation (Barthlott et al., 2005), (de la Rosa et al., 2004), (Pandiyan et al., 2007). Dysregulated Treg cell numbers and/or function are responsible for many diseases. Mice with a naturally occurring mutation in the FOXP3 gene (scurfy mouse), develop fatal autoimmune diseases due to the absence of functional Treg cells (Brunkow et al., 2001). Similar, inactivating mutations in the human homologue of FOXP3 lead to a severe disease termed IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) leading to the early death of newborns (Bennett et al., 2001). Furthermore, Treg cells are thought to be involved in multiple clinical scenarios such as autoimmune disorders, asthma, cancer and infection. Reduced numbers or decreased suppressive activity of Treg cells has been reported in several autoimmune diseases (Baecher-Allan and Hafler, 2004), (Viglietta et al., 2004), (Sugiyama et al., 2005), (Kriegel et al., 2004). In contrast, patients with malignancies show increased Treg cell numbers and function (Beyer and Schultze, 2006), (Liyanage et al., 2002), (Curiel et al., 2004).

4.4 Transforming Growth Factor β

Transforming growth factor- β (TGF β) seems to play a unique role in the control of murine T cell homeostasis, as it can directly control T cell activation but also has an indirect effect on T cell regulation through its important function in development and maintenance of suppressive Treg cells. Like dysregulated Treg cell functions, malfunctions in signaling downstream of TGF β are implicated in serious human diseases such as cancer, fibrosis and wound-healing disorders (Wakefield and Sporn, 1990), (Massague et al., 2000). Deficiency of TGF β 1 results in an early autoinflammatory phenotype in mice (Kulkarni et al., 1993), (Shull et al., 1992), which is associated with a progressive infiltration of lymphocytes into multiple organs (Dang et al., 1995), (Yaswen et al., 1996). T cells appear to be key mediators of the autoimmune disease in these mice, because depletion of CD4⁺ or CD8⁺ T cells decelerates the inflammatory phenotype (Kobayashi et al., 1999), (Letterio et al., 1996).

The TGF β family consists of more than 35 members of regulatory molecules that play an essential role in cell growth, adhesion, migration, cell-fate determination, differentiation and apoptosis (Gorelik and Flavell, 2002), (Li et al., 2006b), (Schmierer and Hill, 2007). There are three TGF β isoforms identified in mammals, TGF β 1-3, which signal through two transmembrane serine-threonine kinase receptors, TGF β receptor I and II (TGF β RI, TGF β RII) (Massague, 1998). Among the three isoforms, TGF β 1 is predominantly expressed in the immune system (Govinden and Bhoola, 2003). TGF β is synthesized in an inactive form, the pre-pro-TGF β precursor. Additional stimuli are required to release active TGF β , enabling it to exert its function by binding to its receptor (Annes et al., 2003), (Munger et al., 1999). Latency associated protein (LAP) or latent TGF β binding protein (LTBP) are non-covalently bound to TGF β , keeping it inactive. Proteolytic processing leads to the dissociation of these inhibitors, leading to the release of active TGF β (Dubois et al., 1995). The active form of TGF β 1 is a 25kD homodimer connected by three disulfide bonds (Shi and Massague, 2003). Active TGF β can function either in a cell surface-bound form or as a soluble factor (Letterio and Roberts, 1998).

4.4.1 The TGF β signaling pathway

Activated TGF β binds to a heterodimeric receptor complex consisting of type I and II transmembrane serine/threonine kinase subunits. Seven type I (activin-like receptor kinase (ALK) family) and five type II receptors have been reported (Chang et al., 2002).

Binding of the TGF β dimer to TGF β RII activates the receptor and leads to the formation of a receptor complex with TGF β RI. Thereby, the active kinase domain of the type II receptor phosphorylates the receptor cytoplasmic GS region of TGF β RI, leading to its activation. Intracellular signaling is then mediated through phosphorylation of the SMAD proteins in the flexible SSXS motif (Massague, 1998). Until now, eight vertebrate SMAD proteins are identified and are divided into three categories: five receptor-associated SMADs (R-SMAD1, 2, 3, 5, 8), one common SMAD (Co-SMAD4), and two inhibitory SMADs (I-SMAD6, 7).

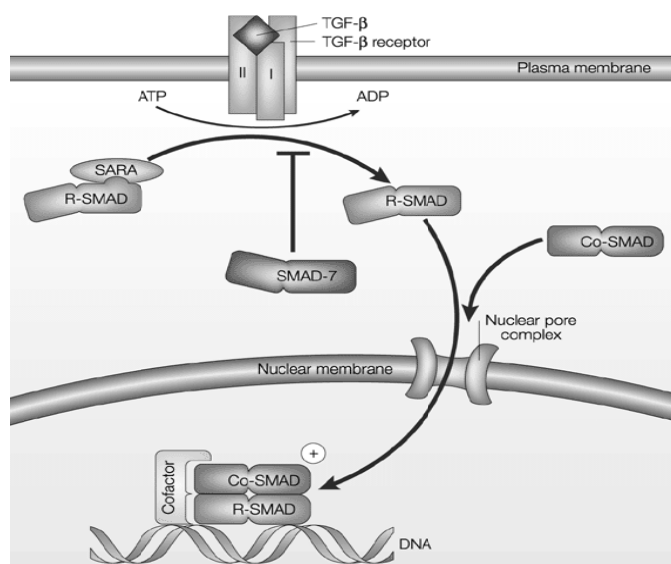


Figure 2: The TGF β signaling pathway

TGF β - binding leads to the formation of two type I and two type II transforming growth factor- (TGF-) receptors. The serine/threonine protein kinase domain of type II receptor then phosphorylates and activates the kinase domain of type I receptor, which catalyses the phosphorylation of regulatory-SMAD (R-SMAD). Phosphorylation reduces the affinity of R-SMADs for SARA (SMAD anchor for receptor activation), and increases their affinity for co-SMADs. The resulting SMAD complex translocates into the nucleus and associates with transcriptional co-activators or co-repressors to regulate gene expression. This scheme was copied from (Gorelik and Flavell, 2002).

Upon stimulation, the active TGF β RI receptor phosphorylates R-SMAD2 and 3, thereby leading to the dissociation of R-SMADs from the inhibitor SARA (SMAD anchor for receptor activation). Phosphorylated R-SMADs form complexes with SMAD4, translocate into the nucleus and bind to DNA containing a SMAD-binding element (SBE) (Inman et al., 2002), (Shi et al., 1998) (Figure 2). SMAD molecules itself can bind to DNA, but effective binding to particular gene-regulatory sites is enabled by specific DNA-binding cofactors. The SMAD complex can interact with multiple co-factors, including co-activators as well as co-repressors, to regulate TGF β target gene expression in a cell-type and cell-state specific manner (Massague, 2000). Both, R-SMAD phosphorylation and SMAD nuclear accumulation are only maintained as long as the receptors are active. Upon decreasing receptor activity, dephosphorylation as well as proteasome-mediated degradation of the R-SMADs occurs. Thereby, the nuclear accumulation of R-SMADs is lost (Nicolas et al., 2004).

Further, the TGF β signaling pathway is negatively regulated by the induction of I-SMADs (Itoh and ten Dijke, 2007). SMAD7 suppresses TGF β signaling through at least two mechanisms: 1. SMAD7 competes with R-SMADs for the binding to the ALK5 receptor. 2. SMAD7 recruits the ubiquitin ligase complex to degrade ALK5 via the proteasome (Kavsak et al., 2000).

4.4.2 TGF β effects on immune cells

The inhibitory effect of TGF β on the immune system was first described in 1986, where TGF β was found to inhibit the proliferation of human B and T cells (Kehrl et al., 1986a), (Kehrl et al., 1986b). Since then, extensive studies were performed in which important and pleiotropic roles of TGF β in the regulation of the immune system were corroborated. The critical role of TGF β in regulating immune suppression under physiological conditions has been demonstrated in TGF β ^{-/-} mice. These mice develop a severe, multifocal inflammatory response associated with

increased inflammatory cytokine production (Shull et al., 1992), (Kulkarni et al., 1993), (Rudner et al., 2003). This phenotype is predominantly mediated through helper T cells, as crossing these mice to mice lacking MHC class II molecules prevented the inflammation (Letterio et al., 1996).

TGF β influences cells of the innate immunity as well as cells of adaptive immunity (Figure 3) (Cazac and Roes, 2000), (Gorelik and Flavell, 2000), (Laouar et al., 2005). This factor prevents the maturation of DCs by maintaining a low expression of co-stimulatory molecules, leading to the reduction of their T cell stimulatory capacity (Geissmann et al., 1999). The cytokine production and cytolytic activity of NK cells is decreased by TGF β (Li et al., 2006b). Immune activating mechanisms of TGF β involve its chemo-attractive role for granulocytes, mast cells and monocytes in inflammatory areas (Li et al., 2006b). In addition, the phagocytic potential of macrophages can be enhanced by this factor (Wahl et al., 2006).

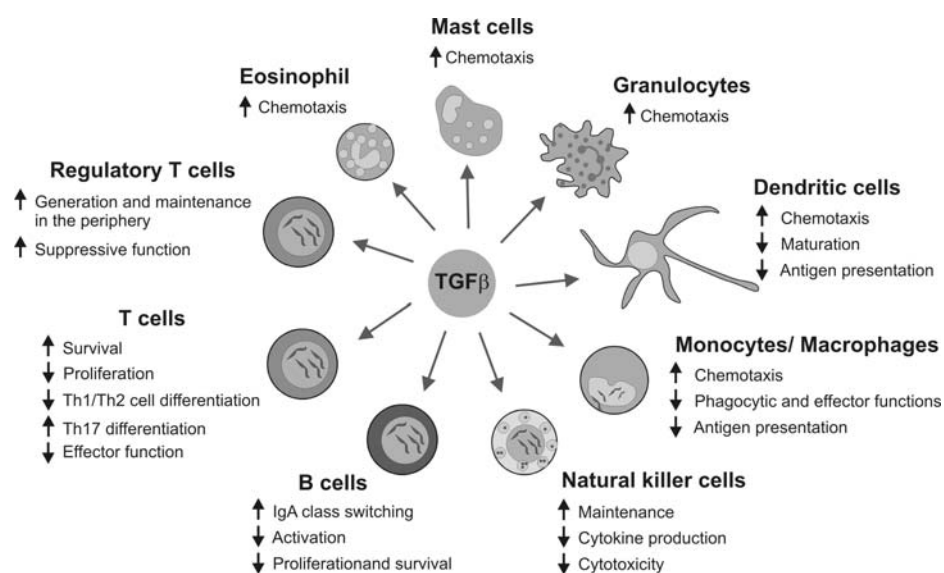


Figure 3: TGF β effects on cells of the innate and the adaptive immunity

Positive or negative effects of TGF β signaling on the indicated aspects of leukocyte biology are marked by arrows pointing up or down. Only cellular processes with a well-documented role for TGF β are indicated in this figure. This scheme was adapted from (Rubtsov and Rudensky, 2007).

Furthermore, TGF β enhances antigen presentation by B cells and induces the switch from IgM to IgA production (Arai et al., 2003). TGF β also decreases B cell activation and proliferation (Lebman and Edmiston, 1999) (Figure 3).

The role of TGF β in controlling T cell functions and immune responses has been studied extensively in murine models (Li et al., 2006b). TGF β suppresses T cell function on multiple levels. TGF β can inhibit T cell proliferation by blocking the production of IL-2, a cytokine known to potently activate T cells, NK cells and other immune cells (Kehrl et al., 1986b), (Das and Levine, 2008), (Nelson et al., 2003). Because addition of exogenous IL-2 did not fully reconstitute T cell proliferation, other mechanisms have to be involved in TGF β -mediated inhibition of T cell proliferation. For example, TGF β 1 inhibits promotes the expression of IL-10,

an anti-inflammatory cytokine, likely through direct activation of the IL-10 promoter by SMAD4 (Kitani et al., 2003).

Additionally, TGF β treatment leads to a block in cell cycle progression through the upregulation of cyclin-dependent kinase inhibitors, e.g. p15, p21 and p27, whereas cell cycle-promoting factors, e.g. c-myc, CDK2 and cyclin E are downregulated (Datto et al., 1995), (Polyak et al., 1994), (Wolfrain et al., 2004).

Moreover, this factor potently inhibits the expression of t-bet and Gata-3, thereby preventing the differentiation into Th1 and Th2 cells, respectively (Gorelik et al., 2002), (Gorelik et al., 2000), (Heath et al., 2000), (Lin et al., 2005). The development of Th2 cells seems to be more sensitive to TGF β -mediated suppression, as TGF β completely inhibits Th2 differentiation under all tested conditions. In contrast, the ability of this factor to suppress Th1 differentiation depends on many conditions, including the mouse strain and the strength of T cell co-stimulation (Sad and Mosmann, 1994), (Hoehn et al., 1995).

TGF β was also shown to be involved in the differentiation of immune cells. TGF β promotes the development and function of inhibitory Treg cells (Chen et al., 2003), (Fantini et al., 2004). But in the presence of IL-6, TGF β induces the development of inflammatory Th17 T cells, which produce IL-17, IL-21 and IL-22 (Veldhoen et al., 2006), (Mangan et al., 2006), (Weaver et al., 2006).

Besides the regulation of T cell function and differentiation, TGF β controls peripheral T cell apoptosis. During apoptosis, TGF β also plays a dual role, as it can suppress as well as induce apoptosis, depending on the cell type and state (Siegel and Massague, 2003). On the one hand, TGF β 1 can inhibit Fas ligand expression and subsequent activation-induced cell death in T cells through downregulation of c-Myc (Genestier et al., 1999). This result is supported by the finding that T cells deficient for TGF β 1 undergo apoptosis (Bommireddy et al., 2003). On the other hand, TGF β can induce apoptosis in lymphocytes by several mechanisms: 1. generation of reactive oxygen species, 2. shifting the balance between anti-apoptotic and pro-apoptotic molecules to pro-apoptotic conditions, 3. downregulation of NF κ B and 4. caspase activation 2, 3, 7, 8 and 9 (Arsura et al., 1996), (Inman and Allday, 2000), (Motyl et al., 1998), (Saltzman et al., 1998). TGF β induced apoptosis is mediated predominantly via the SMAD signaling pathway (Yu et al., 2008).

TGF β also controls cytotoxic CD8⁺ T cells, as it regulates proliferation and effector functions, e.g. the expression of effector molecules, such as IFN- γ and perforin are inhibited by TGF β (Ahmadzadeh and Rosenberg, 2005), (Smyth et al., 1991) (Figure 3).

4.4.3 Role of TGF β in cancer

Dysregulated TGF β function has been implicated in the pathogenesis of cancer, atherosclerosis, and autoimmune and inflammatory diseases (Derynck et al., 2001), (Massague et al., 2000). The role of TGF β in tumorigenesis is particularly complex. In early stages of tumor development, TGF β serves as a tumor suppressor, but once tumors are developing, TGF β enhances tumor progression (Cui et al., 1996). Elevated levels of TGF β were often observed in cancer patients due to overexpression of TGF β by tumor cells (Arteaga, 2006), (Elliott and Blobel, 2005), (Baker et al., 2007). Tumor cells develop several mechanisms to evade the growth inhibitory effect of TGF β , for example mutations in the TGF β R or the complete loss of the receptor (Siegel and Massague, 2003). Furthermore, TGF β promotes the development of a tumor microenvironment that enhances tumor progression (Carmeliet, 2003). In contrast, cells of the immune system are inhibited by TGF β , thereby preventing an anti-tumor response. Additionally, increased levels of TGF β are associated with poor pathological or clinical outcomes including shorter survival time (Reiss, 1999).

Therefore, it is apparent that the TGF β signaling pathway may be a potential therapeutic target to prevent its numerous disease-promoting effects. Several mouse studies already showed that blockade of the TGF β signaling pathway suppressed tumorigenicity and metastasis formation (Muraoka et al., 2002), (Arteaga et al., 1993). Additionally, two groups were able to show, that long-term exposure of TGF β antagonists protected mice against experimental metastasis without significant adverse side events (Bandyopadhyay et al., 2002), (Yang et al.). Furthermore, several studies demonstrated efficacy of small molecules inhibiting the TGF β RI kinase (Yingling et al., 2004), (Singh et al., 2004). Small molecules have the great advantage of precise dosage, high specificity and less unwanted side effects in comparison to other clinical strategies, such as neutralizing antibodies, oligonucleotides or large molecules (Sawyer et al., 2004), (DaCosta Byfield et al., 2004), (Ge et al., 2004). In the case of the small molecule SD-208 (Scios Inc., Fremont, CA), antitumor efficacy correlated with immune-cell infiltration into responding tumors (Uhl et al., 2004). In addition, the TGF β RI kinase inhibitor LY215799 developed by Eli Lilly showed anti-tumor responses in non-small cell lung cancer and breast cancer mouse models (Bueno et al., 2008).

These results highlight the therapeutic potential of preventing TGF β -mediated immune-suppression to generate a significant immunological response. But one has to remember that targeting the TGF β signaling pathway also exhibits a high risk of unwanted side effects, as TGF β null mice showed severe auto-inflammatory diseases and spontaneous tumorigenesis (Kulkarni et al., 1993), (Shull et al., 1992). Thus, one has to precisely control a clinical trial to immediately detect unwanted side effects. Furthermore, a prerequisite of targeted therapy is to

identify the biologically optimal dose rather than the maximum tolerated dose (Eisenhauer, 1998). Therefore, the goal of a clinical trial is a highly controlled therapy with personalized dosage. Both, controlled therapy as well as personalized dosage can be achieved by the implementation of biomarkers. Biomarkers can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Trusheim et al., 2007). mRNA levels as well as protein amounts of either single genes or a group of genes can be monitored as biomarkers.

4.5 TGF β and Treg interaction

Besides the direct immune suppressive mechanism of TGF β this factor also was shown to play an important role in the regulation of the peripheral Treg cell pool and Treg cell function.

In peripheral lymphoid organs the frequency of CD4⁺CD25⁺ T cells and their expression of FOXP3 was increased by the overexpression of TGF β (Huber et al., 2004). Similarly, Treg cells were decreased by impaired TGF β signaling in T cells. Consistently, TGF β 1-deficient mice fail to maintain Treg cells in the periphery due to enhanced proliferation leading to accelerated apoptosis (Marie et al., 2005), (Li et al., 2006a). Recently it was demonstrated that TGF β was not only implicated in the maintenance of peripheral Treg cells, but also in the generation of natural Treg cells in the thymus (Liu et al., 2008). Further studies revealed a role of TGF β in the generation of induced Treg cells, as murine naïve CD4⁺CD25⁻ T cells can be converted into FOXP3 expressing CD4⁺CD25⁺ T cells in the presence of TCR stimulation and TGF β (Chen et al., 2003). These induced Treg cells were anergic and showed suppressive capacities (Chen and Wahl, 2003). Subsequently it was shown that TGF β also induces the expression of FOXP3 in activated human CD4⁺CD25⁻ T cells (Fantini et al., 2004), (Hill et al., 2007), (Davidson et al., 2007). This conversion is due to TGF β -induced *de novo* expression of FOXP3 (Wan and Flavell, 2005). The transcription factors SMAD3 and NFAT are essential for the activation of an enhancer region in the FOXP3 gene, leading to FOXP3 expression (Tone et al., 2008). In contrast, several publications have shown that human CD4⁺CD25⁻ T cells express FOXP3 upon activation even in the absence of TGF β (Pillai et al., 2007), (Wang et al., 2007), (Allan et al., 2007). This transient induction of FOXP3 was implicated with suppressive capacity of those cells in some studies (Walker et al., 2005), (Walker et al., 2003), (Wang et al., 2007), whereas in other studies no inhibitory capacity of activation induced Treg cells was detectable (Tran et al., 2007), (Allan et al., 2007), (Morgan et al., 2005), (Allan et al., 2005). Differences in the suppressive capacity of naturally and induced Treg cells might be explained by the finding that the FOXP3 promoter was demethylated in naturally Treg cells but not in induced Treg cells (Baron et al., 2007).

TGF β seems to be involved not only in the generation and maintenance of Treg cells, but also in their suppressive function. In contrast to naïve T cells, Treg cells produce the active form of surface bound TGF β (Green et al., 2003), (Ostroukhova et al., 2006), (Nakamura et al., 2001). Moreover, active SMAD signalling was observed in the responder T cell upon contact with the Treg cell (Chen and Wahl, 2003), (Nakamura et al., 2001). One study showed that during the contact between Treg cell and responder T cell, TGF β was concentrated at the point of cell-cell contact in response to anti-CTLA-4 treatment (Oida et al., 2006). Thus, besides the direct involvement of CTLA-4 in Treg cell-mediated suppression this study provides an indirect role of CTLA-4, as it may enhance the TGF β signal intensity. Furthermore, Treg cell mediated inhibition was abolished by the blockade of the TGF signalling pathway either by anti-TGF β antibodies or by recombinant latency associated peptide (Nakamura et al., 2004), (Oida et al., 2006). In addition, T cells from CD4-dnTGF β RII mice that are unresponsive to TGF β cannot be controlled by Treg cells, showing an important role of this pathway in suppression (Fahlen et al., 2005), (Chen et al., 2005). On the other hand, studies exist where the administration of anti-TGF β or soluble TGF β receptor did not reverse Treg cell mediated suppression (Piccirillo et al., 2002), (von Boehmer, 2005), (Kullberg et al., 2005), (Shevach et al., 2001). In addition, CD4⁺ T cells with disrupted TGF β signalling were still susceptible to Treg cell-mediated suppression (Piccirillo et al., 2002).

These results lead to the suggestion, that indeed the TGF β pathway is involved in Treg cell-mediated inhibition, but contradicting data reveal the necessity for further examination of the interaction of Treg cells and TGF β in the regulation of immune functions.

4.6 SATB1

As already mentioned before, SATB1 (special AT-rich-binding protein 1) is one of the most prominent chromatin organizers in T cells (Dickinson et al., 1992), (Alvarez et al., 2000), (Cai et al., 2003). SATB1 is localized in the nucleus and it surrounds heterochromatin. It binds to the minor groove of DNA, especially at the matrix attachment regions (MARs) of the DNA. SATB1 seems to anchor loops of chromatin forming a cage-like network structure (Cai et al., 2003). This protein interacts directly with several chromatin-remodeling complexes and is thought to target them to the DNA, thereby regulating chromatin structure and gene expression (Yasui et al., 2002). Unlike classical transcription factors, SATB1 regulates, not only the expression of individual target genes, but orchestrates the transcription of multiple genes even over long-distances. For example, in interaction with the transcription factor promyelotic leukemia (PML), which is attached to the nuclear body, SATB1 organizes the major histocompatibility complex (MHC) class I locus.

SATB1-deficient mice are characterized by a diminished thymus and spleen and die at the age of 3 weeks (Alvarez et al., 2000). Furthermore, they show a block at the double-positive stage of thymocyte development. The few peripheral CD4⁺ T cells that were able to develop, underwent apoptosis and failed to proliferate in response to activating stimuli. SATB1-deficient T cells exhibit a dysregulated expression of many genes. As genes were upregulated as well as downregulated in the absence of SATB1, this protein acts as both, a transcriptional repressor and activator. One gene that is ectopically expressed in SATB1-deficient T cells is *IL2ra*, which displayed an altered nucleosome organization as well as hyperacetylated histones. Yasui et al. could demonstrate, that indeed SATB1 recruits an histone deacetylase complex to the MAR site within the *IL2ra* gene, in order to repress its expression in premature T cells (Yasui et al., 2002).

In addition, post-translational modification of SATB1 may regulate its interaction with different types of chromatin-remodeling complexes and may induce its conversion from a repressor to an activator. Exemplary, the *IL2* promoter is suppressed by phosphorylated SATB1 in resting T cells, whereas T cell activation leads to the dephosphorylation of SATB1. Subsequently, SATB1 gets acetylated and dissociates from the promoter region leading to *IL2* gene transcription (Pavan Kumar et al., 2006).

Upon Th2 cell activation, SATB1 is highly upregulated and leads to the formation of a unique transcriptionally active chromatin structure at the Th2 cytokine locus (Cai et al., 2006). This process includes the binding of GATA3, STAT6 and c-Maf. SATB1 orchestrates the compacting of the chromatin into dense loops at the 200-kb cytokine locus, thereby inducing the expression of the Th2 specific cytokines *IL-4*, *IL-6* and *IL-13*. Thus, SATB1 is an essential factor for the transcriptionally active chromatin structure that forms upon Th2 cell activation (Cai et al., 2006).

These data demonstrate an important role of SATB1 in T cell development and Th2 differentiation. However, whether this factor is implicated also in the differentiation of other CD4⁺ T cell subpopulations, such as Th1 cells, memory cells and Treg cells is not yet investigated. Moreover, the regulation of SATB1 is not yet understood.

4.7 Objectives

Soluble factors, such as PGE₂, CTLA-4 and TGF β , were shown to inhibit T cell proliferation. Besides these factors, also regulatory T cells are involved in the regulation of T cell activation. However, the integration of these soluble inhibitory factors and suppressive Treg cells in the control of CD4⁺ T cells is not completely understood. Especially the interaction of TGF β and Treg cells is complex and many aspects of this interaction are still unknown, particularly in human T cells. Therefore, we were interested in the following aspects of human CD4⁺ T cell immunology:

First, we asked the question: which are the main factors keeping human CD4⁺ T cells at a resting state? T cell homeostasis seems to be mainly regulated by exogenous stimuli. Therefore, we hypothesized that deprivation of resting human T cells of any exogenous signals should reverse intracellular signaling cascades actively controlling the homeostatic state of CD4⁺ T cells. We further postulated that such changes should be recognizable on the genomic level, leading to the identification of the factors controlling CD4⁺ T cell homeostasis.

The transcriptome analysis revealed that TGF β signaling is constantly active in human CD4⁺ T cells. Therefore, we postulated that abrogation of TGF β signaling in response to clinical therapy would be visible in the transcriptional profile of peripheral CD4⁺ T cells. We further hypothesized that this analysis would reveal genes, which can function as biomarkers for anti-TGF β therapy.

Next, we were interested in the inhibitory mechanisms of the soluble factor TGF β and Treg cells and their interplay. Although this has been extensively studied, the results obtained so far by different groups are contradictory. Moreover, data concerning the inhibitory function of TGF β on T cells have mainly been obtained prior to the re-introduction of suppressor or better regulatory T cells by Sakaguchi in 1995. We therefore re-assessed the specific issue, whether TGF β exerts its inhibitory function on human CD4⁺ T cells directly, or whether it always relies on the presence of Treg cells. Analogous, we examined whether the suppressive mechanism of Treg cells is entirely dependent on TGF β or not.

In the human system, the necessity of TGF β in the induction of so-called 'induced' Treg cells is still under debate. We postulated that transcriptional analysis of different kinds of 'induced' Treg cells would provide important insight into the degree of relationship of natural and induced Treg cells.

Within this transcriptome analysis, the chromatin organizer SATB1 was identified to be differentially expressed in Treg cells. We therefore went on to further study the potential mechanisms leading to downregulation of a chromatin-remodeling enzyme in Treg cells.

5 Material and Methods

5.1 Materials

5.1.1 Reagents

Chemicals were purchased from Merck (Darmstadt), Roth (Karlsruhe) or Sigma (Deisenhofen) unless stated otherwise. Solutions were prepared with double distilled water (ddH₂O).

AIM-V	Invitrogen Life Technologies, Karlsruhe DE
β-Mercaptoethanol	Sigma-Aldrich, München, DE
BCA protein assay kit	Pierce, Rockford, US
CFSE	Sigma-Aldrich, München, DE
Chloroform	Sigma-Aldrich, München, DE
Complete, Mini	Roche Diagnostics GmbH, Mannheim, DE
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, München, DE
Dynalbeads [®] M-450	Dynal Biotech, Oslo, NO
Ethylendiamintetraacetat (EDTA)	Sigma, St Louis, US
Ethanol	Roth, Karlsruhe, DE
ExCell 640	JRH Bioscience, Lenexa, US
ExVivo	Lonza, Basel, CH
Ficoll-Paque [™] PLUS	Amersham, Piscataway, US
Fetal calf serum (FCS)	Invitrogen Life Technologies, Karlsruhe, DE
Formaldehyde	Sigma-Aldrich, München, DE
Glutamax	Invitrogen Life Technologies, Karlsruhe, DE
H ₂ O (sterile)	Fresenius Kabi AG, Bad Homburg, DE
Hydrochlorid acid (37 %)	Merck, Darmstadt, DE
Interleukin 2 (IL-2)	Immunotools, Friesoythe, DE
Interleukin 4 (IL-4)	Immunotools, Friesoythe, DE
Interleukin 12 (IL-12)	Immunotools, Friesoythe, DE
Isopropanol	Roth, Karlsruhe, DE
Methanol	Roth, Karlsruhe, DE
MicroBeads CD25 ⁺	Miltenyi Biotech, Bergisch Gladbach, DE
MicroBeads CD45RA ⁺	Miltenyi Biotech, Bergisch Gladbach, DE
NuPAGE [®] Transferpuffer 20x	Invitrogen Life Technologies, Karlsruhe, DE

Paraformaldehyde (PFA)	Sigma-Aldrich, München, DE
PBS	PAA Laboratories GmbH, Pasching, AT
Penicillin/Streptomycin	PAA Laboratories GmbH, Pasching, AT
PKH26 Red	Sigma-Aldrich, München, DE
RNeasy MinElute Cleanup Kit	Qiagen, Hilden, DE
RosetteSep (CD4 ⁺ T cell enrichment kit)	Stem Cell Technologies, London, GB
RPMI 1640	Invitrogen Life Technologies, Karlsruhe, DE
Running buffer 20x	Invitrogen Life Technologies, Karlsruhe, DE
Skim milk powder	Heirler Cenovis GmbH, Radolfzell, DE
Sodium chloride (NaCl)	Roth, Karlsruhe, DE
Sodium hydroxide (NaOH) (32 %)	Merck, Darmstadt, DE
TGFβ1	R&D Systems Europe, Ltd., GB
TRIS (hydroxymethyl)-aminomethane	Carl Roth GmbH Co KG, Karlsruhe, DE
Triton X-100	Promega Corporation, Madison, US
TRIZOL [®]	Invitrogen Life Technologies, Karlsruhe, DE
Trypan blue	Merck, Darmstadt, DE
TaqMan [®] Lightcycler Kit	Roche Diagnostics GmbH, Mannheim, DE

5.1.2 Plastic ware

6-well tissue culture plate	Sarstedt, Nürnberg, DE
24-well tissue culture plate	Sarstedt, Nürnberg, DE
48-well tissue culture plate	Sarstedt, Nürnberg, DE
0,2 - 2 ml Eppendorf tubes	Eppendorf GmbH, Hamburg, DE
cannulae	Medex Medical, Klein-Winterheim, DE
Nitrocellulose-Membrane, Hybond-C Extra	Amersham, Piscataway, US
NuPAGE [®] Novex Bis-Tris Gels, 10 %	Invitrogen, Carlsbad, US
Parafilm	Pechiney, Chicago, US
Petri dish	Becton, Dickinson and company, US
Pipettes 2, 5, 10 and 25 ml	Sarstedt, Nürnberg, DE
Pipette tips, 10, 200, 1000 µl	Sarstedt, Nürnberg, DE
Safe Seal Tips	BIOzym Diagnostik GmbH, DE
Falcon 15 ml	Sarstedt, Nürnberg, DE
Falcon 50 ml	Sarstedt, Nürnberg, DE
Syringe 50 ml	Braun, Melsungen, DE
Sterile filter Ø 2 µm	Sartorius, Hannover, DE

5.1.3 Equipment

Axioplan Mikroskop (63x Vergrößerung)	Carl Zeiss, Göttingen DE
BioPhotometer 6131	Eppendorf GmbH, Hamburg, DE
Centrifuges	
Typ 5402	Eppendorf GmbH, Hamburg, DE
Typ 5415C	Eppendorf GmbH, Hamburg, DE
Varifuge RF	Heraeus Christ Instruments, Düsseldorf, DE
3K30	Sigma Laborzentrifugen GmbH, DE
FACSCanto™	BD Biosciences, Heidelberg, DE
Incubator	Heraeus Christ Instruments, Düsseldorf, DE
Laminar flow (Typ DLF/BSDS 6)	Clean Air, Wreden, NL
LightCycler®1.5	Roche Diagnostics GmbH, Mannheim, DE
Magnet MPC-S	Dynal Biotech, Oslo, NO
Magnet MACS Multi Stand	Miltenyi Biotech, Bergisch Gladbach, DE
Mikroskope SM-LUX	Leitz, Wetzlar, DE
Neubauer chamber	Neubauer, DE
pH-meter	Knick, DE
Pipette boy	IBS Integra Biosciences, CH
Photometer (8,5mm)	Eppendorf GmbH, Hamburg, DE
Roller Mixer SRT 1	Stuart, DE
Scale	Vern
Shaker (type 3011)	GFL, Burgwedel, DE
Thermo cycler T3	Biometra, Göttingen, DE
Thermomixer	Eppendorf GmbH, Hamburg, DE
Vortex Genie2	Bender&Hobein AG, Zürich, CH
Odyssey Scanner	LI-COR Biosciences, Bad Homburg, DE

5.1.4 TGFβ - Inhibitors

LY 2109761	Eli Lilly and Company, Indianapolis, US
SB431542	Tocris Bioscience, Bristol, GB

5.1.5 Software

BD™ CBA	BD Biosciences, Heidelberg, DE
FACSDiva™	BD Biosciences, Heidelberg, DE

Mircosoft Office	Microsoft Deutschland GmbH, DE
LightCycler® 4.05 Software	Roche Diagnostics GmbH, Mannheim, DE
ImageJ	free internet tool, http://rsb.info.nih.gov/ij/
SigmaPlot 10.0	Systat Software GmbH, DE
CorelDRAW X3	Corel Corporation, CA
AxioVision Rel 4.5 software	Carl Zeiss, Göttingen, DE

5.2 Methods

Standard methods of molecular biology were performed – if not otherwise stated – according to protocols described either in Current Protocols in Molecular Biology (Volume 3, 2000), or Current Protocols in Immunology (Volume 2, 1999).

5.2.1 Peripheral blood samples

Following approval by the institutional review board blood samples from healthy blood donors were collected after written informed consent had been obtained. Freshly prepared Buffycoats were provided by the blood bank of the University hospital Cologne.

5.2.2 Isolation of CD4⁺ T cells, CD8⁺ T cells, B cells and monocytes

CD4⁺ T cells were isolated from blood samples using RosetteSep CD4⁺ enrichment kit (StemCell Technologies, Meylan, France) following the manufacturer's protocol. For comparing SMAD2 and SMAD3 phosphorylation in CD4⁺ T cells, CD8⁺ T cells, B cells and monocytes, cells were isolated by positive selection from freshly collected venipuncture blood samples using CD4, CD8, CD19 or CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). In this case, cell populations were labeled with Ab-coupled microbeads (10 µl beads, 90 µl PBS per 10⁷ cells) and separated on LS, MS or LD MACS columns in a magnetic field (Miltenyi et al., 1990). Cell purity was routinely determined by flow cytometry and was always higher than 90%.

5.2.3 Isolation of CD4⁺ T cell subpopulations

In some experiments, CD4⁺ T cells were further separated into subpopulations using CD25 and CD45RA microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereby, conventional CD4⁺CD25⁻ T cells, CD4⁺CD25⁻CD45RA⁺ naïve T cells, CD4⁺CD25⁻CD45RA⁻ memory T cells and CD4⁺CD25⁺ regulatory T cells were isolated. Cell purity was routinely determined by flow cytometry and was always higher than 90%.

5.2.4 Cell counting

Cell numbers were determined using a Neubauer cell chamber. Trypan blue was used to distinguish between viable and non-viable cells, as it enters dead cells via small holes in the membrane and thus, dead cells are stained blue. Only non-stained cells were counted.

cell number: $N \times \text{dilution factor} \times \text{volume} \times 10^4$ N = number of living cells

5.2.5 CD4⁺ T cell culture assays

CD4⁺ T cells were cultured in cell culture plates placed in incubators (Heraeus) at 37°C. The cells were maintained in 95% air atmosphere containing a constant concentration of 5% (v/v) CO₂. All procedures requiring sterility were carried out in sterile hoods (Heraeus) using sterile materials and solutions. Prior to usage, all materials were disinfected with 70% (v/v) ethanol (Bacillol, Bode Chemie, Hamburg). CD4⁺ T cells were always cultured in serum free medium (50% AIM V + 50% ExCell 640 (Invitrogen; JRH Bioscience, Lenexa) unless otherwise stated in the special T cell assay.

5.2.6 Serum deprivation and TGFβ add-back experiments

CD4⁺ T cells were cultured in serum free medium for 2, 8, 12 or 18 hrs (serum deprivation). 10 ng/ml TGFβ1 (R&D Systems, Minneapolis) was added after serum deprivation for 1, 2 or 8 hrs. In other experiments, 50% freshly isolated human serum, in combination with different concentrations of the ALK5 inhibitor SB431542 (Tocris Bioscience, Bristol, UK), was added for 2 hrs to CD4⁺ T cells post serum deprivation.

5.2.7 CD4⁺ T cell treatments

CD4⁺ T cells (either all CD4⁺ T cells or subpopulations) were activated with artificial antigen presenting cells (aAPCs). These aAPCs were comprised of magnetic beads (DynaL Biotech, Oslo, Norway) coated with the following antibodies: 5% anti-CD3 (OKT3), 14% anti-CD28 (9.3, a kind gift of Drs. Carl June and Jim Riley, Abaramson Cancer Research Center, University of Pennsylvania, Philadelphia) and 81% anti-MHC I (W6/32), as previously described (Chemnitz et al., 2004).

Additionally, CD4⁺ T cells were treated in some assays with the inhibitory cytokine TGFβ1 (R&D Systems) in concentrations varying between 0.0001 ng/ml – 30ng/ml (indicated in each figure legend).

Further, the TGF β RI kinase inhibitor LY2109761 (Eli Lilly Inc., US) was added in some experiments. Concentrations varied from 0.6 nM – 2560 nM LY2109761.

In other experiments, 100 U/ml IL-2, 10 ng/ml IL-4, 2.5 ng/ml IL-12 were added to the T cell cultures (Immunotools, Friesoythe, DE).

5.2.8 Induction of Treg cells

CD4⁺CD25⁻CD45RA⁺ naïve T cells were isolated as described in 5.2.3. Afterwards, cells were cultured in serum free medium (AIM V + ExCell 640 1:1 (Invitrogen; JRH Bioscience, Lenexa) for 5 days. Cells were either left untreated (resting control) or stimulated with aAPC (T cell : bead ratio: 1:3) alone or in the presence of either 10ng/ml TGF β 1 or with 10ng/ml TGF β 1 in combination with 100U/ml IL-2. At day 5, CD4⁺CD25⁻CD45RA⁺ naïve T cells stimulated with three different conditions were sorted for CD4⁺CD25^{high} cells. Subsequently, FOXP3 induction was assessed on RNA level (RT-PCR) as well as on protein level (flow cytometry). The suppressive capacity of these induced Treg cells was determined in a functional suppression assay (5.2.12).

5.2.9 Flow cytometry

In a flow cytometer, fluorescent dyes are excited by a laser beam and emitted fluorescent light is detected by photomultipliers. The presence of cell surface proteins can be visualized by staining cells with specific monoclonal antibodies labeled with fluorochromes. The appropriate amount of cells (1-10 x 10⁶ per sample) was surface-stained in 25-100 μ l PBS with combinations of monoclonal antibodies labeled with fluorochromes for 20 min at 4°C. Six different fluorochromes were used: fluorescein isothiocyanate (FITC), phycoerythrin (PE), CychromeTM (Cyc), allophycocyanin (APC), a tandem conjugate: APC and a cyanine Cy7 (APC-Cy7) and a tandem conjugate: PE and a cyanine Cy7 (PE-Cy7). After staining, the samples were washed and resuspended in PBS. Intracellular stainings for FOXP3 were done using the FOXP3 Staining Set from eBioscience (Frankfurt, Germany) according to the manufacturer's instructions.

Stained cells were analyzed on a FACSCantoTM and events in a live lymphocyte gate were analyzed with FACSDivaTM Software (Becton Dickinson Biosciences, USA). Antibodies were purchased from several distributors (listed in Table 1).

Table 1: FACS antibodies

Antibody	Fluorochrome	Company
CD3	PE	BD Biosciences
CD4	APC-Cy7	BD Biosciences
CD8	Per-Cp	BD Biosciences
CD14	FITC	BD Biosciences
CD19	PE	BD Biosciences
CD25	PE-Cy7	BD Biosciences
CD127	PE	BD Biosciences
CCR7	FITC	R&D
CD45RA	PE-Cy5	BD Biosciences
FoxP3	APC	eBiosciences
FoxP3	PE	eBiosciences

5.2.10 FACS sorting

Cell sorting was performed in collaboration with the group of Elmar Endl from the institute of molecular medicine and experimental immunology in Bonn. Cells were stained with the CD4 FITC, CD127 PE and CD25 PE-Cy7 and cells were sorted using a FACSDiva (Becton Dickinson). For RNA isolation, 2×10^6 cells were centrifuged at RT for 5 min (6000 rpm) and resolved in TRIzol[®] (Invitrogen Life Technologies). Afterwards, samples were immediately frozen at -80°C . For FOXP3 intracellular staining and a functional suppression assay, 1×10^6 cells were resuspended in serum free medium (AIM V + ExCell 640 1:1 (Invitrogen; JRH Bioscience, Lenexa).

5.2.11 Proliferation assay

To assess T cell proliferation, CD4^+ T cells were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) prior to stimulation with aAPCs. CFSE is a membrane-permeable fluorescent dye, which is non-fluorescent in its native form, but is rendered highly fluorescent after diffusion into the cell. The dye is then unable to diffuse out of the cell and upon one cycle of cell division the stain is halved in each of the daughter cells, which can be detected by flow cytometry (Figure 4). After washing the cells twice in 10 ml PBS, they were incubated with 0,5 mM CFSE in 1 ml PBS per 10^7 cells at RT for 8 min. To stop the staining reaction 8 ml FCS were added. The cells were then washed twice in 10 ml RPMI, and resuspended in the appropriate volume of medium. After four days of culture CD4^+ T cell proliferation was assessed by flow cytometry.

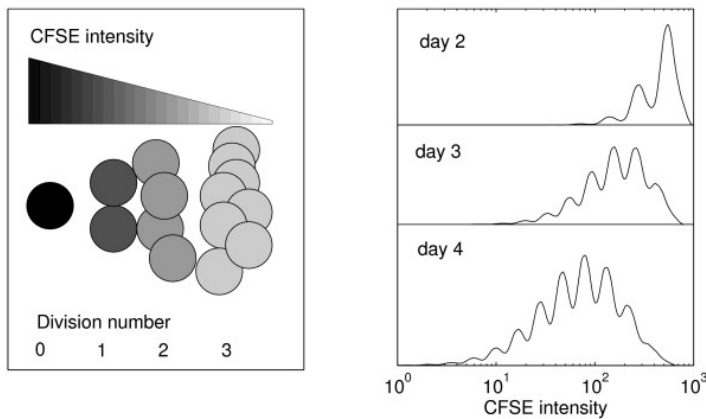


Figure 4: Proliferation of CFSE-labeled cells

CFSE dilution (left) and typical CFSE intensity histograms (right). (Luzyanina et al., 2007)

5.2.12 Suppression assay

To study the suppressive capacity of induced Treg cells, $CD4^+$ T cell proliferation was analyzed. For this experiment, sorted induced Treg cells were rested in serum-free cultures for 24 hrs. Further, freshly isolated naïve $CD4^+CD25^+$ Treg cells were preactivated for 24 hrs in serum free medium (AIM V + ExCell 640 1:1 (Invitrogen; JRH Bioscience, Lenexa) in the presence of 0,5 $\mu\text{g/ml}$ anti-CD3 (Okt3, Ortho Biotech, Bridgewater, US) and 10 U/ml IL-2 (Proleukin, Chiron Corporation, US). These preactivated naïve $CD4^+CD25^+$ Treg cells were used as a positive inhibitory control in this assay. Induced Treg cells as well as naïve $CD4^+CD25^+$ Treg cells were then stained with PKH26 red (Sigma Aldrich Chemie) according to the manufacturer's protocol. $CD4^+CD25^-$ T cells were labeled with CFSE prior to stimulation with aAPCs. The suppression assay was performed in X-Vivo 15 with 10% heat inactivated FCS. $CD4^+CD25^-$ T cells were stimulated with aAPC (5% anti-CD3, 14% anti-CD28, 81% anti-MHC I; T cell : bead ratio: 5:1) alone or in the presence of naïve or induced Treg cells ($CD4^+CD25^-$ T cells: Treg cell: 2:1; 1:1). After three days of culture, $CD4^+CD25^-$ T cell proliferation was assessed by flow cytometry.

5.2.13 Th1/Th2 cytokine measurement

The cytokines $\text{IFN}\gamma$ and IL-6 were measured in the supernatants of cell culture assays using the Human Th1/Th2 Cytokine Kit II (Becton Dickinson). Cytokine amounts were analyzed with the BD CBA software.

5.2.14 Immunofluorescence for SMAD2 and SMAD3

T cells and B cells were isolated from venipuncture blood samples by using RosetteSep T cell enrichment and B cell enrichment kits (StemCell Technologies). Cells were centrifuged on glass cover slides, fixed with 4% paraformaldehyde, and permeabilized in 0,2% Triton X-100

before blocking for 30 min (1% fish skin gelatine (Sigma, Hannover), 10 % goat-serum (Dako Cytomation, Hamburg) in PBS). Slides were incubated with anti-phospho-Smad3 (Merck, Darmstadt, Germany) followed by secondary antibody (Alexa Fluor® 568 goat anti-rabbit IgG (H+L), Invitrogen). Subsequently, cells were incubated with mAbs against CD4 (Novocastra, Newcastle, UK), CD8 (Dako, Hamburg, Germany) or CD19 (Serotec, Düsseldorf, Germany) followed by secondary Ab (Alexa Fluor® 488 goat anti-mouse IgG (H+L), Invitrogen). Afterwards, cells were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for nuclear staining. Pictures were taken with a Zeiss Axioplan microscope (63x magnification) and AxioVision Rel 4.5 software.

For control staining, freshly isolated CD4⁺ T cells were cultured for 24 hrs under serum free conditions and either analyzed directly or treated with 30 ng/ml TGFβ1 for 1 hr.

5.2.15 Cell lysis and Western Blot

Cells were lysed (5 ml Triton 1% (Promega, Mannheim), 750 µl 150 mM NaCl (Roth, Karlsruhe), 250 µl 50 mM Tris-HCl (Invitrogen), 50 µl Phosphatase Inhibitor Cocktail 1 (Sigma), 50 µl Phosphatase Inhibitor Cocktail 2 (Sigma), protease inhibitor (Roche, Complet Mini), 10 µl PMSF 1M). Cell lysates were resolved by SDS-PAGE, proteins were transferred to nitrocellulose and blots were probed with appropriate primary and secondary Ab combinations. The following antibodies were used: anti-phospho-Smad3/Smad1 Ab (Cell Signaling), anti-phospho-Smad2 Ab (Cell Signaling), anti-SMAD2/3 (BD Biosciences), anti-SATB1 (BD Biosciences) anti-β-actin Ab (Chemicon, Hampshire), anti-mouse IgG-HRP (Dako), anti-rabbit IgG-HRP (Dako).

5.2.16 EMSA

Electrophoretic Mobility Shift Assay (EMSA) was performed on the Odyssey® Infrared Imaging System (LI-COR) according to the manufacturer's protocol. Briefly, synthetic oligonucleotides were 5' end-labeled with IRDye™ 700 or IRDye™ 800 phosphoramidite. Oligonucleotides were annealed for 5 min at 100°C to form double-stranded DNA fragments. Nuclear extract of cells and oligonucleotides were incubated in a binding reaction for 20 min at RT. Binding reactions were then loaded on a 5% native polyacrylamide gel to separate the protein-DNA complexes. The gel was then scanned on the Odyssey machine. Oligonucleotides (purchased from MWG Biotech) are listed in Table 2.

Table 2: EMSA oligonucleotides

gene	forward oligonucleotide	reverse oligonucleotide	IRDye
SATB1	GTATACAGTATGCAAACATAACTCACCATT	AATGGTGAGTTATGTTTGCATACTGTATAC	700
SATB1m	GTATACAGTATCGTCGAGCAACTCACCATT	AATGGTGAGTTGCTCGACGATACTGTATAC	800
FKH	TCAAAAATATTGAAGTGTTATCACATACAC	GTGTATGTGATAACACTTCAATATTTTTGA	800

5.2.17 ChIP

1x10⁶ expanded Treg cells were crosslinked with a final concentration of 1% formaldehyde for 10 min at RT. Crosslinking was stopped by the addition of a final concentration of 0.125 M glycine for 5 min at RT. Cells were then washed with ice-cold PBS and pelleted. Afterwards, cells were lysed in 5 ml cell lysis buffer (50 mM Tris-HCl (Invitrogen), pH 8.0, 1% Triton X-100 (Promega, Mannheim), 10 mM KCl, 5 µl PIC (Active Motif), 5 µl PMSF (Active Motif) for 10 min on ice. Nuclei were pelleted by centrifugation and resolved in 1 ml digestion buffer (Active Motif). 100 µl of this suspension was preheated for 5 min on 37°C, then enzymatically digested for 15 min at 37°C (2.5 µl enzyme (Active Motif)). Enzymatic shearing was stopped by the addition of 1µl ice-cold EDTA for 10 min on ice. After centrifugation, supernatant (including sheared chromatin) was transferred into a new tube. Pre-clearing of the chromatin was performed as recommended by the manufacturer's protocol (Active Motif; ChIP-IT). ChIP (Chromatin-immuno-precipitation) was performed with either FOXP3 antibody (eBioscience) or isotype control (BD Biosciences) overnight due to the manufacturer's protocol (Active Motif; ChIP-IT). DNA-antibody complexes were isolated using protein G beads, followed by several washing steps. Afterwards, DNA was eluted, crosslinking was reversed and DNA was purified due to the manufacturer's protocol (Active Motif; ChIP-IT). Purified DNA was analyzed by PCR. Primers (purchased from Sigma-Aldrich) are listed in Table 3.

Table 3: ChIP primer

gene	forward primer	reverse primer
IL7R	CAGGGAATATCCAGGAGGAA	TGTGTGAGCCAGTGTGTATGA
IL7R intron 4	GAGGTGGCAGAAGAGTGGAG	TGCATCACACTGCAAACAAA
SATB1	GAAAGGTGGGTTCTTCTGAAG	GCAATGAATGCAGAATTACCT

5.2.18 RNA Isolation

RNA isolation was performed using TRIzol[®] from Invitrogen Life Technologies following the manufacturer's protocol. Subsequently, RNA was further processed using the RNeasy MinElute Cleanup Kit (Qiagen). RNA quality and quantity was determined by measuring the absorption of the sample at 260 nm and 280 nm, respectively, in a spectrometer (Eppendorf

GmbH, Hamburg, Germany). An OD₂₆₀ of 1 corresponds to approximately 40 µg/ml for RNA. Purity of RNA was estimated by the ratio OD₂₆₀/OD₂₈₀, with 2.0 optimal for RNA.

5.2.19 Real-time-PCR

100 - 500 ng RNA were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). RT-PCR was performed with a LightCyclerTaqman master kit and a Universal ProbeLibrary Assay on a Light Cycler 1.3 instrument (Roche). Analysis was done using LightCycler®4.05 software (Roche). Expression was normalized to that of the 'house-keeping' gene β-2 microglobulin (B2M). Primers (purchased from Sigma-Aldrich) are listed in Table 4.

Table 4: RT-PCR primer

gene	forward primer	reverse primer	Universal Probe Library
CXCR4	TGTTTCCGTGAAGAAAATGCT	TGCCAGTTAAGAAGATGATGGA	#04
KLF10	TTAAAAGTTCCCATCTGAAGG	TCATCAGAACGGGCAAACCT	#02
SLAMF1	ACCGTGAGCAACCCTATCAG	CTGCCCATGGTTTTGTTTCT	# 41
FoxP3	CTACGCCACGCTCATCCGCTG	GTAGGGTTGGAACACCTGCTG	#50
SATB1	CGATGAACTGAAACGAGCAG	CGGAGGATTTCTGAAAGCAA	#14
B2M	TTCTGGCCTGGAGGCTAT	TCAGGAAATTTGACTTTCCATTC	# 42

5.2.20 Microarray procedure

When using the Affymetrix platform RNA isolation, quantification, target preparation and hybridization were performed as described previously (Debey et al., 2004). Biotin-labeled cRNA preparation for the Illumina platform was performed using the Ambion® Illumina RNA amplification kit (Ambion Europe, Huntingdon, Cambridgeshire, UK). Biotin-labeled cRNA (1.5 µg) was hybridized to Sentrix® whole genome bead chips 6 x 2 (Illumina, San Diego, CA, USA) and scanned on the Illumina® BeadStation 500x (Chemnitz et al., 2006). Table 5-7 represents a summary of all microarray experiments performed within this thesis.

Table 5: microarray experiments for TGFβ in T cell homeostasis

	T-0	T-0	T-2	T-2	T-8	T-8	T-12	T-18	T-18 +1h	T-18 +1h TGF	T-18 +2h	T-18 +2h TGF	T-18 +8h	T-18 +8h TGF
time (h)	0	0	2	2	8	8	12	18	19	19	20	20	26	26
celltype	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell
treatment	-	-	-	-	-	-	-	-	-	10ng/ml TGFβ1	-	10ng/ml TGFβ1	-	10ng/ml TGFβ1
cases	10	3	3	3	3	3	4	3	3	3	3	3	3	3
platform	Affy	Illumina	Affy	Illumina	Affy	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina

Notes: TGFβ1 was added at time point 18hrs; the samples used are given. T-0, T-8, etc. are abbreviations for CD4⁺ T-cells assessed at the given time points. Furthermore, the time point, the cell type, the treatment, the number of cases and the platform used is given.

Table 6: microarray experiments for TGFβRI kinase inhibitor analysis

	resting	TGF	TGF + LY	activated	act + TGF	act + TGF + LY
time (h)	12h + 8h	12h + 8h	12h + 8h	12h + 8h	12h + 8h	12h + 8h
celltype	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell
treatment	-	-	-	CD3CD28 (3 beads : 1 T cell)	CD3CD28 (3 beads : 1 T cell)	CD3CD28 (3 beads : 1 T cell)
		10ng/ml TGFβ1	10ng/ml TGFβ1		10ng/ml TGFβ1	10ng/ml TGFβ1
			2560nM LY2109761			2560nM LY2109761
cases	3	3	3	3	3	3
platform	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina

Notes: cells were rested for 12hrs followed by 8hrs of treatment; the samples used are given. Resting, TGF, etc. are abbreviations for CD4⁺ T-cells treated under different conditions. Furthermore, the time point, the cell type, the treatment, the number of cases and the platform used is given.

Table 7: microarray experiments for induced Treg cell analysis

	CD4 ⁺ CD25 ⁻	CD4 ⁺ CD25 ⁺	CD4 ⁺ CD25 ⁺ CD45RA ⁺	resting	activated	act + TGF
time (days)	0	0	0	5	5	5
celltype	T-cell	T-cell	T-cell	T-cell	T-cell	T-cell
treatment	-	-	-	-	CD3CD28 (3 beads : 1 T cell)	CD3CD28 (3 beads : 1 T cell)
						10ng/ml TGFβ1
cases	3	3	3	3	3	3
platform	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina

Notes: the samples used are given. CD4⁺CD25⁻; CD4⁺CD25⁺, etc. are abbreviations for CD4⁺ T-cells assessed at the given time points treated. Furthermore, the time point, the cell type, the treatment, the number of cases and the platform used is given.

5.2.21 Data analysis and software

For data collection, assessment and statistical analysis we used Affymetrix Microarray Analysis Suite 5.0 (MAS5.0), Affymetrix Data Mining Tool 3.0 (DMT), Illumina® BeadStudio and R language (Bioconductor project). In R language the vsn method and the quantile method were used for data normalization of Affymetrix and Illumina microarrays, respectively. Unpaired t-tests were calculated as appropriate. For visualization and gene ontology assessment we used GenMAPP® and MAPPfinder® (Dahlquist et al., 2002). All heatmaps were visualized using MAYDAY (Dietzsch et al., 2006). For further gene ontology analysis the R platform was used.

To better understand changes in transcriptional regulation an algorithm was developed that integrates Gene Ontology information provided by the international Gene Ontology (GO) consortium, a quantitative distance analysis between different experimental groups (here time points), a calculation of the statistical power of the method based on a permutation approach and a visualization of the data. The algorithm will be described in more detail elsewhere (D. E., T.Z. and J.L.S., manuscript in preparation, as well as in the PhD thesis of Daniela Eggle). All microarray data can be accessed using the National Center for Biotechnology Information (GEO accession number).

5.2.22 Statistics

For statistical evaluation the 2-tailed Students t-Test was used. Differences were considered statistically significant when $p < 0.05$.

6 Results

6.1 TGF β controls T cell homeostasis

6.1.1 Transcriptional changes in human CD4⁺ T cells after serum deprivation

In mice, stimulatory signals (e.g. self-peptide–MHC ligands, IL-2, IL-4, IL-7, IL-9, and IL-15) as well as inhibitory signals (e.g. CTLA-4, PGE2 and TGF β) were shown to be involved in the control of T cell homeostasis. However, the integration of these signals is not yet completely understood. In humans, the identification of factors keeping T cells at a resting state is even more complex, since in contrast to murine models, no knock-out strategy can be applied when studying biological mechanisms. To circumvent this limitation, we designed an approach, in which we exposed purified human CD4⁺ T cells to an environment depleted of blood-derived soluble factors present in serum (Figure 5). We expected that deprivation of resting human CD4⁺ T cells of any exogenous signals should reverse intracellular signaling cascades actively keeping CD4⁺ T cells at a resting state. We further hypothesized that these changes should be recognizable on the genomic level in a genome-wide transcription analysis.

Thus, human CD4⁺ T cells were cultured under serum-free conditions for different time length followed by the analysis of genome-wide transcriptional changes using Affymetrix microarrays.

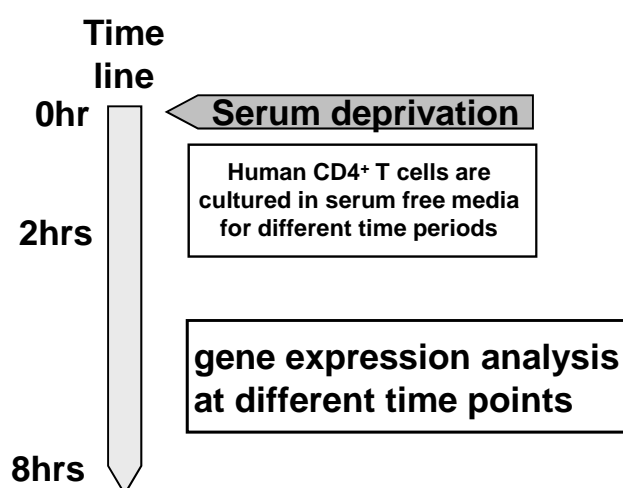


Figure 5: Experimental setup to address the role of exogenous factors

Filtering based on fold changes (FC) and significance (variable probe sets, FC > 1.5 or FC < -1.5 and p-value < 0.05) revealed a high number of genes (878 genes, 443 up- and 435

downregulated) with altered transcription after 2 hrs of serum deprivation in highly purified CD4⁺ T cells. Changes of transcription even further increased at a later time point (910 genes at 8 hrs; 593 up- and 317 downregulated) (Figure 6A). When hierarchical clustering was performed using all variable probe sets, time of serum withdrawal was the major factor separating the sample groups (Figure 6B).

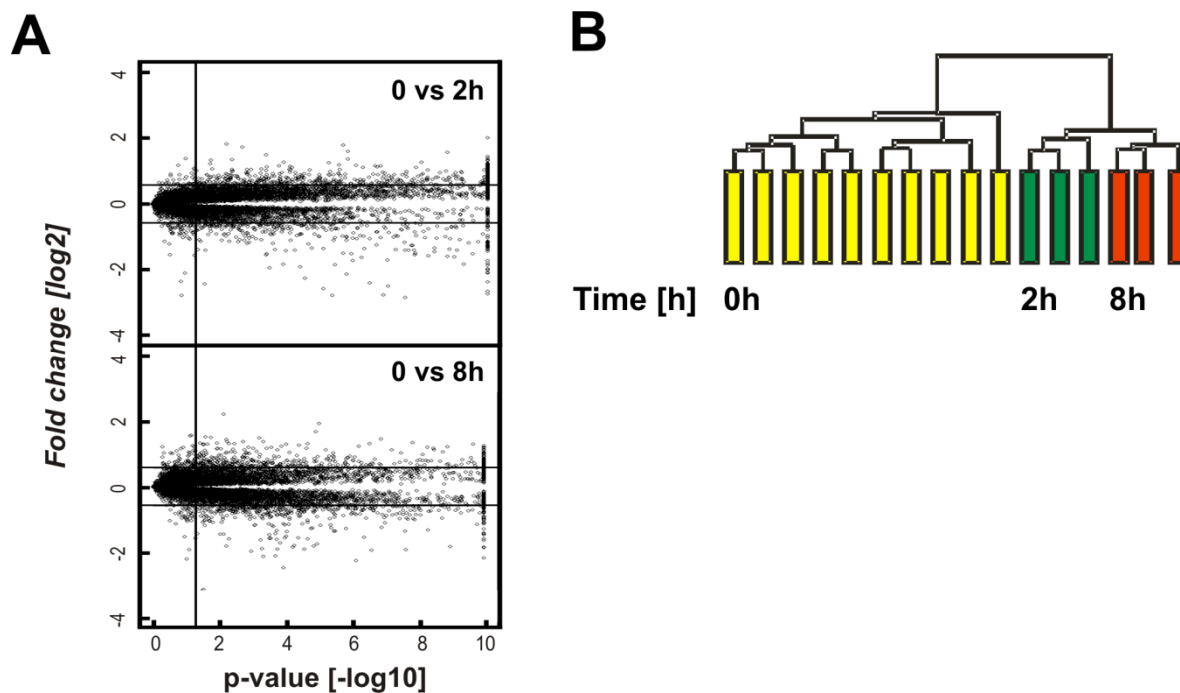


Figure 6: Time of serum withdrawal separates the sample groups

(A) To visualize significant changes in gene expression volcano plots were used. The fold change (\log_2 FC) in gene expression was plotted against the negative p-value (\log_{10}). All CD4⁺ T cell samples assessed on the Affymetrix platform are included. Plotted are genes changed between t=0 (n=10) and t=2 hrs (n=3) as well as t=0 and t=8hrs (n=3). Genes significantly changed are defined by FC < -1.5 or FC > 1.5 and a p-value < 0.05 (see respective lines). (B) Hierarchical clustering of all T cell experiments on the Affymetrix platform. Prior to clustering, genes were filtered using all variable probesets ($0.5 < \text{sd}/\text{mean} < 10$).

6.1.2 The TGF β signaling pathway is a major target after serum deprivation

Next we were interested, which biological systems mainly contribute to these changes of gene expression. Therefore we applied a systems biology approach based on Gene Ontology. In short, a set of ‘gene spaces’ was defined as the group of genes given by one gene ontology (GO) ID in the category ‘biological process’ (www.geneontology.org). Only those GO IDs were used which are represented with at least 5 probesets on the HGU133A Affymetrix array (Figure 7A). Out of the 18455 currently known GO IDs 9805 comprise biological processes, 2616 are present on the HGU133A array, but only 1336 of them include at least 5 probe sets (Figure 7A). Within these 1336 predefined gene spaces Euclidean distances were calculated between the different sample groups (time points t = 0, 2, and 8 hrs). Significance of the

obtained distances was validated by permutation analysis. As demonstrated in Figure 7B, 384 GO IDs were identified to be affected on a significance level below 0.1%, 180 GO IDs between 0.1 and 1% and 230 GO IDs between 1 and 5% after 8 hours of serum deprivation.

When analyzing the most significant GO IDs, it became apparent that genes belonging to the biological terms 'cell cycle', 'cell growth' and 'transcription regulation' were major contributors to differences in gene expression after serum deprivation (Figure 7B). Surprisingly, 31 out of 56 'cell cycle' related GO IDs were affected on a significance level below 0.1% and only 5 'cell cycle' related GO IDs did not reach the 0.05 significance level (error rate >5%). To identify signals that might account for these changes in T cells after serum deprivation, we next searched for potential extrinsic signals upstream of 'cell cycle', 'cell growth' and 'transcription regulation'. This analysis identified the TGF β pathway to be the most significantly changed exogenous signaling cascade (error rate <1%). To corroborate the Gene Ontology based approach, we compiled a set of genes containing previously described as TGF β 1 target genes (Renzoni et al., 2004; Siegel and Massague, 2003). We postulated that these TGF β target genes should again reveal significant changes in gene expression associated with serum deprivation. Indeed, this set of genes was even more significantly changed in human primary CD4⁺ T cells (error rate <0.1%) (Figure 7B).

To further evaluate the specificity of our results, GO IDs containing genes associated with immune regulation were studied. Strikingly, none of these GO IDs reached a level of significance exceeding 1% (3 GO IDs with error rate between 1 and 5%, and 8 GO IDs with error rate >5%). Taken together, the genome wide screen for transcriptional changes and a quantitative bioinformatics approach revealed that changes in TGF β related genes are major contributors to the overall transcriptional changes observed after serum deprivation in human CD4⁺ T cells.

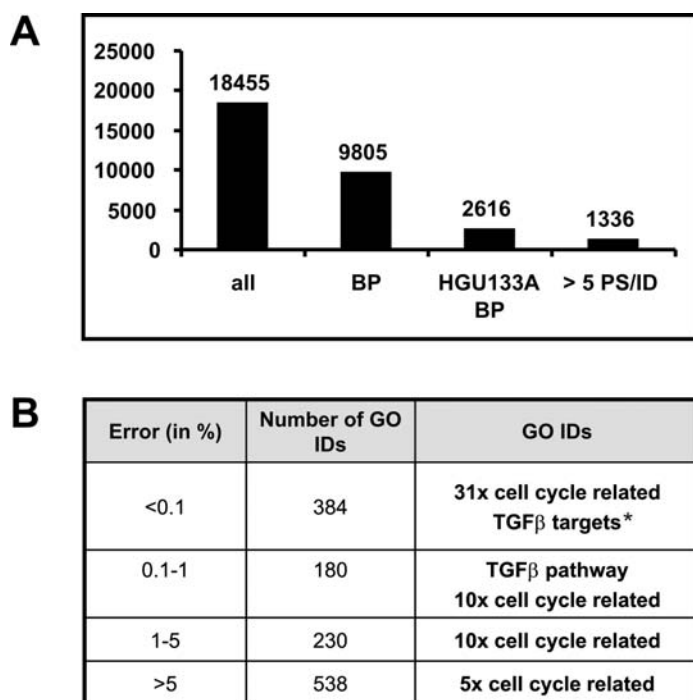


Figure 7: The TGF β signaling pathway is a major target after short-term serum deprivation

(A) All GO IDs ('all') were filtered first on the category 'biological process' (BP), next filtered on presence within the HGU133A array (HGU133A/BP) and finally on those represented with at least 5 probesets (> 5 PS/ID). (B) Gene ontology analysis revealed several significantly changed GO IDs (number of GO IDs) in CD4⁺ T cells after 8 hrs of incubation in serum free medium. The number of cell cycle related GO IDs is given; highlighted on the right site are 'offspring' of the overall GO term 'cell cycle', *"TGF β targets" is a set of known TGF β target genes. Significance levels are presented as error rates (in %).

6.1.3 Serum-deprived human CD4⁺ T cells present a 'TGF β loss signature'

To visualize the impact of serum deprivation on TGF β related genes we adapted the TGF β signaling map provided by GenMAPP[®] adding target genes significantly changed in gene expression (FC > 1.5 or FC < -1.5, p < 0.05 at t = 8h) post serum deprivation (Figure 8). After 2 hrs, inhibitors of TGF β signaling (SMURF1 and 2, SMAD7, TGIF, and SKI) were significantly upregulated while at the same time many genes known to be induced by TGF β including JUN, JUNB, GADD45B, ZFP36L2, ID2, BHLB2, and KLF11 were downregulated. This regulation was further pronounced after 8 hrs: 13 known target genes of TGF β were significantly reduced in expression, while three genes usually repressed by TGF β (ID3, MYB and PIGF) were induced (Figure 8).

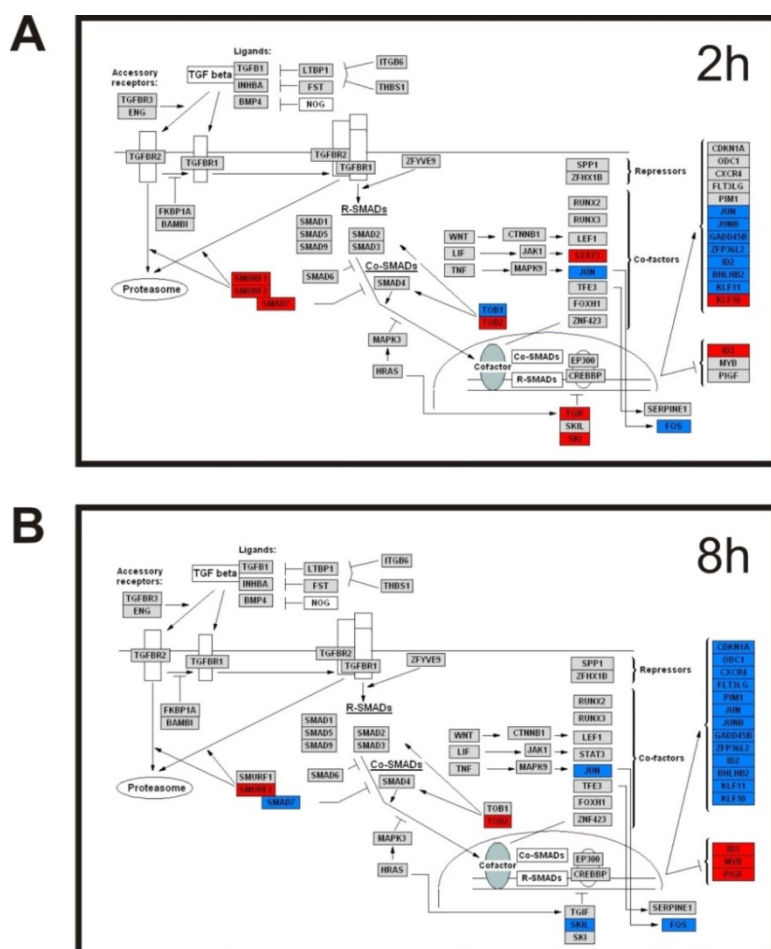


Figure 8: Expression changes in the TGF β signaling pathway in response to serum deprivation

Changes in gene expression within the TGF β signal transduction pathway and exemplified target genes after 2 (**A**) and 8 (**B**) hrs of culturing CD4⁺ T cells were analyzed using the GenMAPP[®] software. Genes with increased expression (FC \geq 1.5, $p < 0.05$) are highlighted in red, those with a decreased expression (FC \leq -1.5, $p < 0.05$) in blue color. The mean expression value was used for each time point (0h: n=10; 2hrs: n=3; 8hrs: n=3).

To further support these findings we performed an additional experiment extending the time of serum deprivation to 12 and 18 hrs, (Figure 9, plotted are only genes differentially expressed after 8 hrs). For those TGF β target genes we observed a similar expression pattern at the later time points further supporting that the transcriptional control of these genes by TGF β is lost post serum deprivation.

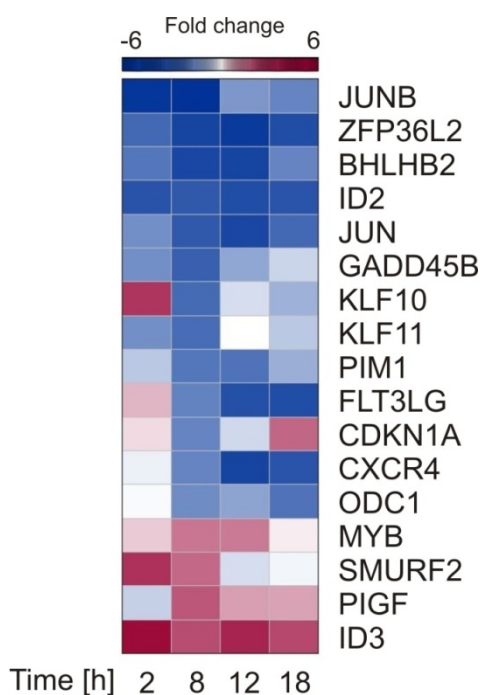


Figure 9: Fold changes of known TGFβ target genes in response to serum deprivation

Heatmap visualizing fold changes of known TGFβ target genes at the 2, 8, 12 and 18 hrs time points. Only genes differentially expressed after 8 hrs are depicted. All fold changes were computed in comparison to the 0 hrs time point and color coded. Genes with increased expression (FC ≥ 1.5, p < 0.05) are shown in red, those with decreased expression in blue color (FC ≤ -1.5, p < 0.05). Changes in gene expression were assessed using two different microarray platforms (Affymetrix, Illumina). Prior to visualization, data were normalized and cross annotated.

6.1.4 Identification of novel TGFβ target genes in resting CD4⁺ T cells

To investigate, which genes are main targets of TGFβ in resting CD4⁺ T cells, human CD4⁺ T cells were first serum-deprived for 18 hrs followed by a single pulse of TGFβ1 (10 ng/ml). The experimental setup is shown in Figure 10.

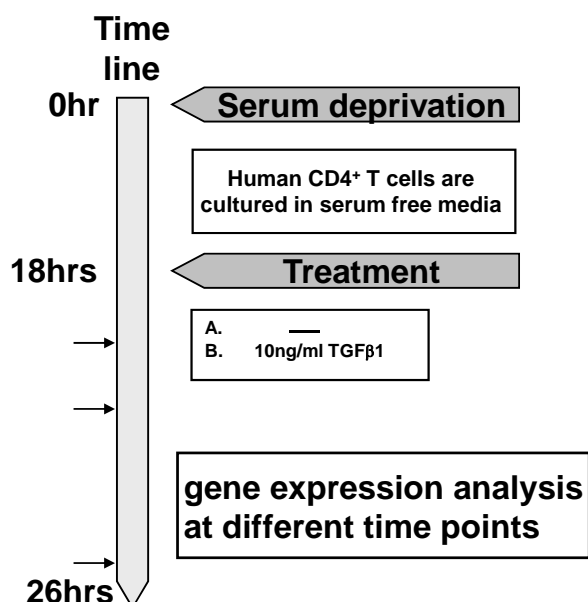


Figure 10: Experimental setup to identify TGFβ target genes

TGFβ target genes were defined as genes showing transcriptional changes post serum deprivation (FC > 1.5 or FC < -1.5, p-value < 0.05 at t = 8 hrs post serum deprivation) and

counter-regulation after addition of TGF β 1 (FC > 1.25 or FC < -1.25, p-value < 0.05 at t = 2 hrs post addition of TGF β 1). As demonstrated in Figure 11A, most of the known TGF β target genes identified as significantly regulated during serum deprivation (see Figure 9) were indeed counter-regulated post addition of TGF β 1 with only few exceptions.

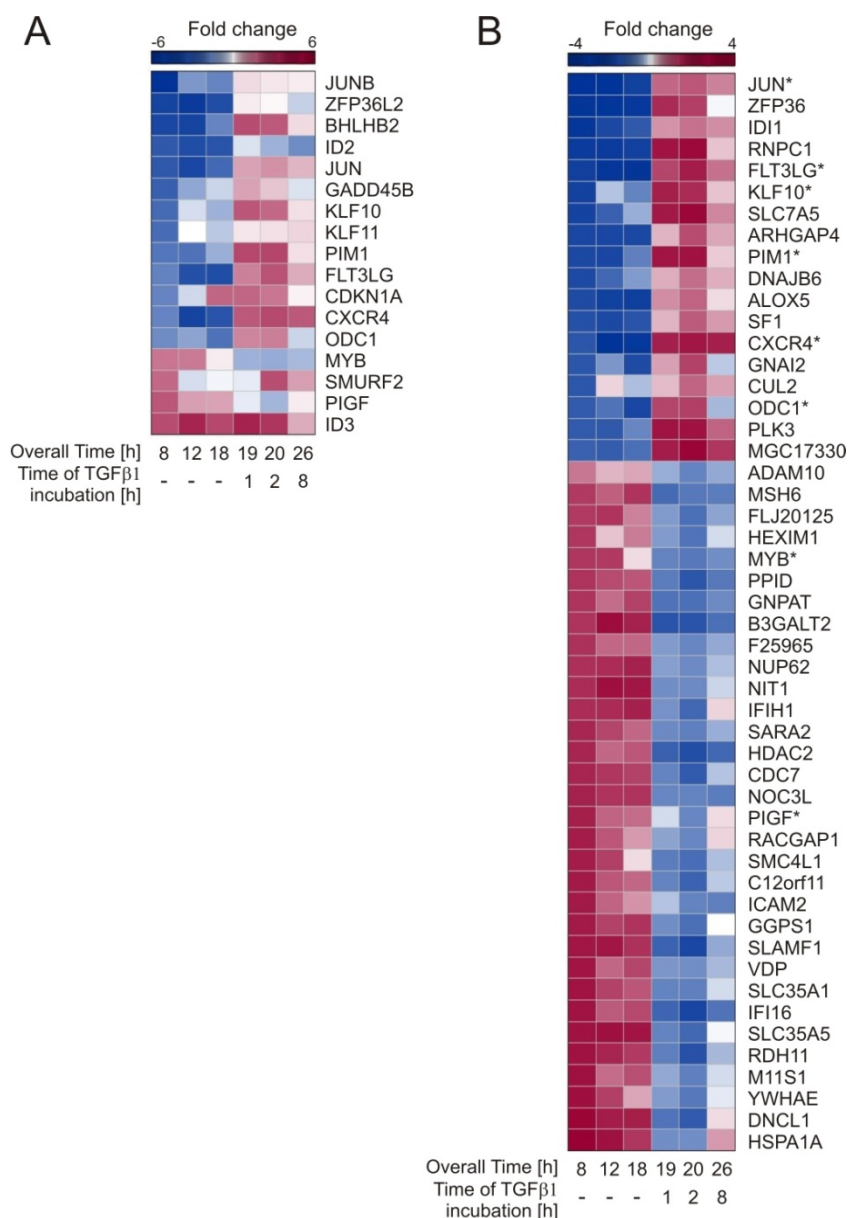


Figure 11: Identification of new TGF β target genes

Heatmap displaying fold changes of known (**A**) and novel (**B**) TGF β target genes. Genes were selected based on differential expression (FC > 1.5 or FC < -1.5, p-value < 0.05) at 8 hrs after serum deprivation compared to the 0 hrs time point. Fold changes for the 8, 12 and 18 hrs after serum deprivation were computed in comparison to the 0 hrs time point. Fold changes for the 1, 2 and 8 hrs post addition of TGF β 1 were computed in comparison to the 18 hrs time point of serum deprivation. Fold changes are color-coded showing genes being up- and down-regulated in red and blue, respectively. Genes marked with an asterisk in Figure B are known TGF β target genes.

In addition to known TGF β target genes we identified 42 novel genes that – so far – have not been recognized as TGF β target genes in other cellular systems (Figure 7B). While most of the known TGF β target genes were downregulated during serum deprivation and restored post TGF β 1 pulse (Figure 7A), the majority of the new target genes were found to be suppressed by TGF β 1 (Figure 7B). The identification of numerous new TGF β target genes might be explained by previous strategies that mainly identified TGF β target genes solely by exposing cells to increased concentrations of TGF β 1 (Renzoni et al., 2004). Applying a gene ontology

approach on those TGF β target genes lead to the identification of four major groups of genes: genes encoding for membrane associated proteins (transport and signaling), proteins with nuclear localization (transcriptional regulation), proteins involved in cell cycle regulation, and genes of unknown function.

Six genes (RDH11, SLC35A1, VDP, PIGF, B3GALT2, GNPAT) associated with intracellular membranes especially of the Golgi apparatus and the endoplasmic reticulum are repressed by TGF β . Except for PIGF (phosphatidylinositol glycan class F), a key enzyme involved in glycosylphosphatidylinositol (GPI) anchor biosynthesis, the function of the other genes in T cells is still elusive. Expression of three extracellular membrane proteins, CXCR4, FLT3LG, and SLC7A5, is significantly reduced upon serum deprivation, while ICAM2, an adhesion molecule, and SLAMF1 (CD150) a costimulatory molecule, are suppressed by TGF β . SLAMF1 has been shown to be constitutively expressed in T cells and increased expression of SLAMF1 is clearly associated with T cell activation (Cocks et al., 1995). Our data further suggest that the level of constitutive SLAMF1 expression is under the control of TGF β with increased expression in the absence of TGF β .

The second group of genes codes for proteins with nuclear localization. In addition to regulators of transcription known to be TGF β target genes (KLF10, JUN and MYB), expression of seven novel genes involved in transcriptional regulation (HDAC2, SF1, ZFP36, RNPC1, RACGAP1, YWHAE and IFI16) was shown to be altered by TGF β in T cells. ZFP36, which is decreased upon serum deprivation, can bind to AU-rich elements in mRNAs coding for inflammatory cytokines such as TNF α or CSF2, thereby increasing the lability of these mRNAs (Carballo et al., 1998).

In addition to known TGF β target genes involved in cell cycle regulation, such genes were also identified among the novel TGF β targets, namely CDC7, CUL2, SMC4L1, PLK3, and RACGAP1. Several newly identified TGF β target genes were genes with yet unknown function, particularly in T cells (C12orf11, F25965, FLJ20125, MGC17330, M11S1, NOC3L, and SLC35A5).

To verify these findings by a second technique we performed real time-PCR for exemplary target genes (Figure 12). CD4⁺ T cells were cultured under the same culture conditions. Similar to the microarray data, mRNA for CXCR4 and KLF10 was significantly downregulated following serum deprivation and stayed low over the whole culture period (up to 26 hrs). In contrast, after a single pulse of TGF β 1 (10 ng/ml) at 18 hrs the expression levels of both genes were counter-regulated almost restoring the levels of CXCR4 expression to base line and significantly exceeding base line levels for KLF10. Exemplary for a gene suppressed by TGF β 1 RT-PCR results obtained for SLAMF1 are shown. mRNA for SLAMF1 was significantly

upregulated following serum deprivation and was highly transcribed over the whole culture period (up to 26 hrs). Addition of TGF β 1 (10 ng/ml) 18 hrs after serum deprivation reduced the expression to baseline levels.

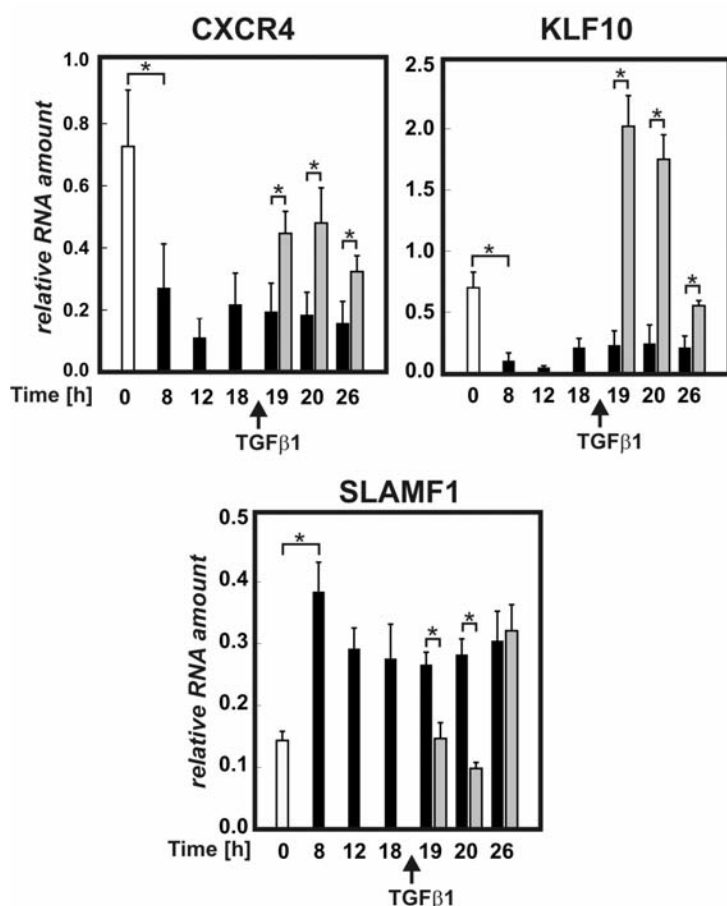


Figure 12: RT-PCR of exemplary TGF β target genes

Regulation of CXCR4, KLF10 and SLAMF1 mRNA were assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments. White bars: T cells prior to culture; black bars: T cells under serum free conditions; grey bars: T cells after addition of TGF β 1 (10 ng/ml). Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

6.1.5 CD4⁺ T cell display an activated TGF β signaling pathway

The genome-wide transcription analysis suggested that constitutive TGF β signaling controls resting T cells on the transcriptional level. Phosphorylation of receptor regulated SMAD2 and SMAD3 is an early event following binding of TGF β to its cognate receptor complex. Loss of transcriptional control by TGF β should therefore be accompanied by loss of SMAD phosphorylation. To study SMAD3 phosphorylation on single cell level, immunofluorescence analysis was performed on human lymphocytes from venipuncture blood immediately after isolation. As depicted in Figure 13, all freshly isolated CD4⁺ T cells contained significant amounts of phosphorylated nuclear SMAD3 (Figure 13B). CD4⁺ T cells cultured under serum free conditions for 24 hrs completely lost phosphorylation of SMAD3 (Figure 13A) while addition of TGF β 1 restored SMAD3 phosphorylation. In contrast, SMAD3 was not significantly phosphorylated in CD8⁺ T cells while CD19⁺ B cells showed only moderate amounts of phosphorylated SMAD3 (Figure 13B).

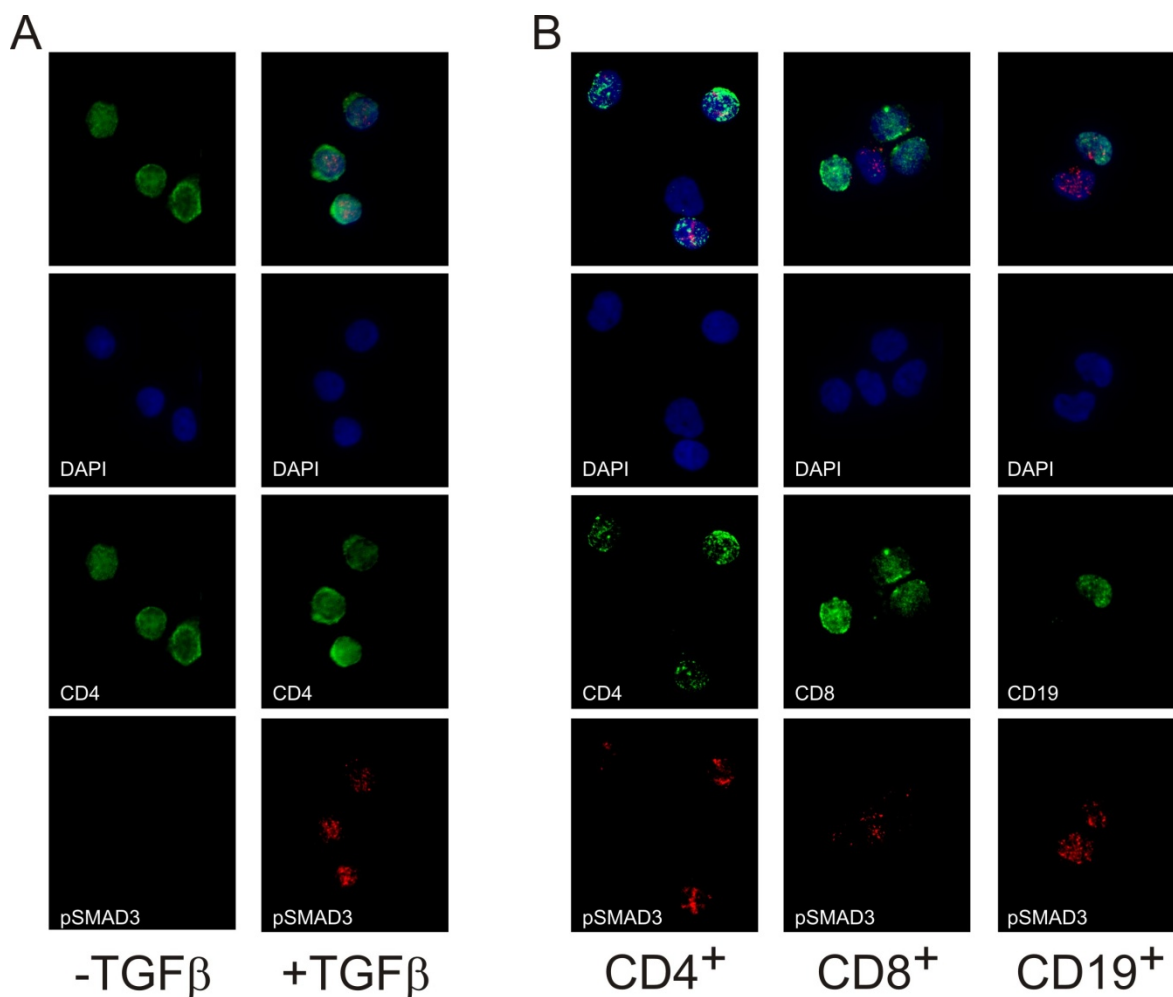


Figure 13: SMAD3 phosphorylation in human lymphocytes

Immunofluorescence analysis of venipuncture blood samples; cells were stained either for CD4, CD8, or CD19 (green, Alexa 488) and p-SMAD3 (red, Alexa 568). The nucleus was stained with DAPI (blue). The overlay is depicted in the top row.

(A) CD4⁺ T cells analyzed after 24 hrs of serum deprivation (-TGFβ1) and after an additional incubation period of 1 hr with 30ng/ml TGFβ1 (+TGFβ1). (B) Freshly isolated cells were either stained with antibodies against CD4, CD8 or CD19 as well as p-SMAD3 mAb. One representative experiment out of four is shown.

SMAD2 and SMAD3 phosphorylation was also assessed by Western blot analysis of highly purified CD4⁺ T cells, CD8⁺ T cells, CD14⁺ monocytes and CD19⁺ B cells (Figure 14A). While both SMAD molecules are phosphorylated in CD4⁺ T cells, only SMAD2 phosphorylation was detectable in CD8⁺ T cells. In contrast, monocytes only expressed background levels of phosphorylated SMAD2 and SMAD3. In accordance with the immunofluorescence analysis, B cells showed only weak SMAD3 phosphorylation. To assess the variability of SMAD phosphorylation in human T cells, CD4⁺ T cells derived from 5 healthy individuals were analyzed by Western blot analysis. SMAD2 and SMAD3 were constitutively phosphorylated in T cells of all donors analyzed *ex vivo* (Figure 14B).

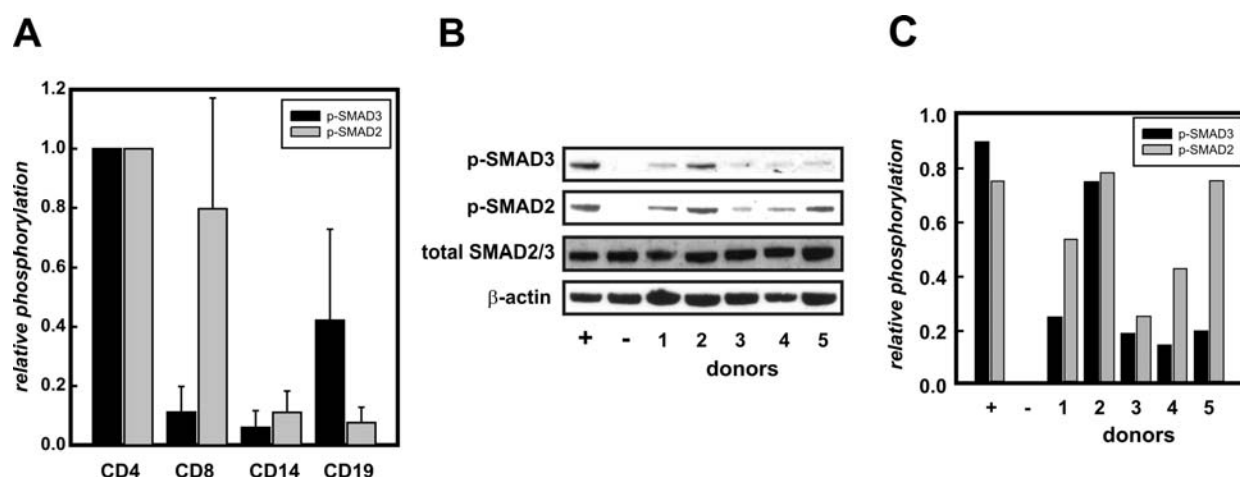


Figure 14: SMAD2 and SMAD3 are phosphorylated in primary human CD4⁺ T cells

(A) p-SMAD2 and p-SMAD3 were analyzed in highly purified CD4⁺, CD8⁺, CD14⁺ and CD19⁺ cells by Western blotting using antibodies specifically detecting phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) (n=4). The relative amount of p-SMAD2 and p-SMAD3 in comparison to β -actin was measured by densitometry. Shown here are mean \pm standard deviation of the data after normalization to CD4⁺ cells. (B) p-SMAD2, p-SMAD3, and total SMAD2/3 were analyzed in highly purified CD4⁺ T cells derived from peripheral blood of five healthy individuals by Western blotting. “-“ indicates the negative control (T cells post serum deprivation), “+“ the positive control (T cells stimulated with 10 ng/ml TGF β 1). (C) The relative amount of p-SMAD2 and p-SMAD3 in comparison to β -actin was measured by densitometry.

The TGF β signal intensity among different individuals was heterogeneous (Figure 14C) which is not due to donor specific differences in the amounts of total SMAD protein, as protein levels are comparable in all tested donors (Figure 14B).

Further T cell subpopulations were purified and SMAD phosphorylation was assessed to address whether all CD4⁺ T cell subsets are influenced by TGF β .

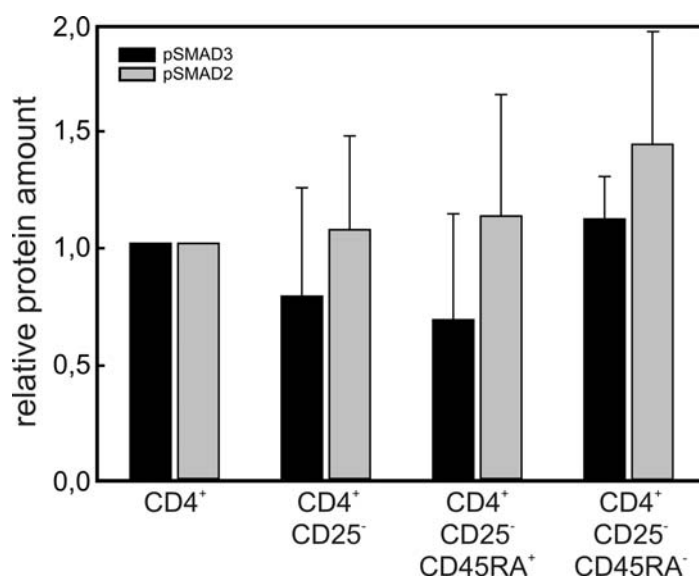


Figure 15: SMAD-phosphorylation in CD4⁺ T cell subpopulations

p-SMAD2 and p-SMAD3 were analyzed in highly purified CD4⁺ subpopulations by Western blotting using antibodies specifically detecting phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) (n=3). The relative amount of p-SMAD2 and p-SMAD3 in comparison to β -actin was measured by densitometry. Shown are mean \pm standard deviation of the data after normalization to CD4⁺ T cells.

As shown in Figure 15, CD4⁺CD25⁻CD45RA⁺ naïve T cells, CD4⁺CD25⁻CD45RA⁻ memory T cells, as well as conventional CD4⁺CD25⁻ T cells show similar amounts of phosphorylated

SMAD2 and SMAD3. Additionally, no difference in the phosphorylation levels of SMAD2 and SMAD3 in CD4⁺CD25⁺ regulatory T cells was detectable, indicating that the TGF β signaling pathway is also active in resting regulatory T cells (Figure 16).

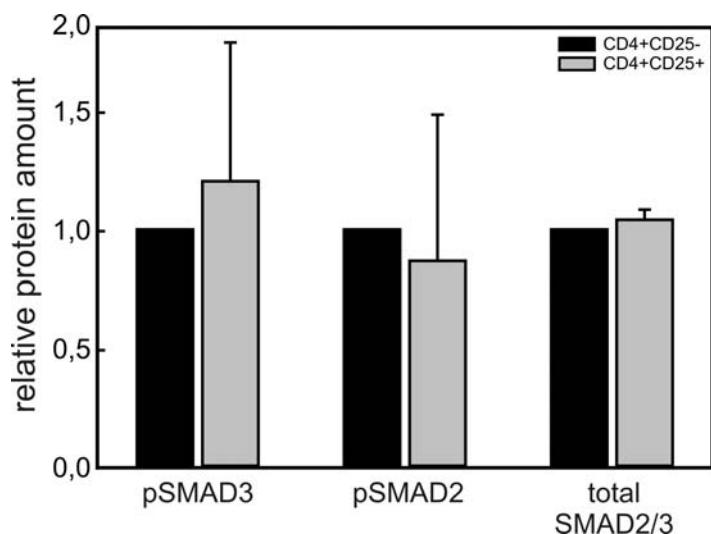


Figure 16: SMAD-phosphorylation in regulatory T cells

p-SMAD2, p-SMAD3 and total SMAD2/3 were analyzed in highly purified CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells by Western blotting using antibodies specifically detecting phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) and total SMAD2/3 (n=3). The relative amount of p-SMAD2, p-SMAD3 and total SMAD2/3 in comparison to β -actin was measured by densitometry. Shown are mean \pm standard deviation of the data after normalization to CD4⁺CD25⁻ T cells.

6.1.6 Ablation of TGF β signaling is associated with reduced SMAD phosphorylation

Next, we determined if serum deprivation would be accompanied by loss of phosphorylation of SMAD molecules. Indeed, phosphorylation of SMAD2 and SMAD3 was significantly reduced after 2 hrs and basically undetectable after 8 hrs (Figure 6A and 6B) while the amount of total SMAD protein remained constant during serum deprivation, ruling out that loss of total SMAD protein is responsible for the decrease of SMAD phosphorylation.

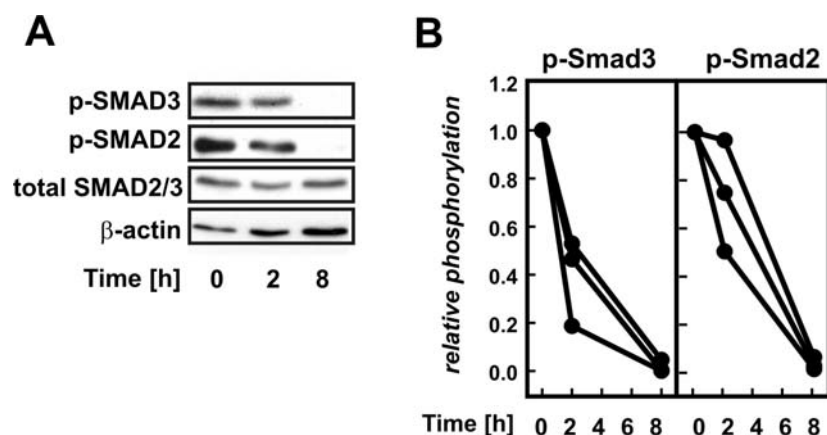


Figure 17: Kinetics of de-phosphorylation of SMAD2 and SMAD3

(A) CD4⁺T cells were serum deprived for up to 8 hrs. p-SMAD2, p-SMAD3 and total SMAD2/3 were analyzed by Western blotting using antibodies specifically detecting phosphorylated SMAD2 (p-SMAD2), SMAD3 (p-SMAD3) and total SMAD2/3. Shown is one representative experiment out of three. (B) The relative amount of p-SMAD2 and p-SMAD3 in comparison to β -actin was measured by densitometry (n=3).

Therefore, signaling events distal of the TGF β receptor become inactive in human resting CD4⁺ T cells shortly after removal of TGF β . If removal of TGF β leads to loss of SMAD phosphorylation reconstitution with TGF β should restore SMAD phosphorylation in serum-deprived resting CD4⁺ T cells. To address this hypothesis, CD4⁺ T cells were serum deprived for 18 hrs to reduce SMAD phosphorylation to undetectable levels and afterwards stimulated with increasing concentrations of TGF β 1.

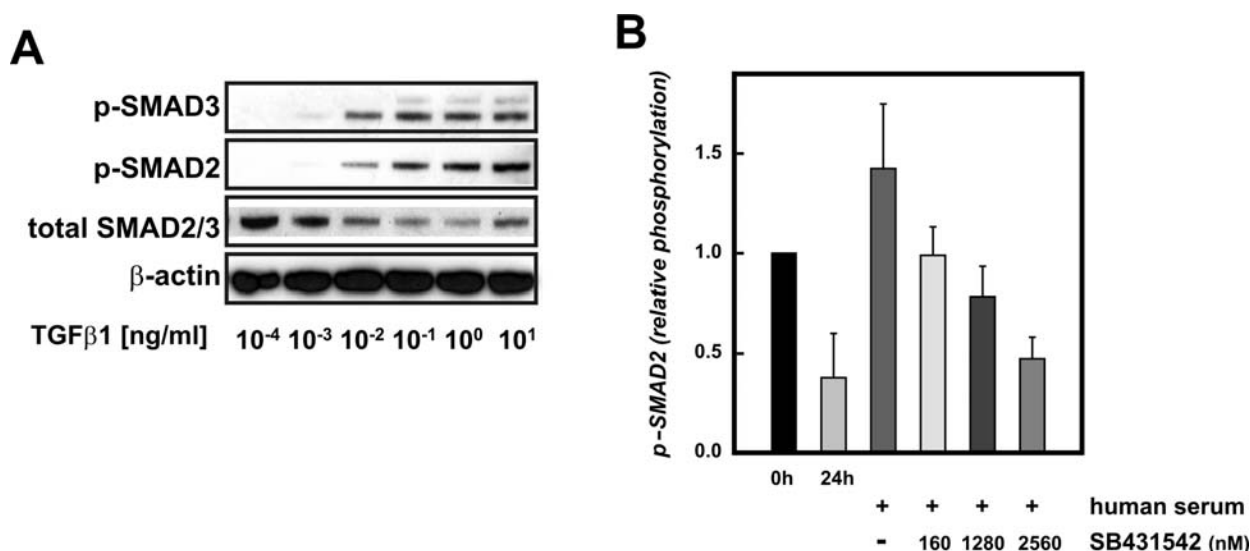


Figure 18: Dose dependency of SMAD2 and SMAD3 re-phosphorylation

(A) CD4⁺ T cells were deprived of serum for 16 hrs followed by incubation for 2 hrs with increasing concentrations of TGF β 1. SMAD2/SMAD3 phosphorylation was measured by Western blotting. Data is representative for three independent experiments. (B) CD4⁺ T cells were serum deprived for 24 hrs followed by 2 hrs of incubation with freshly isolated human serum and increasing concentrations of SB431542. SMAD2 phosphorylation was measured by Western blotting. Data represent mean \pm standard deviation of three independent experiments.

Figure 18A demonstrates that already as little as 0.001 ng/ml TGF β 1 induced significant phosphorylation of SMAD3 and at 0.01 ng/ml TGF β 1 SMAD2 phosphorylation was clearly detectable 2 hrs post TGF β 1 addition. As we have shown constitutive phosphorylation of SMAD molecules in resting CD4⁺ T cells from peripheral blood, we postulated that freshly isolated human serum, containing active TGF β , should also restore SMAD phosphorylation post serum deprivation. Hence, T cells were cultured under serum-free conditions for up to 24 hrs (Figure 18B) with subsequent addition of serum to the culture for 2 hrs. At this time point, SMAD phosphorylation was significantly increased and exceeded base line levels. To demonstrate that this effect was due to signaling via the TGF β -RII we used the TGF β receptor kinase inhibitor SB431542. As shown in Figure 18B, serum induced SMAD phosphorylation was decreased by this inhibitor in a concentration-dependent manner, further supporting that SMAD phosphorylation in human resting T cells is controlled by TGF β . The observed signaling

events downstream of TGF β are independent of other signals such as TCR stimulation or co-stimulation, since the effects were observed in the absence of further T cell stimulation.

6.1.7 Presence of constitutive TGF β signaling leads to decreased T cell proliferation

The transcriptional analysis revealed significant changes of gene expression associated with cell cycle regulation (Figure 7B). Does this have a functional consequence for CD4⁺ T cells? Ablating TGF β signaling in resting CD4⁺ T cells should lead to a more pronounced activation and proliferation since entry of the cells into the cell cycle should be enhanced in the absence of TGF β dependent regulators.

To address the net effect of TGF β removal prior to activation of resting human CD4⁺ T cells, T cells were deprived from serum for 16 hrs and then stimulated. T cell stimulation was performed using increasing concentrations of artificial antigen presenting cells (aAPC) (ratios aAPC : T cell from 1:10 to 2:1) comprised of magnetic beads coated with suboptimal concentrations of anti-CD3 and anti-CD28 mAbs (Chemnitz et al., 2004) in the presence or absence of TGF β 1 (1 ng/ml). When only low concentrations of aAPC were present (1:10 ratio), approximately half of the T cells underwent cell division (46,5%) in the absence of TGF β 1, while addition of TGF β 1 decreased the percentage of proliferating cells to 25% (Figure 19A). Even more important, while a significant number of cells reached 4 or 5 cell divisions in response to low antigen (1:10 ratio) in the absence of TGF β 1, virtually no cells had divided 5 times and only 4% had divided 4 times in presence of TGF β 1 (Figure 19B).

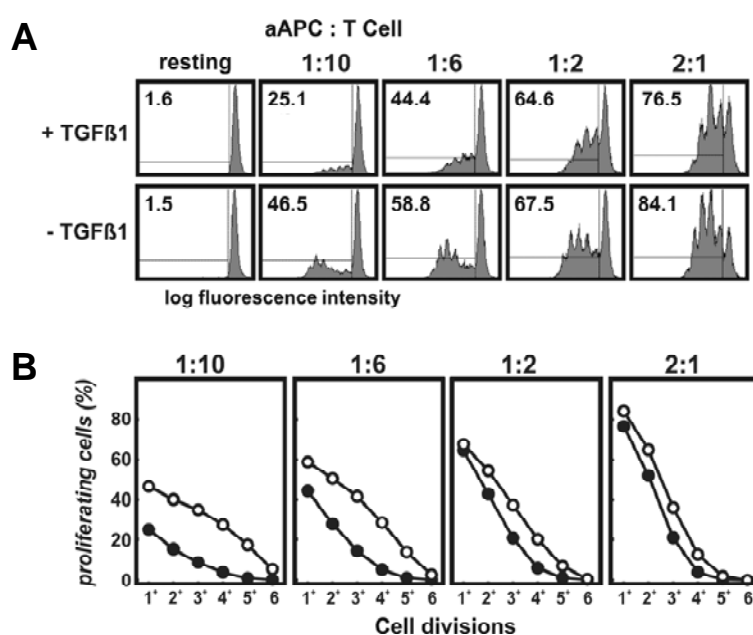


Figure 19: CD4⁺ T cell proliferation is decreased in the presence of TGF β 1

CD4⁺ T cells were serum deprived for 16 hrs and then stimulated with the indicated ratios of CD3/CD28/MHC-I aAPC in the presence or absence of 1 ng/ml TGF β 1.

(A) CD4⁺ T cell proliferation was assessed by CFSE labeling. The percentage of cells dividing at least once is indicated inside the respective histogram plot. (B) Displayed is the percentage of cells dividing at least for the indicated numbers of cycles (e.g.: 1+ = all dividing cells, 2+ = cells that divided 2 or more times, 5+ = cells that divided 5 or more times) depending on the presence (black circles) or absence (white circles) of TGF β 1 for the indicated ratios of aAPC : T cells.

When increasing the amount of aAPC to very high concentrations, aAPC still were not able to activate TGF β 1-treated CD4⁺ T cells to the same extent as untreated T cells. Albeit CD4⁺ T cell proliferation was always lower in presence of TGF β 1, this difference was less pronounced at higher concentrations of aAPC suggesting that TGF β 1 is particularly able to inhibit T cell proliferation at low levels of antigen and/or co-stimulation. Taken together, the lack of TGF β 1 signaling, as a consequence of serum deprivation, leads to an enhanced capacity of human resting T cells to respond to low antigen concentrations.

6.2 Identification of biomarkers for a new clinical TGF β R kinase inhibitor

In recent years, TGF β is getting an attractive target for the development of cancer therapeutics as the inhibition of TGF β signaling simultaneously attacks the tumor and its microenvironment. Concerning the emerging role of TGF β as a target in cancer therapies, our results are important for clinical applications of this class of novel compounds. We postulated that abrogation of TGF β signaling in response to clinical therapy would be measurable in the transcriptional profile of peripheral CD4⁺ T cells. To evaluate this hypothesis, we tested a novel small molecule compound developed by Eli Lilly Inc. targeting the TGF β signaling pathway. This TGF β receptor kinase inhibitor was designed to abrogate the TGF β signaling pathway in clinical therapy by blocking the kinase domain of the TGF β receptor type I (Yingling et al., 2004), (Singh et al., 2004). Functional assays as well as transcriptome analyses were performed to study the effects of this inhibitor on peripheral human CD4⁺ T cells.

6.2.1 The TGF β R kinase inhibitor restores TGF β -mediated T cell inhibition

One major consequence of active TGF β 1 signaling is the inhibition of T cell proliferation (see Figure 19). Hence, it was tested whether and at which concentrations the TGF β receptor kinase inhibitor (LY2109761) can restore T cell proliferation.

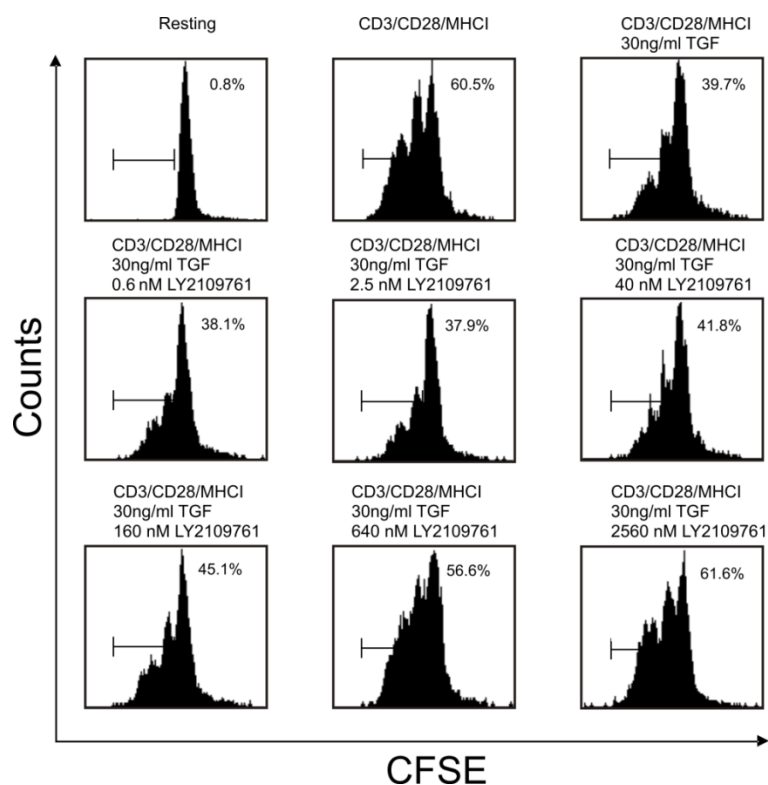


Figure 20: TGF β -mediated T cell inhibition is restored by LY2109761

Freshly isolated CD4⁺ T cells were serum deprived for 16 hrs and then stimulated CD3/CD28/MHC-I aAPC (1 T cell : 3 beads) in the presence or absence of 30 ng/ml TGF β 1 and increasing concentrations of LY2109761.

CD4⁺ T cell proliferation was assessed by CFSE labeling. The percentage of cells dividing at least once is indicated inside the respective histogram plot.

T cell activation led to the proliferation of 60% of the cells, while addition of TGF β 1 reduced this percentage to 40% (Figure 20). The presence of low inhibitor concentrations (0.6, 2.5 and 40 nM LY2109761) did not show any influence on TGF β mediated T cell inhibition, as the rate of dividing cells (approximately 40%) did not increase. Further increase of the inhibitor dose reduced the inhibitory effect of TGF β 1 in a concentration dependent manner. When adding 2560 nM LY2109761, T cell proliferation was completely restored, as 61% of the cells underwent proliferation. These results demonstrate the potential of the compound to block the TGF β signaling pathway efficiently and the blockade was concentration dependant.

CD4⁺ T cells consist of several subpopulations. In contrast to CD4⁺CD25⁻CD45RA⁺ naive T cells, CD4⁺CD25⁻CD45RA⁻ memory T cells are antigen experienced leading to a more pronounced activation in response to an appropriate antigen stimulation. Therefore, it was analyzed whether antigen experienced memory T cells respond similar to TGF β -mediated inhibition of T cell proliferation as naïve T cells do and whether it is possible to block the TGF β -mediated suppression in both cell types using the TGF β inhibitor LY2109761?

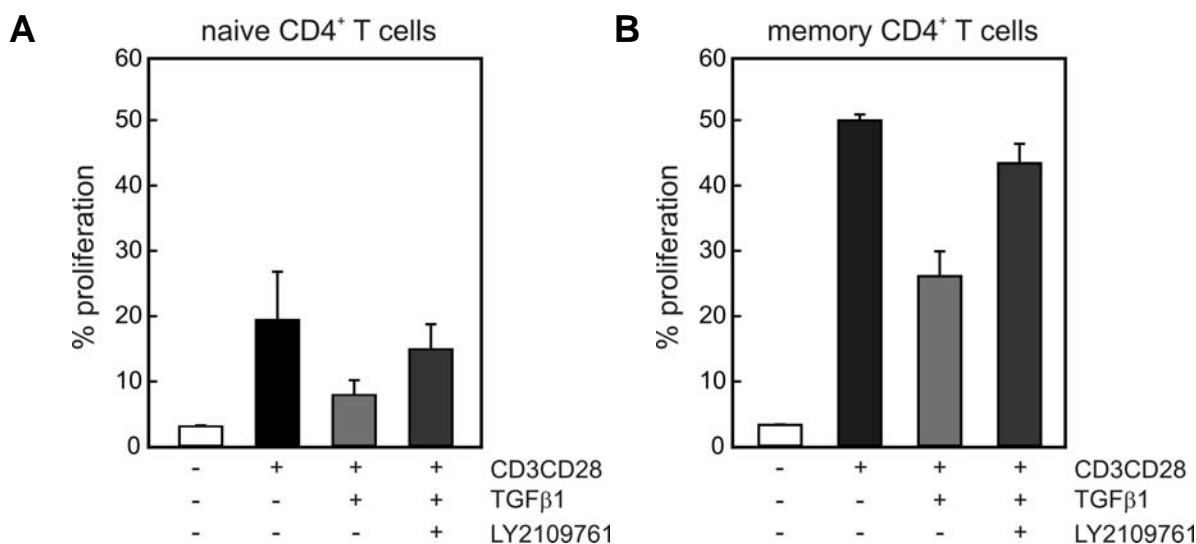


Figure 21: LY2109761 restores the TGF β -mediated block of proliferation in naïve and memory T cells

CD4⁺CD25⁻ T cells were subdivided in CD4⁺CD25⁻CD45RA⁺ naïve (A) and CD4⁺CD25⁻CD45RA⁻ memory (B) T cells. Cells were stimulated with CD3/CD28/MHC-I aAPC (3 T cells : 1 bead) in the presence or absence of 10 ng/ml TGF β 1 and 2560nM LY2109761. T cell proliferation was assessed by CFSE labeling. Shown are the percentages of dividing cells. Data represent mean +/- standard deviation of three independent experiments.

As expected, the overall proliferation was decreased in CD4⁺CD25⁻CD45RA⁺ naïve T cells due to the prolonged activation time of naïve T cells (Figure 21). Interestingly, the suppressive effect of TGF β on CD4⁺CD25⁻CD45RA⁺ naïve and on CD4⁺CD25⁻CD45RA⁻ memory T cells was similar. The inhibitory effect of TGF β could be restored in both T cell subpopulations using the TGF β RI kinase inhibitor. Therefore, the blocking effect of the TGF β RI kinase inhibitor on TGF β signaling is comparable in naïve and memory CD4⁺ T cells.

6.2.2 The inhibitor counter-regulates most TGF β -mediated transcriptional changes

Next, microarray experiments were performed to identify gene expression changes in response to LY2109761 treatment. In these experiments, a concentration of 2560 nM of LY2109761 was chosen to study the effect of a complete TGF β signaling blockade.

Human CD4⁺ T cells were serum deprived for 12 hrs and then left untreated or treated with either 10 ng/ml TGF β 1 or the combination of TGF β 1 and LY2109761 for further 8 hrs (Figure 22). Subsequently, gene expression analysis was performed using the Illumina whole-genome microarray platform.

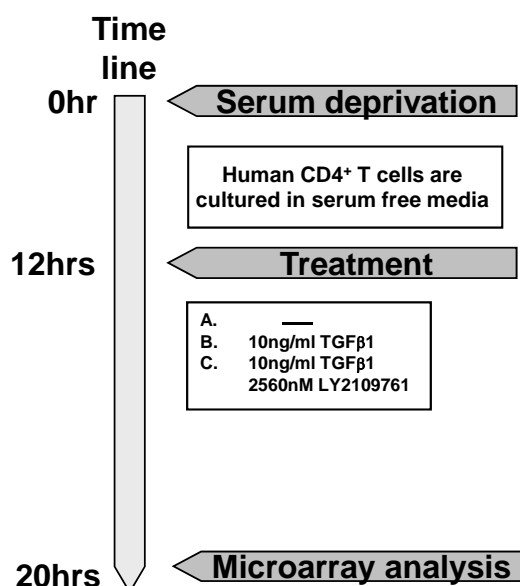


Figure 22: Setup to study LY2109761 induced expression changes in resting CD4⁺ T cells

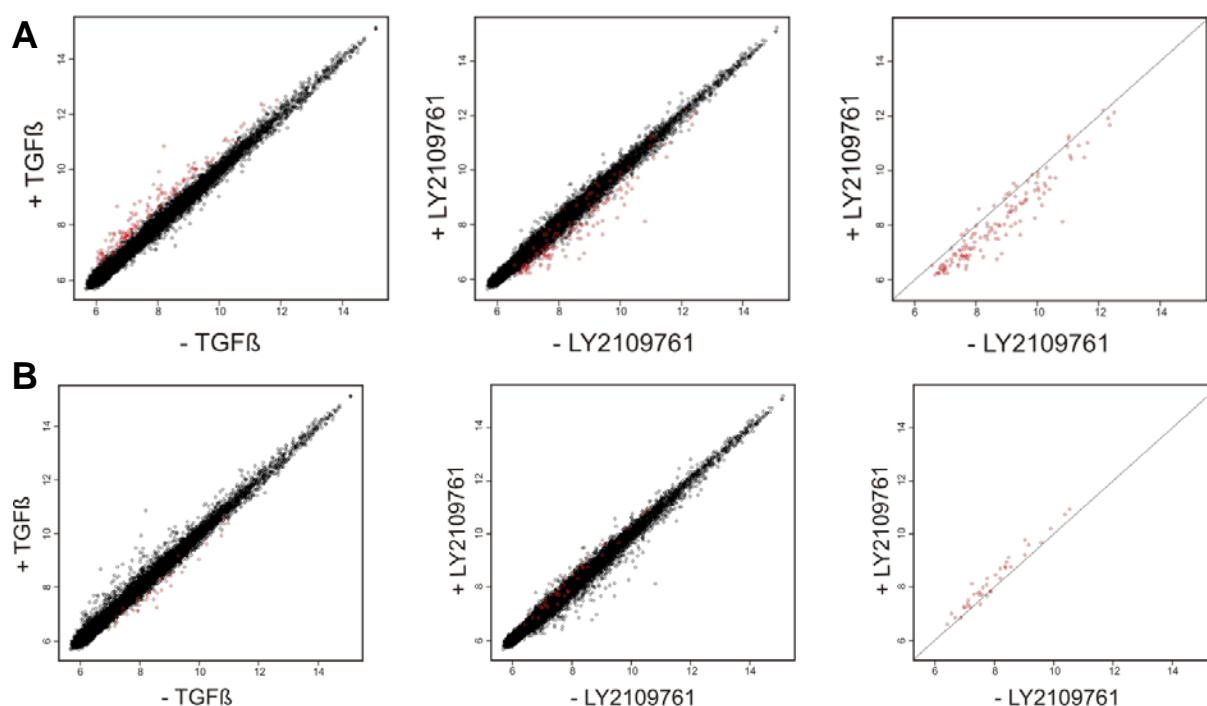


Figure 23: TGFβ1 regulated genes are counter-regulated by LY2109761 in resting CD4⁺ T cells

CD4⁺ T cells were serum deprived for 12 hrs. Cells were either left untreated or treated with 10 ng/ml TGFβ1 in the presence or absence of 2560nM LY2109761 for further 8 hrs. Gene expression changes were measured using the Illumina-platform. Shown here are scatter plots displaying gene expression changes of all genes. In the left plots expression changes in response to TGFβ1 treatment are depicted, but only genes significantly upregulated (**A**, FC ≥ 1.5, p < 0.05) and downregulated (**B**, FC ≤ -1.5, p < 0.05) in response to TGFβ1 treatment are highlighted in red. In the middle plots expression changes of all genes in response to LY2109761 are shown, whereas on the right plots only those genes in response to LY2109761 are visualized that were regulated by TGFβ1 (marked in red in the first plot).

In Figure 23, only genes significantly upregulated (**A**, FC ≥ 1.5, p < 0.05) and downregulated (**B**, FC ≤ -1.5, p < 0.05) in response to TGFβ treatment are highlighted in red in the far left

picture. Not all genes significantly regulated by TGF β are also significantly counter-regulated by the inhibitor using stringent filter criteria. Therefore, we decided to envision transcriptional changes in response to LY2109761 treatment using scatter plots, which enables to show the general tendency of gene expression changes in response to LY2109761 treatment. The far right scatter plot indeed demonstrates that, even if not significant (as judged by our experimental setting with low sample numbers), mostly all genes regulated by TGF β in resting CD4⁺ T cells were counter-regulated by 2560nM LY2109761.

Since resting and activated CD4⁺ T cells co-exist *in vivo*, it is important to study additionally the transcriptional effect of the inhibitor on activated CD4⁺ T cells.

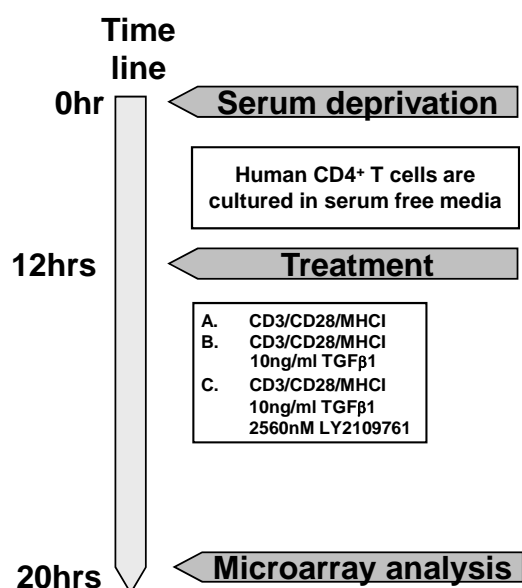
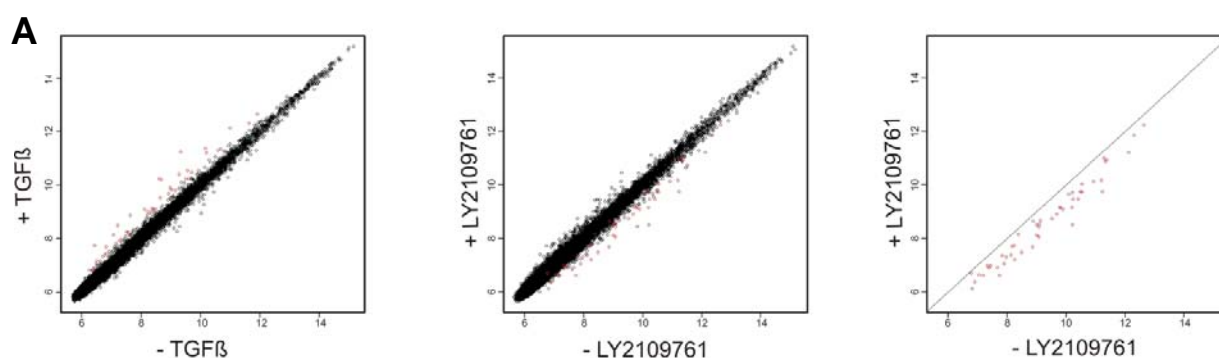


Figure 24: Setup to study LY2109761 induced expression changes in activated CD4⁺ T cells

Thus, human CD4⁺ T cells were serum deprived for 12 hrs and then activated with aAPCs in the presence or absence of 10 ng/ml TGF β 1 or the combination of TGF β 1 and LY2109761 for further 8 hrs (Figure 24). Afterwards, microarray analysis was performed using the Illumina whole-genome microarray platform.



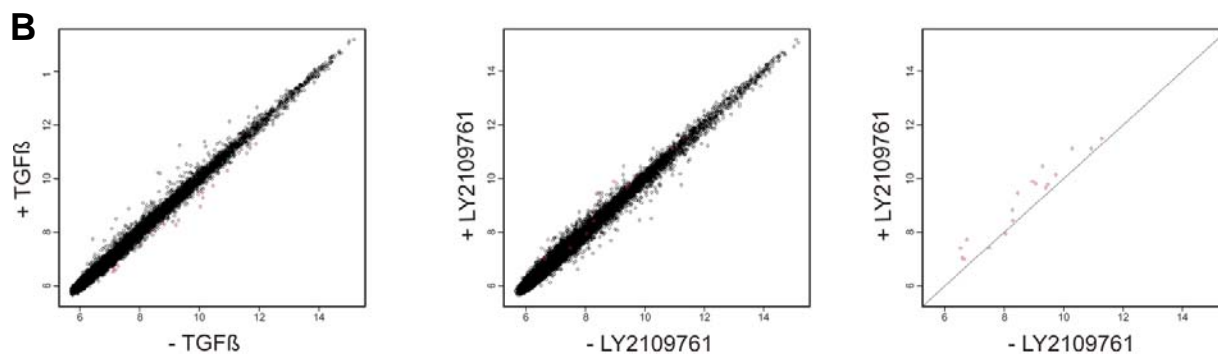


Figure 25: TGFβ1 regulated genes are counter-regulated by LY2109761 in activated CD4⁺ T cells

CD4⁺ T cells were serum deprived for 12 hrs. Cells were stimulated with aAPCs (3 beads: 1 T cell) and either left untreated or treated with 10 ng/ml TGFβ1 in the presence or absence of 2560nM LY2109761 for further 8 hrs. Shown are scatter plots displaying gene expression changes of all genes. In the left plots expression changes in response to TGFβ1 treatment are depicted, but only genes significantly upregulated (**A**, FC ≥ 1.5, p < 0.05) and downregulated (**B**, FC ≤ -1.5, p < 0.05) in response to TGFβ1 treatment are highlighted in red. In the middle plots expression changes of all genes in response to LY2109761 are shown, whereas in the right plots only those genes in response to LY2109761 are visualized that were regulated by TGFβ1 (marked in red in the first plot).

In activated CD4⁺ T cells, fewer genes were regulated by TGFβ1 than under resting conditions. This result can be explained by the fact that in this experimental setting two signals influenced the CD4⁺ T cells: 1. aAPC induced T cell activation and 2. TGFβ1 mediated T cell inhibition. The expression changes induced by these two factors can be separated into four groups: 1: Genes targeted by activation, 2: Genes influenced by TGFβ1, 3: Genes regulated by both factors in the same direction and 4: genes counter-regulated by these two factors. Therefore, the regulation of fewer genes by TGFβ1 under T cell activating conditions was expected, as expression changes in response to two factors lead to a less pronounced effect than studying only one factor. Nevertheless, equally to resting conditions, mostly all genes regulated by TGFβ1 were counter-regulated by LY2109761 in activated CD4⁺ T cells (Figure 25).

Taken together, the scatter plots demonstrate the high efficiency of the inhibitor to counter-regulate TGFβ1-mediated transcription changes in resting as well as activated CD4⁺ T cells.

6.2.3 Biomarker candidates for clinical TGFβRI kinase inhibitor therapy

Biomarkers are considered as a useful tool in clinical therapy, as they can provide information about the efficiency and the required dosage of a therapeutic drug. In fact, the transcriptional profile obtained in CD4⁺ T cells in response to TGFβR kinase inhibitor treatment revealed information about the general efficiency of this therapeutic drug. Moreover, transcriptional profiles can be used to identify those genes, which are regulated strongest by TGFβRI kinase

inhibitors. The most significantly regulated genes comprise the potential to function as biomarkers for the TGF β receptor kinase inhibitor in clinical applications. The requisites of clinical biomarkers for the TGF β receptor kinase inhibitor are 1. biomarker genes should be TGF β target genes that are strongly regulated by the inhibitor and 2. the biomarker genes should be regulated by the TGF β receptor kinase inhibitor in resting as well as activated CD4⁺ T cells, since especially in cancer patients the amount of activated T cells may be increased. Therefore, in the next step of the analysis, stringent filter criteria (FC > 1.5 or FC < -1.5, p-value < 0.05) were used to detect only those genes that were significantly regulated by TGF β and significantly counter-regulated by the inhibitor in resting as well as activated CD4⁺ T cells.

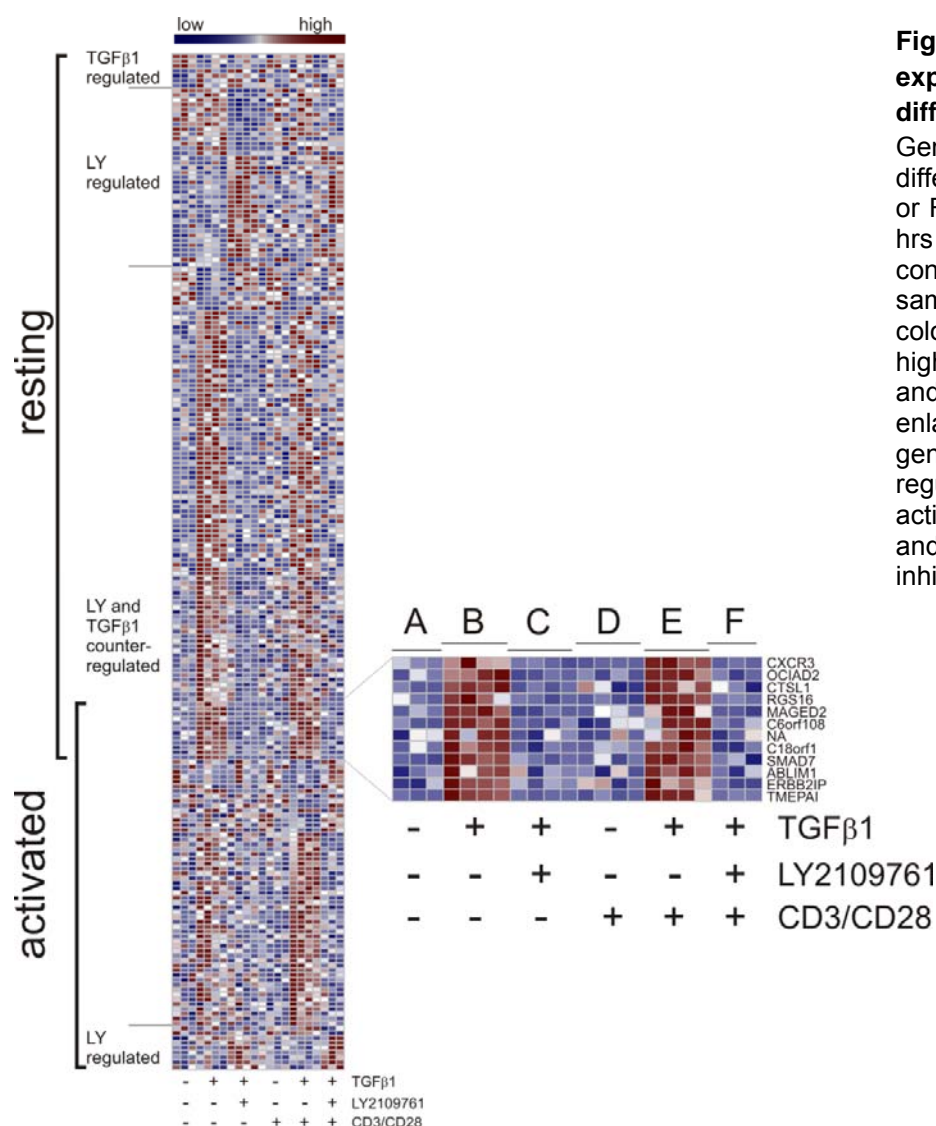


Figure 26: Heatmap displaying expression values of differentially expressed genes

Genes were selected based on differential expression (FC > 1.5 or FC < -1.5, p-value < 0.05) at 8 hrs after treatment under different conditions compared to untreated samples. Expression values are color-coded showing genes being high- and low-expressed in red and blue, respectively. The enlargement represents those genes that were significantly regulated in resting as well as activated CD4⁺ T cells by TGF β 1 and counter-regulated by the inhibitor.

Altogether, 12 genes were identified to be significantly upregulated in response to TGF β 1 and counter-regulated by LY2109761 in resting as well as activated CD4⁺ T cells (Figure 26). These 12 genes contain the potential to serve as biomarkers for clinical drugs targeting the TGF β signaling pathway.

6.2.4 Further development of biomarkers for clinical TGF β RI kinase inhibitor therapy

Biomarker candidates obtained by transcriptional profiling have to be further tested for their clinical application and robustness. Therefore, I designed a workflow to address the applicability of these biomarkers. Initially, further *in vitro* experiments are needed to define the regulation of these biomarker genes (Figure 27).

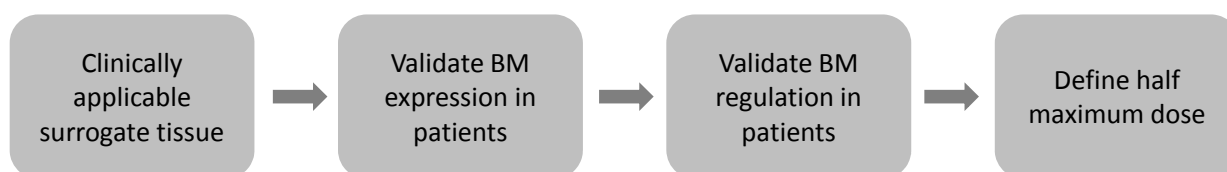


Figure 27: Schematic workflow for *in vitro* biomarker validation experiments

Notes: 'BM' stands for biomarker

Validation experiments were performed in the laboratory of Prof. Schultze during the diploma thesis of Christine Muth ('Entwicklung von Biomarkern für Theraphieverlaufkontrollen mit TGF β Rezeptor Kinase Inhibitoren in peripherem Blut und Charakterisierung eines unbekanntes Gens'), Institute for Genetics, University of Cologne.

Briefly, out of these 12 biomarker candidate genes, four genes (OCIAD2, TMEPAI, CTSL1 and SMAD7) were chosen for further development since they were regulated most significantly in response to TGF β R kinase inhibitor treatment in CD4⁺ T cells. Next, regulation of these four genes was studied in a more clinically applicable surrogate tissue, namely peripheral blood mononuclear cells (PBMC). PBMCs can be isolated much easier, faster and with much lower costs than CD4⁺ T cells. Out of these four genes, three genes (OCIAD2, TMEPAI and SMAD7) showed similar regulation in PBMCs. This result allows biomarker monitoring in PBMCs as surrogate tissue and thereby highly increases the practicability in the clinics.

In the next step, expression levels of these three genes in PBMCs of lung cancer patients were assessed to exclude that cancer patients have altered expression levels of these biomarker candidate genes. This analysis revealed no significant difference of the expression levels of these three genes in lung cancer patients when compared to healthy volunteers. Even more important, regulation of these three genes in response to TGF β R kinase inhibitor treatment in PBMCs of lung cancer patients was comparable to regulation in healthy individuals. These results further support the potential to use these genes as biomarkers during TGF β R kinase inhibitor therapy of cancer patients.

In addition, the sensitivity of these biomarkers was investigated. Regulation of the expression levels of the biomarker genes was highly sensitive, as already 320 nM of the TGF β R kinase inhibitor were sufficient to downregulate biomarker expressions significantly independent of the present TGF β concentrations (0ng/ml – 10ng/ml).

Since the TGF β R kinase inhibitor will be catabolized *in vivo*, we questioned how long the effect of the TGF β R kinase inhibitor is still detectable by monitoring the regulation of the biomarkers after removal of the drug. Within this study we were able to show that 2 hrs after TGF β R kinase inhibitor removal the biomarkers still were significantly downregulated. In contrast, 8 hrs afterwards, biomarker expression levels reached basal expression levels.

Taken together, these *in vitro* data demonstrate the high potential of the three newly identified biomarkers to provide information about the effects and activity of a therapeutic drug targeting the TGF β signaling pathway in cancer patients. However, these promising data have to be corroborated *in vivo* (Figure 28).

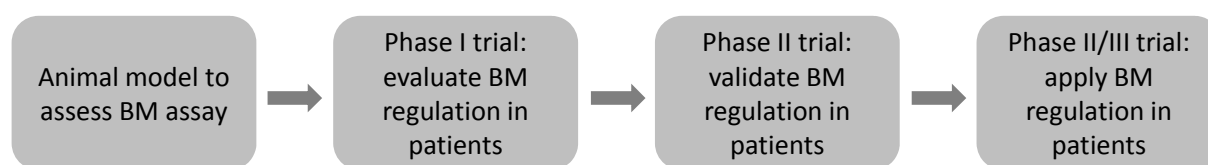


Figure 28: Schematic workflow for *in vivo* biomarker validation experiments

Notes: 'BM' stands for biomarker

To evaluate whether the three biomarkers also provide information about the TGF β R kinase inhibitor activity *in vivo*, the inhibitor was administered to mice. Regulation of the genes was then studied in peripheral PBMCs at different time points after treatment. All three biomarker genes (OCIAD2, TMEPAI and SMAD7) were significantly downregulated in mice in response to TGF β R kinase inhibitor treatment. This effect was measurable for up to 24 hrs after treatment. Thus, similar regulation of these three biomarker genes was validated in the murine *in vivo* model. These data formed the basis for the incorporation of monitoring the three novel biomarkers in cancer patients within a phase I clinical trial assessing a TGF β R-kinase-specific inhibitor planned by Eli Lilly Inc. to be started within the second half of 2008.

Taken together, by combining an unbiased transcriptome approach with molecular and immunological experiments novel biomarkers were identified for monitoring therapies targeting the TGF β signaling pathway. Using these three biomarkers during clinical therapy might help to determine the therapeutic dosage and to identify whether or not patients show clinical response to the compound (Classen et al., manuscript in preparation).

6.3 Interaction of TGF β and Treg cells

As outlined in chapter 6.1, we demonstrated that TGF β is an important factor controlling the homeostasis of CD4⁺ T cells. Furthermore, TGF β signaling was active in all tested CD4⁺ T cell subpopulations (naïve T cells, memory T cells and Treg cells) in the resting state (Figure 15). However, TGF β seems to exert different functions in CD4⁺ T cell subpopulations. Whereas TGF β controls the activation of T cells in response to stimulation (Gorelik and Flavell, 2002), this factor is also implicated in the generation, maintenance and function of inhibitory Treg cells (Liu et al., 2008), (Marie et al., 2005), (Nakamura et al., 2001).

Treg cells as well as the soluble factor TGF β both have suppressive capacity on T cell activation. Mice deficient either for Treg cells or for active TGF β signaling (e.g. TGF β 1^{-/-}, DN TGF β RII) develop similar severe autoinflammatory diseases (Brunkow et al., 2001), (Kulkarni et al., 1993), (Shull et al., 1992). Since TGF β 1-deficient mice cannot maintain Treg cells in the periphery (Marie et al., 2005), it is not yet clear what causes the inflammatory response in these mice: 1. Is the inflammatory phenotype in TGF β 1-deficient mice due to the loss of direct inhibition of T cell activation by TGF β 1 or 2. Is the phenotype due to an indirect loss of TGF β -induced inhibitory Treg cells or 3. is the loss of both mechanisms implicated in the inflammatory disease (Figure 29; (Li et al., 2006a))?

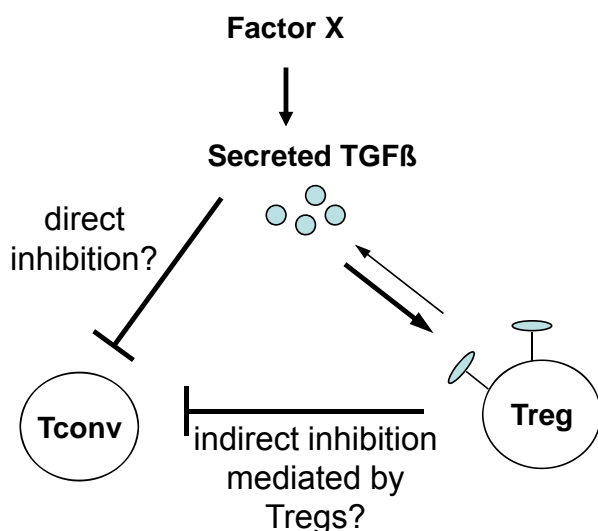


Figure 29: TGF β -Treg interaction scheme

In human T cell biology, there are some obstacles when studying the interaction of soluble factors such as TGF β and rare cells such as Treg cells. For example, the well-integrated knock-out and knock-in strategies used in the murine system to define the role of single genes certainly cannot be applied to the human system. In humans, knock-out or overexpression of

selected genes can only be performed by *in vitro* approaches. It is rather difficult to integrate the influence of the micro milieu on individual cells in such *in vitro* systems. Nevertheless, direct effects of soluble factors such as TGF β on individual cells and downstream effects can be studied in human cells. Therefore, we focused on these aspects first.

6.3.1 TGF β mediates T cell inhibition in the absence of Treg cells

The inhibitory potential of TGF β on T cell activation was studied around 1990 (Kehrl et al., 1986b), (Ahuja et al., 1993). At that time, previously described suppressor cells were not accepted anymore in the scientific community and therefore, TGF β -mediated T cell inhibition was always analyzed on whole CD4⁺ T cells. Since the seminal findings by Sakaguchi et al. in 1995 the presence of suppressive Treg cells has been re-established within the CD4⁺ T cell population (Powrie et al., 1994), (Sakaguchi et al., 1995). Interestingly, the direct inhibitory role of TGF β on all CD4⁺ T cell subpopulations has still not been re-addressed adequately. Therefore, it needs to be further clarified, whether the inhibitory effect of TGF β on activation of human T cells is 1.) directly mediated by TGF β , 2.) indirectly by activation of Treg cells (Figure 29) or, alternatively, 3.) by both mechanisms. To further elucidate this question, we first studied the inhibitory capacity of TGF β on human T cell proliferation in the absence of regulatory T cells.

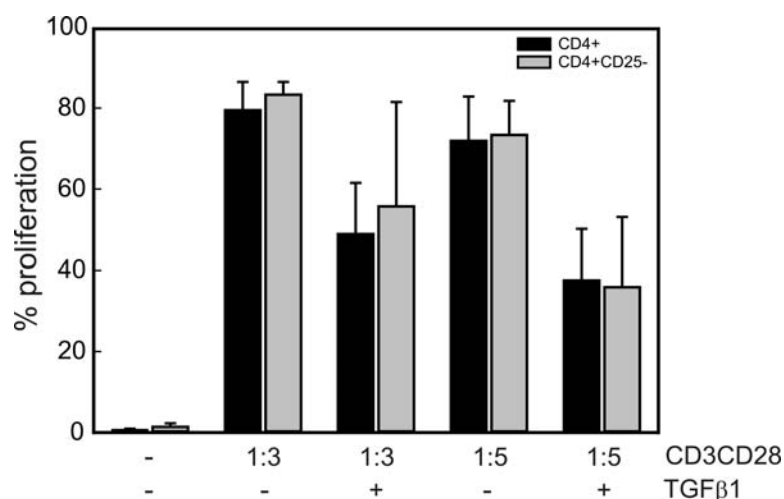


Figure 30: TGF β mediates T cell inhibition in the absence of Treg cells

CD4⁺ and CD4⁺CD25⁻ T cells were isolated and stimulated with the indicated ratios of CD3/CD28/MHC-I aAPC in the presence or absence of TGF β 1 (10 ng/ml). T cell proliferation was assessed by CFSE labeling. Shown are the percentages of dividing cells. Data represent mean \pm standard deviation of three independent experiments.

In this experiment, T cell proliferation was assessed in whole CD4⁺ T cells in comparison to CD4⁺ T cells depleted of Treg cells (CD4⁺CD25⁻ T cells) (Figure 30). T cell proliferation in response to T cell stimulation was similar in these two groups, independent of the strength of T cell stimulation (3 T cells : 1 aAPC; 5 T cells : 1 aAPC). Additionally, the TGF β -mediated inhibition of T cell proliferation was comparable in whole CD4⁺ T cells and CD4⁺ T cells

depleted of Treg cells under both conditions (Figure 30). Therefore, the suppressive effect of TGF β is attained independently of Treg cells and TGF β acts directly on the target cell.

6.3.2 The TGF β RI kinase inhibitor reduces the suppressive effect of Treg cells

In the previous experiment, we were able to demonstrate that TGF β can inhibit T cell activation in a Treg cell-independent way. However, whether TGF β is involved in the suppressive mechanism of Treg cells on T cell activation is not yet clear. So far, conflicting results concerning the role of TGF β on the suppressive function of Treg cells have been reported. While Treg cell-mediated inhibition of T cell activation was abolished by the blockade of the TGF β signalling pathway in some studies, (Nakamura et al., 2004), (Oida et al., 2006), (Fahlen et al., 2005), (Chen et al., 2005), other studies exist where blockade of TGF β signalling did not reverse Treg cell-mediated suppression (Piccirillo et al., 2002), (von Boehmer, 2005), (Kullberg et al., 2005), (Shevach et al., 2001).

These results were achieved by *in vitro* assays using either TGF β neutralizing antibodies or soluble TGF β RII-Fc. Thereby, TGF β signaling was abrogated by trapping the ligand outside the cell. This strategy has the disadvantage that it cannot be entirely excluded that some TGF β molecules still bind to their receptors, thereby leading to (low-level) TGF β signaling.

Interestingly, direct blockade of TGF β signaling in human T cells by compounds targeting the TGF β signaling pathway within the cell has not been reported to date. We had the opportunity to assess the effect of the small molecule inhibitor to study the role of TGF β as a mediator of the suppressive mechanism of Treg cells *in vitro*. The TGF β RI kinase inhibitor is characterized by a highly specific abrogation of TGF β signaling inside the cell. In addition, the effectiveness of the TGF β signaling blockade can be precisely controlled by the administered dosage of the TGF β R kinase inhibitor, as it is a stochastic process that all receptors are targeted inside the cell.

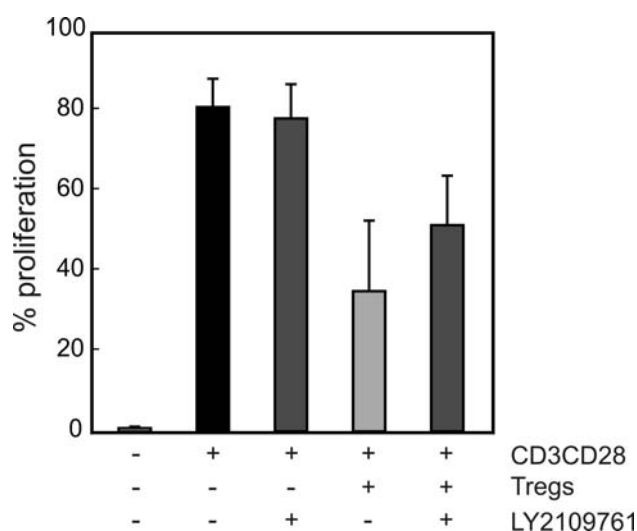


Figure 31: The TGF β R kinase inhibitor partially reduces Treg cell-mediated suppression

CD4⁺CD25⁻ T cells were stimulated with CD3/CD28/MHC-I aAPC (3 T cells : 1 bead) in the presence Treg cells (2 T cells : 1 Treg cells) and in combination with 2560 nM LY2109761. T cell proliferation was assessed by CFSE labeling. Shown are the percentages of dividing cells. Data represent mean \pm standard deviation of three independent experiments.

Flow cytometric analysis of CFSE-labeled CD4⁺ T cells revealed that approximately 80% of all cells proliferated in response to CD3CD28 treatment (Figure 31). T cell proliferation was more than 2-fold reduced in the presence of inhibitory Treg cells (38%). The suppressive capacity of Treg cells was partially reduced in the presence of the TGF β RI kinase inhibitor (53%). This results supports the notion that human Treg cells may suppress T cell proliferation not only by activating the inhibitory TGF β signaling pathway in the target cell, but also by using other mechanisms, such as signaling via IL-10 and CTLA-4.

6.4 Implication of TGF β on 'induced' human Treg cells

It has been established over the last years in the murine system that naïve T cells can convert into FOXP3⁺ Treg cells in the presence of TGF β under T cell activating conditions (Chen et al., 2003), (Wan and Flavell, 2005). These induced Treg cells are anergic and show suppressive capacities (Chen and Wahl, 2003). Thus, FOXP3 expression and suppressive capacity are directly linked in mice.

In contrast to the murine system, the conversion of human CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ Treg cells seems to be more complex. Comparable to murine studies, several publications demonstrated the possibility to convert human CD4⁺CD25⁻ T cells into FOXP3 Treg cells under T cell activating conditions in the presence of TGF β (Fantini et al., 2004), (Hill et al., 2007), (Davidson et al., 2007). However, recently several groups suggested that in humans, activation of conventional CD4⁺CD25⁻ T cells alone is sufficient to induce FOXP3⁺ expression (Pillai et al., 2007), (Allan et al., 2007), (Wang et al., 2007). Furthermore, FOXP3 expression and suppressive function are not directly correlated in human induced Treg cells in all experiments. In some studies, FOXP3⁺ induced Treg cells showed suppressive capacity (Pillai et al., 2007), (Walker et al., 2005), (Walker et al., 2003), (Wang et al., 2007), whereas in

other studies no inhibitory capacity after activation of FOXP3⁺ T cells was detectable (Tran et al., 2007), (Allan et al., 2007), (Morgan et al., 2005), (Allan et al., 2005).

Taken together, in humans, contradicting results exist whether T cell activation alone or in combination with TGF β results in the induction of FOXP3⁺ Treg cells. Moreover, it is not clear, whether all these cells expressing FOXP3 exert suppressive function or not.

One important issue has to be considered when studying the induction of Treg cells. If indeed all human T cells upregulate FOXP3 and other Treg specific markers in response to T cell activation, and these cells even gain suppressive function, these findings might contradict the present understanding of immunity. One paradigm of adaptive immunity is that upon T cell stimulation, T cells get activated and start proliferation in order to destroy the invading pathogen. Regarding this point, two important questions need to be considered: how can an activation of conventional CD4⁺CD25⁻ T cells result in a gain of suppressive capacity by these T cells and how can these findings be integrated into the current understanding of immunity? We hypothesize here that induction of FOXP3 and gain of suppressive function might be a rather late event during T cell activation. The following experiments were designed to elucidate this further.

6.4.1 FOXP3 induction is a late activation response

To better define the role of T cell activation and TGF β in the induction and function of human Treg cells, we first studied the expression levels of FOXP3 in CD4⁺CD25⁻CD45RA⁺ naïve T cells on RNA and protein level after different time length of T cell culture (2,3,4,5 and 6 days). Shown here are the results obtained after 3 and 5 days of T cell stimulation. Day 3 represents the time point, on which T cell activation is normally measured, since T cell activation is most advanced at day 3. Day 5 is a late time point of T cell activation.. Furthermore, freshly isolated CD4⁺ T cell subpopulations were integrated into the analysis, to compare the degree of FOXP3 induction to FOXP3 expression levels present in natural Treg cells.

As shown in Figure 32B, 3 days of T cell stimulation induced FOXP3 expression only slightly in comparison to FOXP3 expression levels present in natural CD4⁺CD25⁺ Treg cells (Figure 32A). The addition of TGF β to the T cell cultures increased FOXP3 RNA levels but still FOXP3 expression was low in comparison to expression levels in natural Treg cells (Figure 32A). 5 days of T cell activation in the absence of TGF β enhanced FOXP3 expression but still only to approximately 10% of the levels detectable in natural Treg cells. In contrast, CD4⁺CD25⁻CD45RA⁺ naïve T cells activated for 5 days in the presence of TGF β enhanced FOXP3 expression levels to levels obtained in natural Treg cells (Figure 32B).

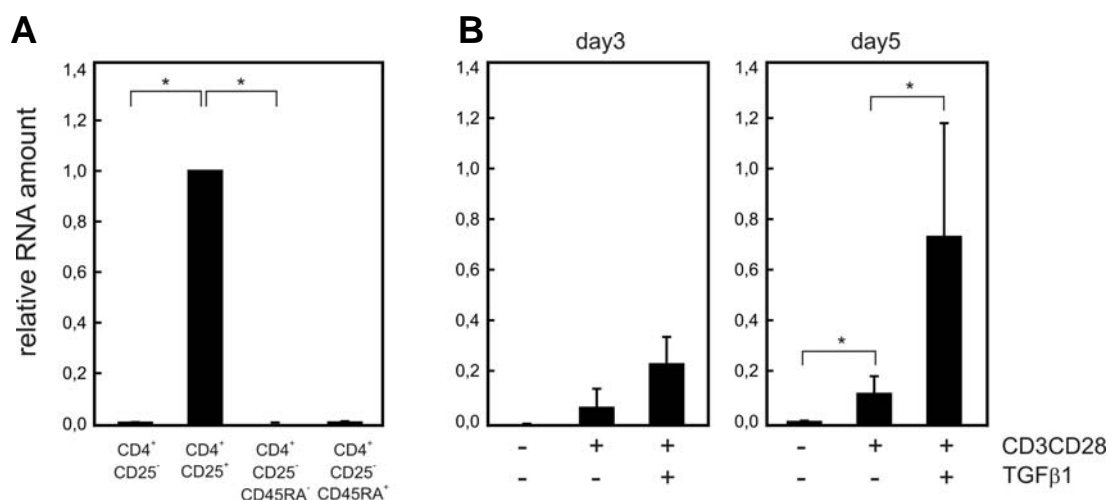


Figure 32: FOXP3 expression is induced upon T cell stimulation in naïve T cells

(A) FOXP3 RNA levels were assessed in freshly isolated CD4⁺CD25⁻, CD4⁺CD25⁺, CD4⁺CD25⁻CD45RA⁻ and CD4⁺CD25⁻CD45RA⁺ T cells by RT-PCR. (B) CD4⁺CD25⁻CD45RA⁺ T cells were cultured for 3 days or 5 days in the absence or presence of CD3/CD28/MHC-I aAPCs (3 T cells : 1 beads); and 10 ng/ml TGFβ. FOXP3 RNA levels were assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean +/- standard deviation of three independent experiments. Statistically significant differences (paired t-test p<0.05) are marked with an asterisk.

These data reveal an important difference in the induction of FOXP3 RNA expression in activated CD4⁺CD25⁻CD45RA⁺ naïve T cells depending on the presence of TGFβ. Therefore, we investigated, whether this difference is also detectable on the protein level.

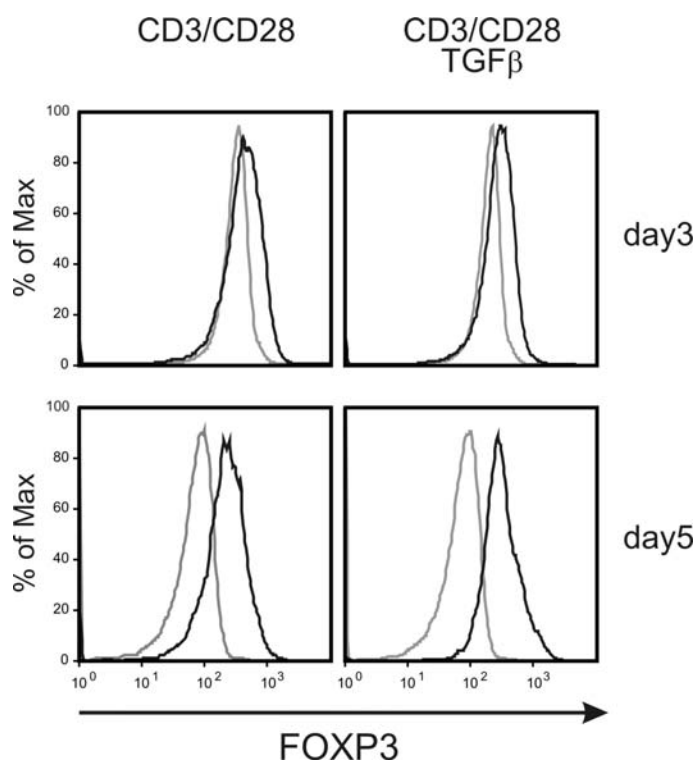


Figure 33: FOXP3 protein levels are upregulated in response to T cell stimulation

CD4⁺CD25⁻CD45RA⁺ naïve T cells were cultured for 3 or 5 days in the presence of CD3/CD28/MHC-I aAPCs (3 T cells : 1 beads) (left picture) with or without 10 ng/ml TGFβ (right picture). At day 3 and 5, FOXP3 protein levels were assessed by flow cytometry (black line). The grey line represents the negative control (untreated CD4⁺CD25⁻CD45RA⁺ naïve T cells). Shown here is one representative experiment out of six.

Comparable to FOXP3 RNA levels, FOXP3 protein levels were only slightly increased in activated $CD4^+CD25^-CD45RA^+$ naïve T cells (black line) in comparison to untreated $CD4^+CD25^-CD45RA^+$ naïve T cells (grey line) after 3 days of stimulation (Figure 33, left picture in the upper row). Again, FOXP3 protein levels were highly increased in activated $CD4^+CD25^-CD45RA^+$ naïve T cells at day 5 of T cell activation (black line, left picture in the lower row). However, in contrast to RNA expression levels, no further upregulation of FOXP3 protein levels could be detected in the presence of $TGF\beta$ (Figure 33, right pictures).

Our experimental settings resulted in an upregulation of FOXP3 expression in nearly all T cells in response to T cell activation, independent of the presence of $TGF\beta$. Since natural $FOXP3^+$ Treg cells as well as murine induced $FOXP3^+$ Treg cells are characterized by an anergic phenotype (Sakaguchi, 2004), (Chen et al., 2003), we tested, whether T cell stimulation led to proliferation of $CD4^+CD25^-CD45RA^+$ naïve T cells or whether these cells are anergic.

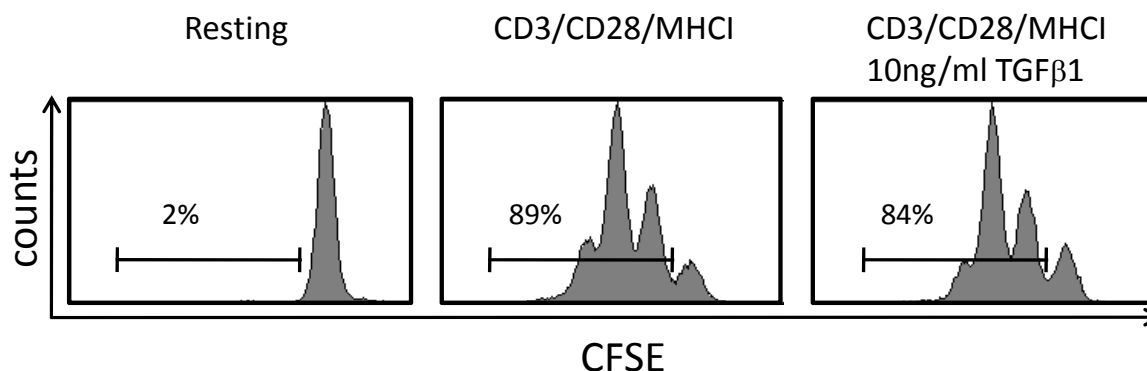


Figure 34: Induced Treg cells proliferate in response to T cell activation

(Freshly isolated $CD4^+CD25^-CD45RA^+$ T cells were cultured for 3 days in the absence or presence of CD3/CD28/MHC-I aAPCs (3 T cells : 1 beads); and 10 ng/ml $TGF\beta$. T cell proliferation was assessed by CFSE labeling. Shown is one representative experiment out of three.

CFSE-labeled human $CD4^+CD25^-CD45RA^+$ naïve T cells were cultured in the absence or presence of aAPCs as well as $TGF\beta$. T cell proliferation was assessed after 3 days of culturing by flow cytometry. $CD4^+CD25^-CD45RA^+$ naïve T cells strongly proliferated in response to stimulation and did not show an anergic phenotype, independent on the presence of $TGF\beta$ (Figure 34). Under these experimental conditions, $TGF\beta$ -mediated inhibition on T cell proliferation was only weak, but still detectable (Figure 34, right panel).

In summary, $CD4^+CD25^-CD45RA^+$ naïve T cells strongly proliferate in response to T cell stimulation and do not display an anergic phenotype, although nearly all cells upregulate FOXP3 expression. Whereas FOXP3 induction is only weak upon 3 days of T cell activation, after 5 days of T cell stimulation FOXP3 expression levels were as strong as in natural Treg cells. Therefore, FOXP3 induction seems to be a late event in response to strong and constant T cell activation.

6.4.2 Human induced Treg cells show suppressive capacity

Since both conditions, T cell activation alone or in combination with TGF β , resulted in the upregulation of FOXP3 in human naïve CD4⁺CD25⁻CD45RA⁺ T cells, we studied these cells in more detail. To simplify the following paragraphs, CD4⁺CD25⁻CD45RA⁺ T cells that upregulate FOXP3 already in response to T cell activation are from now on termed 'activation-induced' Treg cells. Human CD4⁺CD25⁻ T cells that upregulate FOXP3 expression in response to T cell activation in the presence of TGF β , are termed 'TGF β -induced' Treg cells.

In the next step, we examined, whether FOXP3 upregulation is accompanied by the induction of suppressive function in these induced Treg cells. The suppressive capacity was investigated in a suppression assay. The layout of this experiment is depicted in Figure 35. Briefly, naïve CD4⁺CD25⁻CD45RA⁺ T cells were cultured for 5 days in the absence or presence of aAPCs as well as TGF β . Following this treatment, the suppressive activity of these induced Treg cells was investigated in a suppression assay, in which CFSE-labeled CD4⁺CD25⁻ T cells were stimulated with aAPC. Moreover, natural CD4⁺CD25⁺ Treg cells were used in this assay as the positive control of suppressive activity. CD4⁺CD25⁻ T cell proliferation was assessed by flow cytometry.

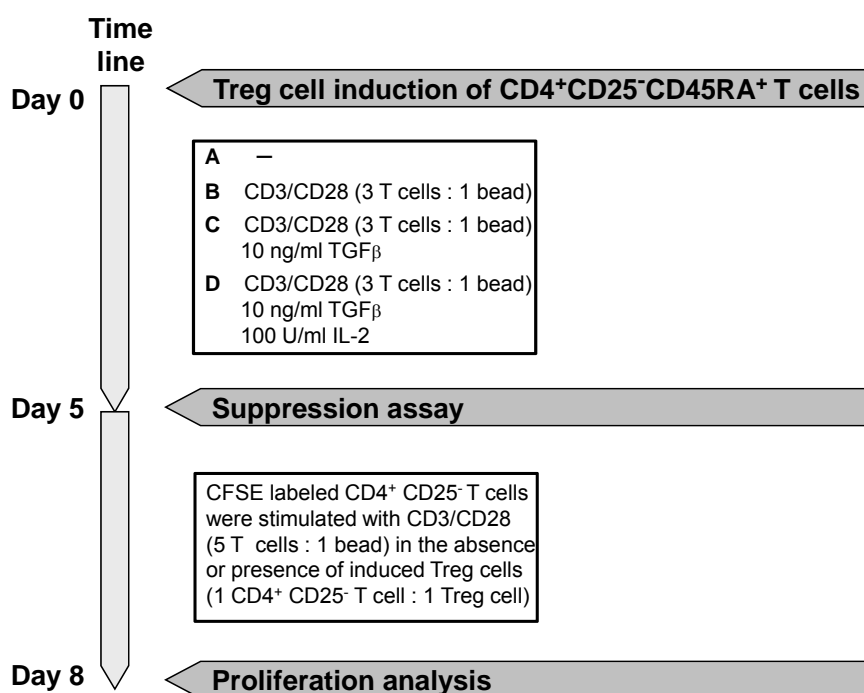


Figure 35: Experimental setup to test the suppressive capacity of induced Treg cells

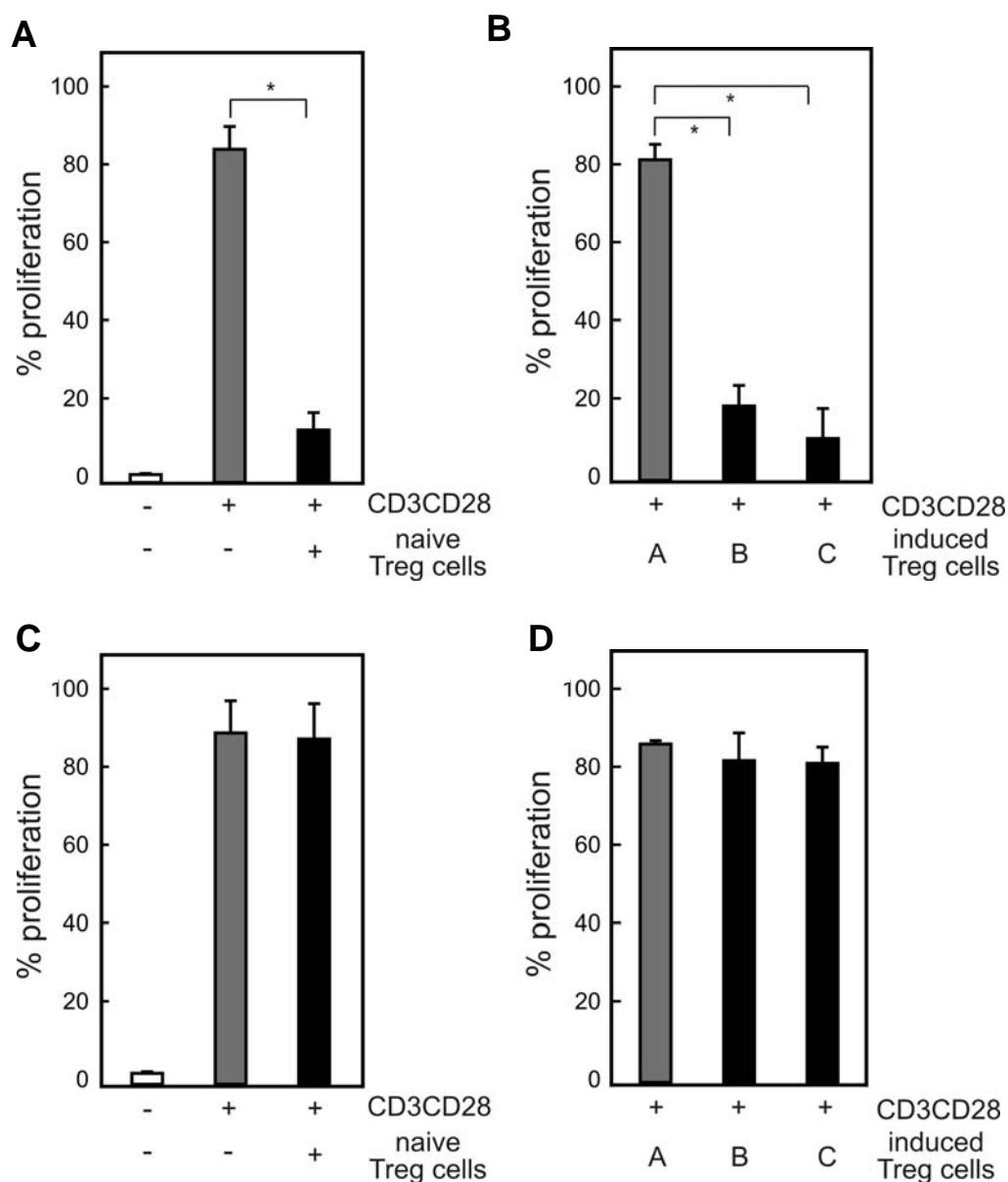


Figure 36: Induced Treg cells show cell-contact dependent suppressive capacity

CD4⁺CD25⁻ T cells were stimulated with CD3/CD28/MHC-I aAPC (5 T cells : 1 bead) in the presence or absence of either naïve Treg cells (plot **A + C**) or induced Treg cells (plot **B + D**) (1 CD4⁺CD25⁻ T cell : 1 Treg cell). 'Induced Treg cells' in this picture terms CD4⁺CD25⁻CD45RA⁺ T cells treated for 5 days with different conditions: A: untreated; B: aAPCs (3 T cells : 1 beads); C: aAPCs (3 T cells : 1 beads) and 10 ng/ml TGFβ. In plots **C + D**, CD4⁺CD25⁻ T cells and Treg cells were separated during the suppression assay in a transwell. T cell proliferation was assessed by CFSE labeling. Data represent mean +/- standard deviation of three independent experiments. Statistically significant differences (paired t-test p<0.05) are marked with an asterisk.

As shown in Figure 36A, natural Treg cells reduced T cell proliferation by a factor of six. In contrast, CD4⁺CD25⁻CD45RA⁺ naïve T cells that were cultured only in serum for 5 days did not show any suppressive capacity (Figure 36B, line A). Interestingly, activation-induced Treg cells gained suppressive capacity and were able to inhibit T cell activation to the same extent as natural Treg cells. Similar suppressive capacity was obtained in TGFβ-induced Treg cells. As both conditions of Treg cell induction led to a comparable suppressive activity in these cells, these data demonstrate that the gain of suppressive capacity was independent of the

presence of the co-inhibitory molecule TGF β in the culture but dependent on T cell activation – at least under these experimental settings.

In the literature, natural Treg cells were described to mediate suppression in a cell-contact dependent manner (Nakamura et al., 2001), (Thornton and Shevach, 2000), (Piccirillo and Shevach, 2001). Thus, we tested, whether induced Treg cells also suppress T cell proliferation via cell-contact. To examine this point, the same suppression assay was performed in a transwell plate. In a transwell assay, Treg cells and CD4⁺CD25⁻ T cells do not have any contact, and therefore, if cell-contact is required for suppressive function, no suppression of T cell proliferation should occur.

Figure 36C clearly demonstrates that naïve Treg cells were not able to inhibit CD4⁺ T cell proliferation in a transwell assay. Similarly, activation- as well as TGF β -induced Treg cells did not reduce the proliferative capacity of T cells in a transwell (Figure 36D). Hence, we conclude that both, naïve as well as induced Treg cells, mediate their suppressive capacity via cell-contact dependent mechanisms.

Taken together, the experimental setting chosen in these studies indeed led to the conversion of CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ cells after 5 days of T cell stimulation. Furthermore, these cells gained suppressive capacity. Since these induced Treg cells did not show an anergic phenotype but strongly proliferated after three days of T cell activation, these results lead to the hypothesis, that in the first days of an immune response, T cells get activated and proliferate in order to destroy the pathogen. However, in later stages of the immune response, T cells gain suppressive function, which may lead to a negative regulation of the immune response.

6.4.3 The transcriptional profile of induced Treg cells is related to natural Treg cells

Since T cell stimulation leads to the conversion of CD4⁺CD25⁻CD45RA⁺ naïve T cells into T cells with Treg cell-specific characteristics, we postulated that the analysis of the transcriptional profile of induced Treg cells would reveal important information about the degree of relationship between natural and induced Treg cells. Until now, this point has not been addressed in humans.

Within this analysis, we were interested in the question whether the transcriptional profile of naïve CD4⁺CD25⁻CD45RA⁺ T cells treated for 5 days with different conditions (sample A-C) is still related to naïve CD4⁺CD25⁻CD45RA⁺ T cells or whether T cell treatment led to a transcriptional profile more related to natural CD4⁺CD25⁺ Treg cells.

For this purpose, we first evaluated those genes, which are differentially expressed between conventional $CD4^+CD25^-$ T cells and natural $CD4^+CD25^+$ Treg cells ($FC \geq \pm 1.5$, p -value < 0.05). Altogether, 597 genes were included into this gene list, from now on termed 'Treg gene profile'.

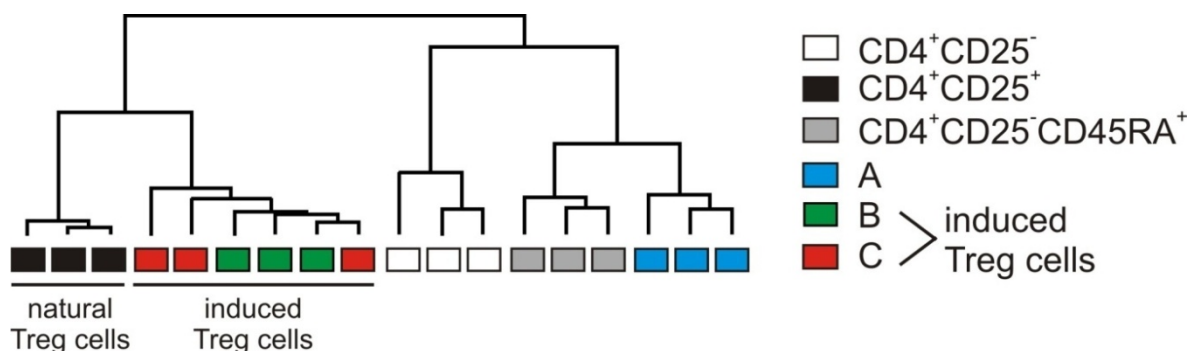


Figure 37: Hierarchical clustering revealed induced Treg cells to cluster with natural Treg cells

Hierarchical clustering of natural $CD4^+CD25^+$ Treg cells, $CD4^+CD25^-$ T cells and naïve $CD4^+CD25^-CD45RA^+$ T cells. Samples A-C in this picture terms $CD4^+CD25^-CD45RA^+$ T cells treated for 5 days with different conditions: A: untreated; B: aAPCs (3 T cells : 1 beads); C: aAPCs (3 T cells : 1 beads) and 10 ng/ml $TGF\beta$. Hierarchical clustering was performed on genes differentially expressed in natural $CD4^+CD25^+$ Treg cells ($FC \geq \pm 1.5$, p -value < 0.05) in comparison to conventional $CD4^+CD25^-$ T cells.

Interestingly, when sample clustering was performed on the Treg gene profile, activation- as well as $TGF\beta$ -induced Treg cells clustered together with natural Treg samples. In contrast, naïve $CD4^+CD25^-CD45RA^+$ T cells cultured only in media for 5 days (sample A) clustered together with conventional $CD4^+CD25^-$ T cells and naïve $CD4^+CD25^-CD45RA^+$ T cells (Figure 37). Similar to FOXP3 protein levels and the gain of suppressive function, clustering of induced Treg cells to natural Treg cells was independent of the presence of the co-inhibitory molecule $TGF\beta$ in the culture but dependent on T cell activation. These data demonstrate that the transcriptional profile of naïve $CD4^+CD25^-CD45RA^+$ T cells activated for 5 days is more related to natural Treg cells than to untreated naïve $CD4^+CD25^-CD45RA^+$ T cells.

The result that activation- as well as $TGF\beta$ -induced Treg cells were closely related to natural Treg cells in their transcriptional profile was surprising and needed further investigation. Therefore, gene expression changes of the Treg gene profile in all samples were displayed in a color-coded heatmap (Figure 38). Downregulated genes were color-coded in blue, whereas upregulated genes were shown in red. Visualizing gene expression changes clearly reveals that there were approximately half of the genes regulated in natural and induced Treg cells in the same direction, either up- or -downregulated. Among the genes that were upregulated in natural as well as induced Treg cells, there were genes considered as the best-studied Treg

cell-specific genes: FOXP3, CTLA-4, GITR and IL2Ralpha and beta. In addition, CD127 expression was low in both, natural and induced Treg cells.

However, half of the genes were not expressed similarly in natural and induced Treg cells. These genes were regulated in natural Treg cells in one direction and regulated in the other direction in induced Treg cells. None of the in the literature described Treg cell-specific genes was found within this gene group.

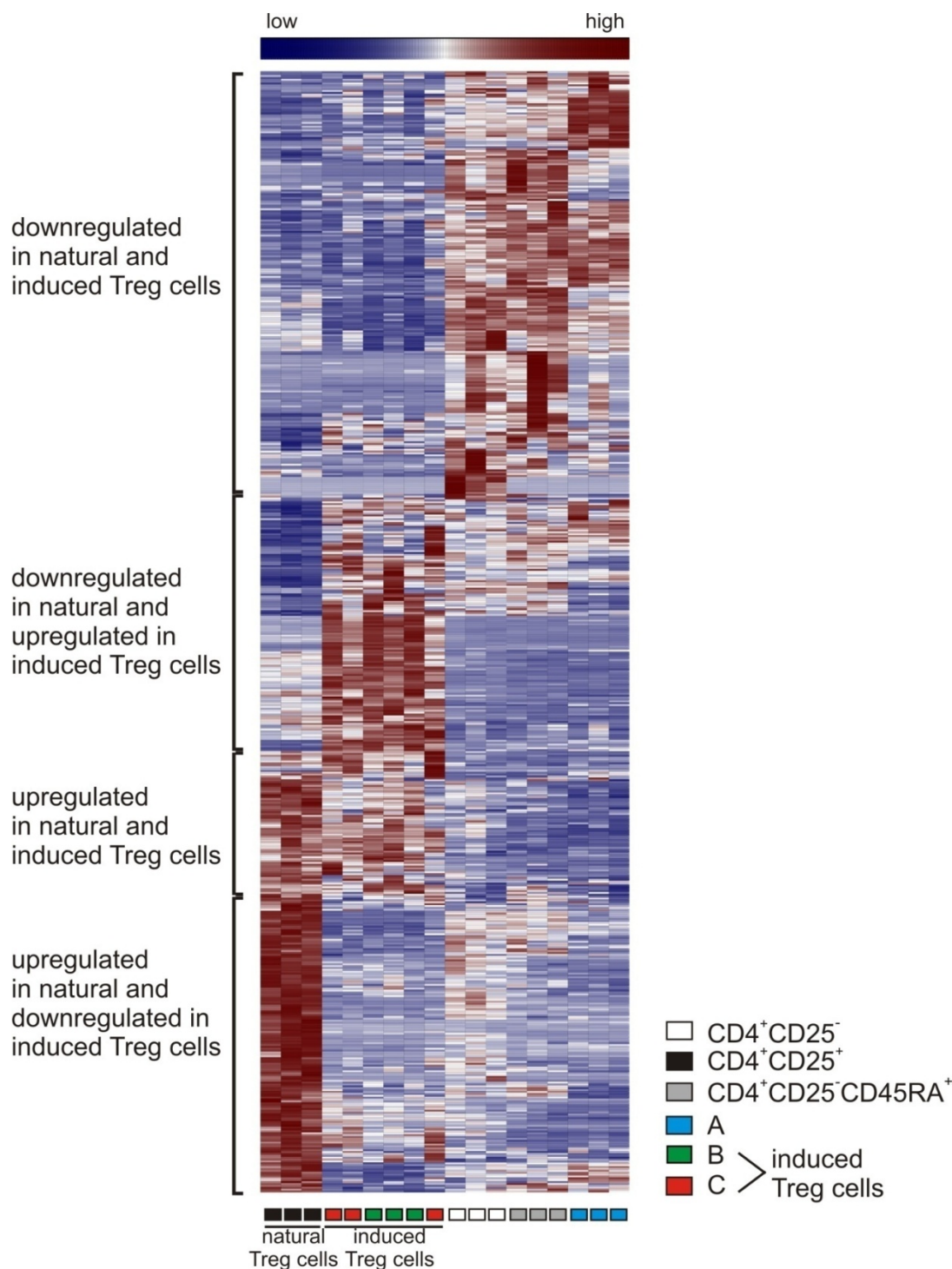


Figure 38: Heatmap displaying expression values of differentially expressed genes

Genes were selected based on differential expression ($FC > 1.5$ or $FC < -1.5$, $p\text{-value} < 0.05$) of natural CD4⁺CD25⁺ Treg cells compared to conventional CD4⁺CD25⁻ T cells. Expression values are color-coded showing genes being high- and low-expressed in red and blue, respectively.

As shown before, clustering of induced Treg cells to natural Treg cells was independent of the presence of the co-inhibitory molecule TGF β in the culture but only dependent on T cell activation. In the next step of the analysis, we were interested in the question, whether the presence of TGF β during the process of Treg cell induction, had any influence on the expression of genes comprised in the Treg gene profile. For this purpose we evaluated, which of the genes included in the Treg gene profile (597 genes) are differentially expressed between activation-induced Treg cells (sample B) and TGF β -induced Treg cells (sample C). Altogether, out of those 597 genes, 66 genes revealed to be differentially expressed by the presence of TGF β during culture (FC $\geq \pm 1.5$, p-value < 0.05). Among those 66 genes, 16 genes were differentially regulated in the similar direction in both, natural and TGF β -induced Treg cells (Table 8).

Table 8: Differentially expressed genes in natural and TGF β -induced Treg cells

gene	natural Treg cells	TGF β -induced Treg cells
GZMA	-3,11	-2,45
SATB1	-2,46	-1,64
STOM	-2,06	-1,78
CEBPB	-1,90	-1,71
XBP1	-1,72	-1,94
SSH3	1,72	1,68
GLB1	1,83	1,75
TBC1D4	1,86	1,81
ID3	2,09	1,67
TRAF1	2,10	1,84
CCR4	2,40	3,37
IL2RB	2,50	1,53
ATP1B1	2,96	2,38
NCF4	3,30	1,65
F5	3,45	1,89
CTLA4	3,90	1,78

Notes: 1. genes were filtered based on differential expression (FC > 1.5 or FC < -1.5, p-value < 0.05) in natural CD4⁺CD25⁺ Treg cells in comparison to conventional CD4⁺CD25⁻ T cells (natural Treg cells). 2. genes differentially expressed (FC $\geq \pm 1.5$, p-value < 0.05) in activation-induced Treg samples B: aAPCs (3 T cells : 1 beads) versus TGF β -induced Treg samples C: aAPCs (3 T cells : 1 beads) and 10 ng/ml TGF β (TGF β -induced Treg cells). These gene lists were compared and only those genes were depicted which were differentially expressed in both lists and expression changes were observed in the same direction.

Among these 16 genes two genes were already upregulated in naïve CD4⁺CD25⁻CD45RA⁺ T cells upon 5 days of activation, CTLA-4 and IL2Rbeta. However, the presence of TGF β enhanced the expression levels of these two genes significantly. Surprisingly, within these 16 genes, four genes were identified which are known to be implicated in the regulation of gene transcription. Three genes were downregulated in these cells (CEBPB, XBP1 and SATB1), whereas ID3 was upregulated in natural as well as TGF β -induced Treg cells. The

accumulation of genes involved in the regulation of transcription lead to the suggestion that these genes may play an important role in the biology of Treg cells.

Among these 4 regulators of transcription, SATB1 was of special interest, as it is an ATP-dependent chromatin-remodeling enzyme predominantly expressed in T cells (Cai et al., 2003), (Alvarez et al., 2000), (Dickinson et al., 1992). This protein directly interacts with several chromatin-remodeling complexes and it is thought that SATB1 targets them to DNA. Thereby, in contrast to transcription factors, SATB1 is involved in the regulation of gene expression over long distances (Yasui et al., 2002). SATB1 seems to play an important role in T cell development, as SATB1-deficient mice show a block at the double-positive stage of thymocyte development (Alvarez et al., 2000). Additionally it is involved in T cell differentiation, as under Th1 and Th2 activating conditions SATB1 is highly upregulated and particularly induces the expression of the Th2 specific cytokines *IL-4*, *IL-5* and *IL-13* (Cai et al., 2006). Following these interesting findings, SATB1 regulation and function was studied in more detail.

6.4.4 SATB1 levels are reduced in natural and TGF β -induced Treg cells

Microarray experiments revealed a significant reduction of SATB1 expression levels in natural CD4⁺CD25⁺ Treg cells as well as in TGF β -induced Treg cells. The microarray results obtained for SATB1 were validated by RT-PCR (Figure 39).

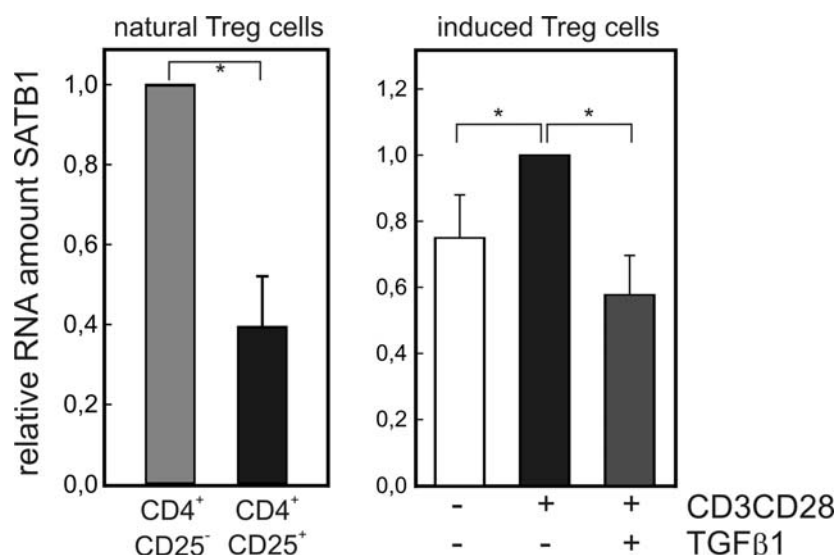


Figure 39: SATB1 expression is decreased in natural and TGF β -induced Treg cells

(A) SATB1 RNA levels were assessed in CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells by RT-PCR. (B) CD4⁺CD25⁻ CD45RA⁺ T cells were cultured for 5 days in the absence or presence of CD3/CD28/MHC-I aAPCs (3 T cells : 1 beads) and 10 ng/ml TGF β . SATB1 RNA levels were assessed in these cells at day 5. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

In the next step, we examined, whether reduced SATB1 expression levels lead to reduced SATB1 protein levels. For this purpose, SATB1 protein levels were investigated in natural and induced Treg cells by western blotting.

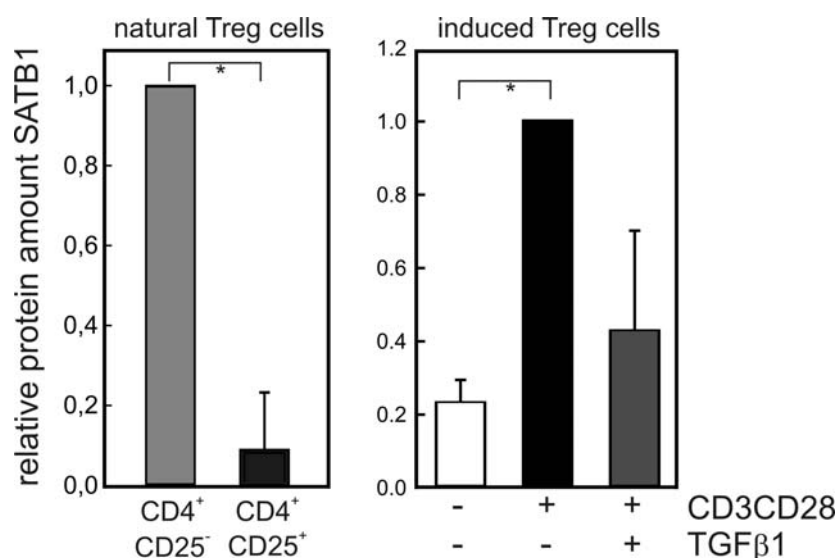


Figure 40: SATB1 protein levels are reduced in natural Treg cells

(A) SATB1 RNA levels were assessed in CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells by Western blotting. (B) CD4⁺CD25⁻CD45RA⁺ T cells were cultured for 5 days in the absence or presence of CD3/CD28/MHC-I aAPCs (3 T cells : 1 beads) and 10 ng/ml TGFβ. SATB1 protein levels were assessed in these cells at day 5. The relative protein amount of SATB1 in comparison to β-actin was measured by densitometry and plotted. Data represent mean +/- standard deviation of three independent experiments. Statistically significant differences (paired t-test p<0.05) are marked with an asterisk.

In accordance to SATB1 RNA levels, SATB1 protein levels were significantly reduced in natural Treg cells (Figure 40, left picture). Activation-induced Treg cell showed increased SATB1 protein levels, whereas the presence of TGFβ led to the downregulation of SATB1.

This result allows to hypothesize that the downregulation of SATB1 may be an important mechanism in Treg cell biology and raises the following questions: 1. Is downregulation of SATB1 in natural Treg cells also TGFβ1-mediated or are other factors involved in this regulation? 2. Does SATB1 regulation occur under resting and/or under T cell activating conditions? 3. How is SATB1 expressed in other CD4⁺ T cell subpopulations?

6.5 TGF β is a key player of SATB1 regulation in activated CD4⁺ T cells

6.5.1 T cell activation leads to SATB1 upregulation

To understand SATB1 regulation, we performed time courses on whole CD4⁺ T cells. First, we analyzed the influence of T cell stimulation on SATB1 expression.

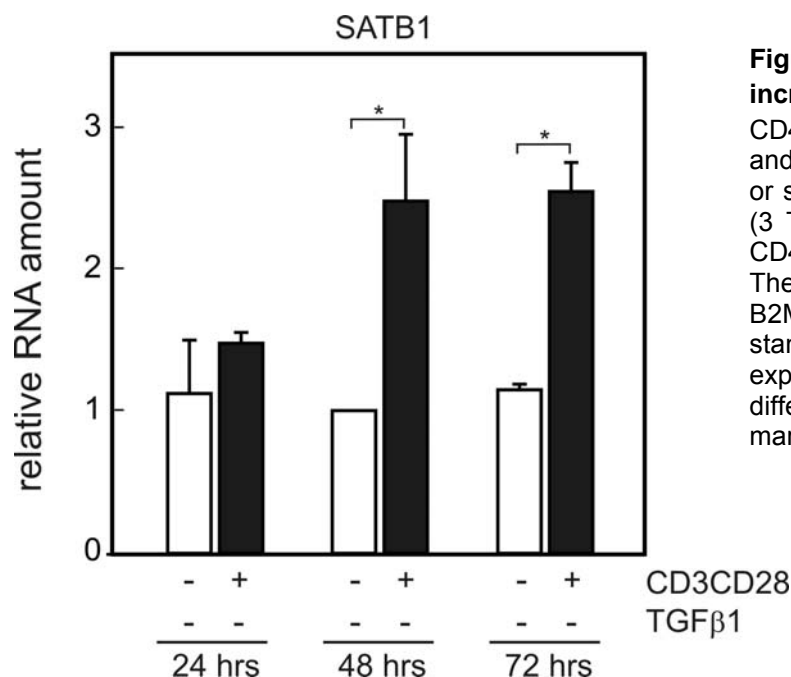


Figure 41: SATB1 expression is increased in response to T cell activation

CD4⁺ T cells were cultured for 24 hrs, 48 hrs and 72 hrs. Cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead) SATB1 RNA levels in CD4⁺ T cells were assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

As shown in Figure 41, the expression level of SATB1 was slightly upregulated already 24 hrs after CD4⁺ T cell stimulation under neutral conditions. This upregulation was even more pronounced 48 hrs and 72 hrs post CD4⁺ T cell activation (Figure 41).

6.5.2 SATB1 is downregulated by TGF β in activated CD4⁺ T cells

Subsequently, the influence of TGF β on SATB1 regulation was assessed. The same activation assay as shown in Figure 41 was performed, but additionally TGF β 1 was added to the T cell cultures. In contrast to activation-induced SATB1 upregulation, the additional TGF β 1 treatment led to a downregulation of SATB1 expression at all measured time points (Figure 42).

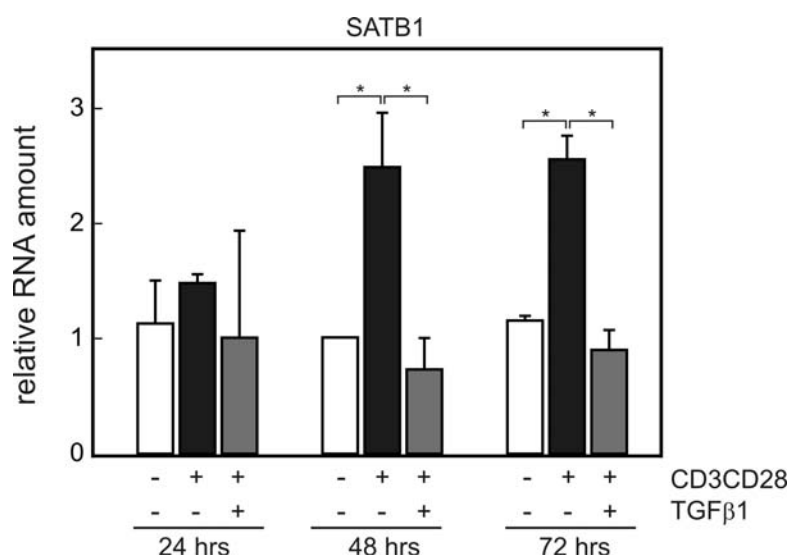


Figure 42: TGFβ downregulates SATB1 in activated CD4⁺ T cells

CD4⁺ T cells were cultured for different time length: 24 hrs, 48 hrs and 72 hrs. Cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead) in the presence or absence of 10ng/ml TGFβ1. Regulation of SATB1 mRNA was assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean +/- standard deviation of three independent experiments. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

Since SATB1 is downregulated in activated T cells in response to TGFβ signaling we studied the role of this cytokine on SATB1 expression in resting CD4⁺ T cells. For this purpose, CD4⁺ T cells were cultured for 72 hrs in the absence or presence of TGFβ1. Afterwards, SATB1 expression levels were investigated.

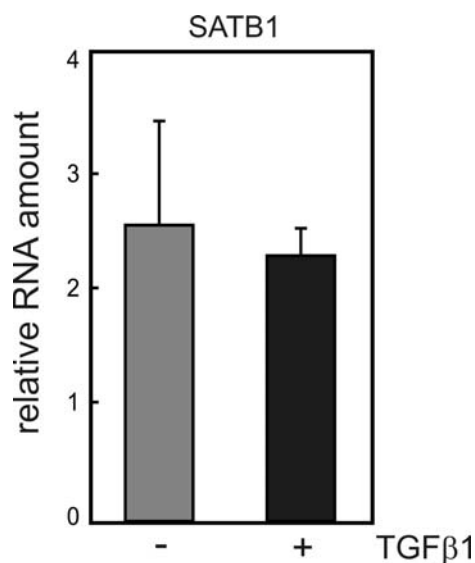


Figure 43: SATB1 expression is not altered by TGFβ in resting CD4⁺ T cells

CD4⁺ T cells were cultured for 72 hrs in the presence or absence of 10ng/ml TGFβ1. Regulation of SATB1 mRNA was assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean +/- standard deviation of three independent experiments.

Figure 43 demonstrates that the presence of TGFβ1 alone was not sufficient to reduce SATB1 expression levels under resting conditions.

6.5.3 SATB1 levels of expression are reduced in memory CD4⁺ T cells

CD4⁺ T cells are composed of several subgroups. The decrease of SATB1 RNA as well as protein levels in natural CD4⁺CD25⁺ Treg cells in comparison to conventional CD4⁺CD25⁻ T cells already showed differences in SATB1 expression levels in these subgroups. Therefore,

SATB1 expression levels were also assessed in $CD4^+CD25^-CD45RA^+$ naïve and $CD4^+CD25^-CD45RA^-$ memory T cells.

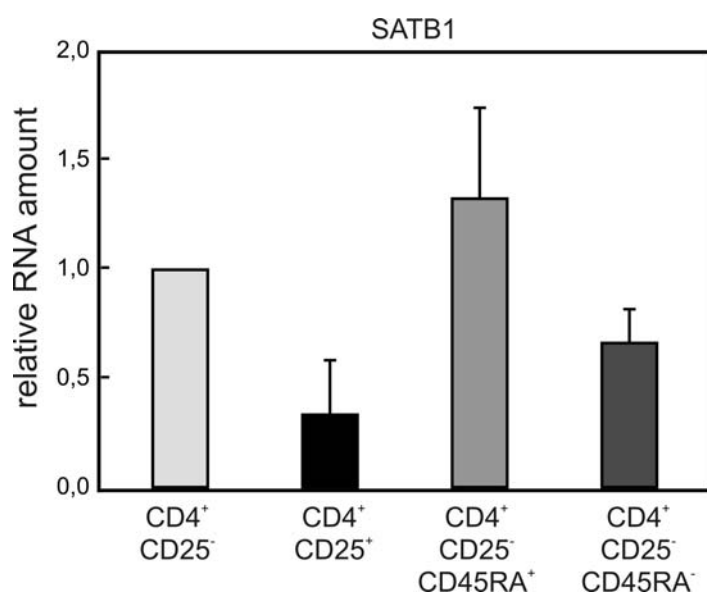
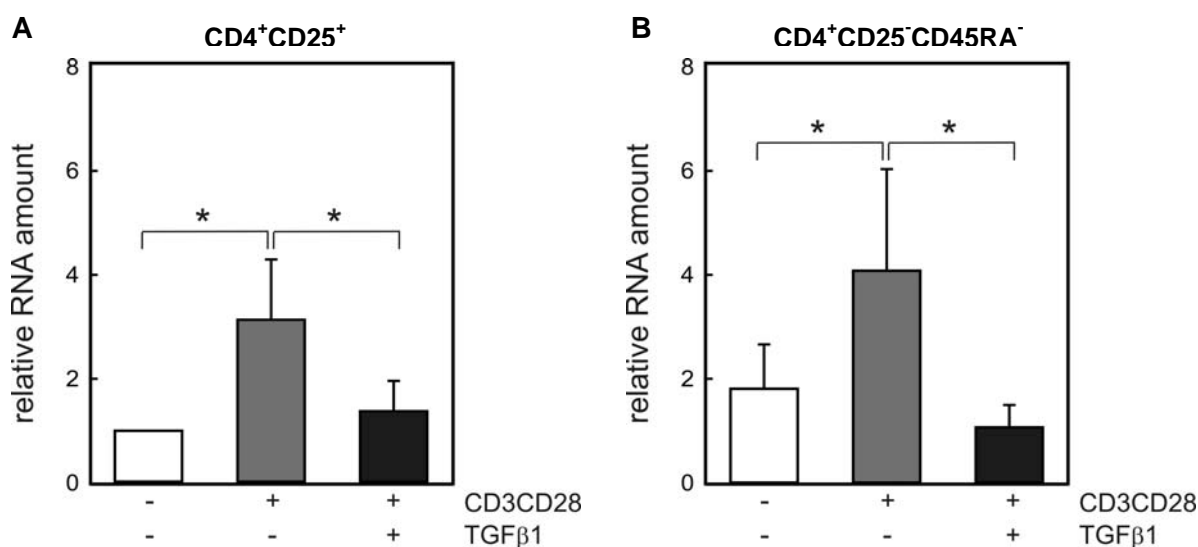


Figure 44: SATB1 expression is reduced in memory $CD4^+$ T cells

SATB1 RNA levels in freshly isolated peripheral $CD4^+CD25^-$, $CD4^+CD25^+$, $CD4^+CD25^-CD45RA^+$ and $CD4^+CD25^-CD45RA^-$ T cells were assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of five independent experiments.

As already shown in Figure 39, expression levels of SATB1 were reduced in $CD4^+CD25^+$ Treg cells. Surprisingly, SATB1 was also decreased in antigen-experienced $CD4^+CD25^-CD45RA^-$ memory T cells in comparison to $CD4^+CD25^-CD45RA^+$ naïve T cells, but less than in Treg cells (Figure 44).

We questioned whether these differing SATB1 expression levels in $CD4^+$ T cell subpopulations under resting conditions also influence the regulation of SATB1 in response to T cell activation and/or $TGF\beta$ treatment. To address this question, $CD4^+CD25^+$ Treg cells, $CD4^+CD25^-CD45RA^+$ naïve and $CD4^+CD25^-CD45RA^-$ memory T cells were isolated and cultured for 72 hrs under neutral T cell activating conditions. As shown in Figure 45, all three $CD4^+$ T cell subpopulations responded to T cell stimulation by SATB1 upregulation.



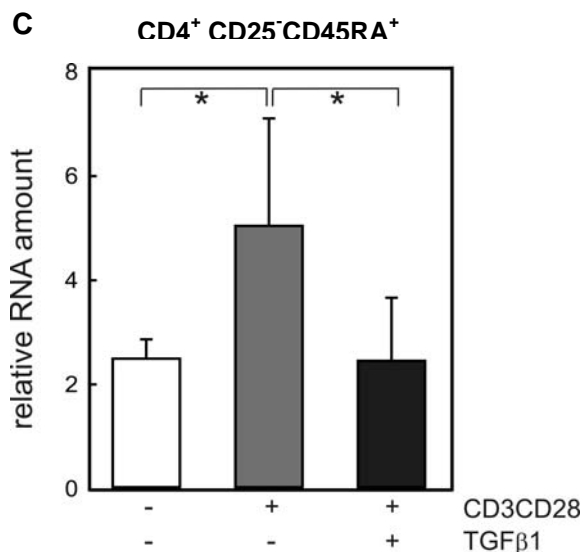


Figure 45: SATB1 RNA regulation in activated CD4⁺ T cell subpopulations

CD4⁺CD25⁺ (A), CD4⁺CD25⁻CD45RA⁻ (B) and CD4⁺CD25⁻CD45RA⁺ (C) T cells were cultured for 72 hrs. Cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead). Further, 10ng/ml TGFβ with or without 100U/ml IL-2 were added to the cultures. SATB1 RNA levels were assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean +/- standard deviation of three independent experiments. Statistically significant differences (paired t-test p<0.05) are marked with an asterisk.

Further, the influence of TGFβ on SATB1 regulation was assessed in these CD4⁺ T cell subpopulations under neutral activating conditions. SATB1 was downregulated in all tested cell types in response to TGFβ treatment (Figure 45).

Taken together, the basal expression levels of SATB1 show strong variation in CD4⁺ T cell subpopulations. Nevertheless, these differences had no impact on the expression changes of SATB1, since T cell activation led to upregulation of SATB1 in all tested CD4⁺ T cell subpopulations, whereas further addition of TGFβ reduced these expression levels.

6.5.4 TGFβ downregulates SATB1 under Th1 and Th2 promoting conditions

SATB1 functions in chromatin-remodeling to regulate gene expression over long distances, thereby regulating for example the expression of Th1 and Th2 cytokines (Yasui et al., 2002), (Cai et al., 2006). This protein is highly upregulated in T cells that undergo differentiation towards Th1 or Th2 (Lund et al., 2003), (Cai et al., 2006).

Furthermore, it was demonstrated that SATB1 expression is downregulated in the presence of TGFβ under Th1 and Th2 promoting conditions (Lund et al., 2003). However, within this study it was also shown that SATB1 RNA levels were downregulated in response to neutral T cell stimulation (Lund et al., 2003). In contrast to these results, our experiments revealed an upregulation of SATB1 expression already after 24 hrs under neutral T cell activating conditions (Figure 42). This discrepancy can be explained by the experimental setup used by Lund et al. In their T cell cultures, 1% human AB serum including high amounts of active TGFβ as well as other not defined factors was added, whereas experiments reported here

were performed under serum-free conditions. Indeed, presence of TGF β 1 under neutral T cell activating conditions also led to downregulation of SATB1 in our experiments (Figure 42).

Since the presence of human serum has such a great influence on SATB1 expression, we performed the experiments only using serum-free culture conditions. Thereby, we wanted to ensure that indeed the presence of TGF β and not any other factor present in serum mediates downregulation of SATB1 under Th1 and Th2 promoting conditions. Moreover, we evaluated whether the presence of TGF β under Th1 and Th2 promoting conditions not only decreases SATB1 RNA expression but also influences SATB1 protein levels, leading to the block of Th1 and Th2 specific cytokine secretion.

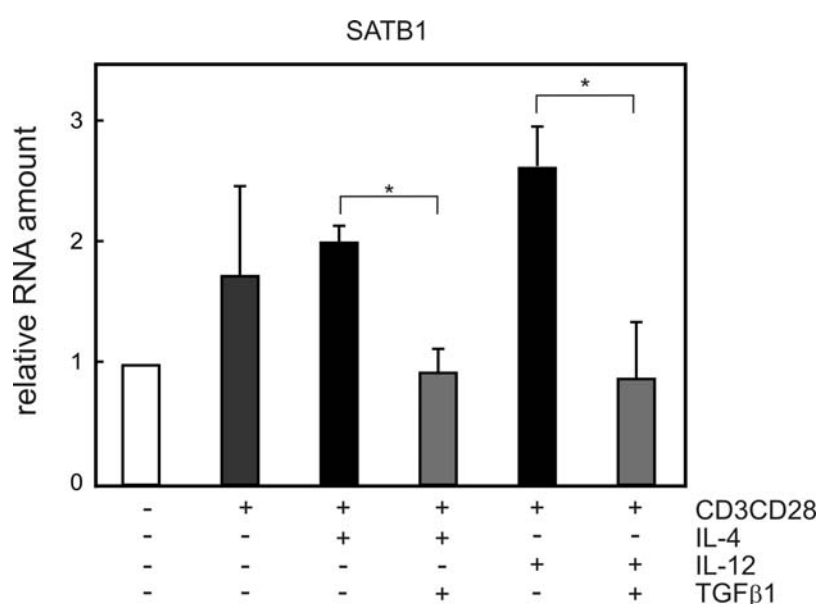


Figure 46: SATB1 is downregulated by TGF β under Th1/Th2 promoting conditions

CD4⁺ T cells were cultured for 72 hrs and either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (1 bead: 3 T cells), in combination with 10ng/ml IL4 or 2.5 ng/ml IL-12 in the presence or absence of 10ng/ml TGF β 1. Regulation of SATB1 mRNA was assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

As shown in Figure 46, SATB1 RNA levels were upregulated in response to Th1 as well as Th2 promoting T cell stimulation under serum free culture conditions for 48 hrs. The addition of TGF β 1 led to a downregulation of SATB1 at all measured time points and under all T cell activating conditions.

Altered RNA levels do not automatically result in altered protein levels due to translational regulation. Therefore, we investigated SATB1 protein levels under the before described conditions.

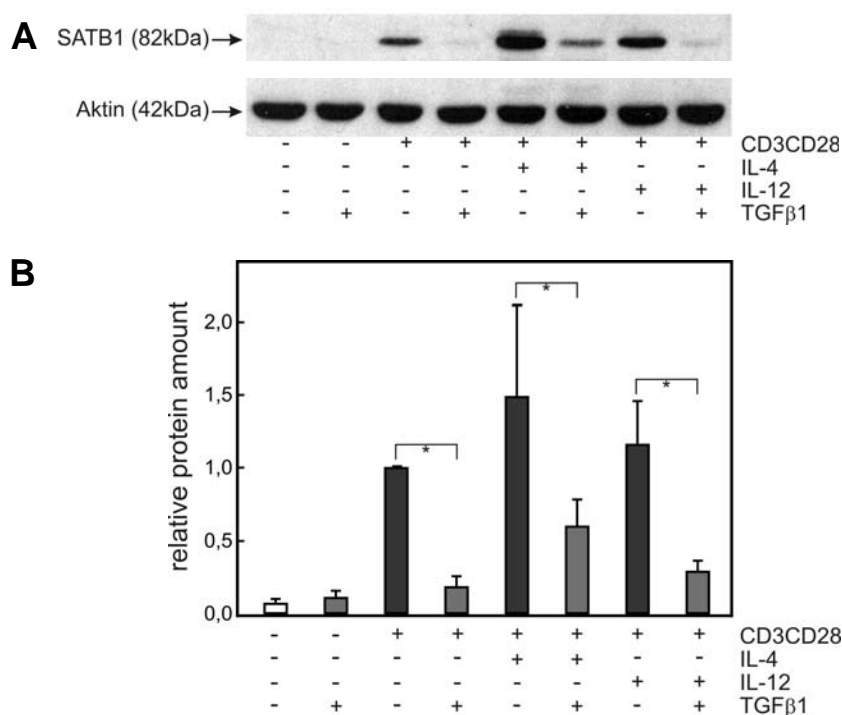


Figure 47: The protein level of SATB1 is reduced by TGFβ under Th1/Th2 promoting conditions

CD4⁺ T cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead), in combination with 10ng/ml IL4 or 2.5 ng/ml IL-12 in the presence or absence of 10ng/ml TGFβ1 for 72 hrs. SATB1 protein levels were assessed by Western blotting. In (A) an exemplary Western Blot of one donor is shown. (B) The relative protein amount of SATB1 in comparison to β-actin was measured by densitometry. Shown are mean +/- standard deviation of the data of four independent experiments after normalization to stimulated CD4⁺ cells. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

Figure 47 depicts that SATB1 protein regulation was similar to the regulation obtained for the RNA expression levels. T cell activation (either neutral, Th1 or Th2 promoting conditions) resulted in the strong upregulation of SATB1 in comparison to protein levels measured in resting CD4⁺ T cells (left bar). The presence of TGFβ blocked this upregulation.

These results demonstrate the potential of TGFβ to downregulate SATB1 under neutral, Th1 and Th2 promoting conditions on RNA as well as on protein level (Figure 46 and Figure 47).

In the next step, the secretion of IFN-γ (Th1) and IL-6 (Th2) was analyzed by cytometric bead array to confirm by a functional assay the TGFβ-mediated blockade of Th1/Th1 differentiation under these culture conditions.

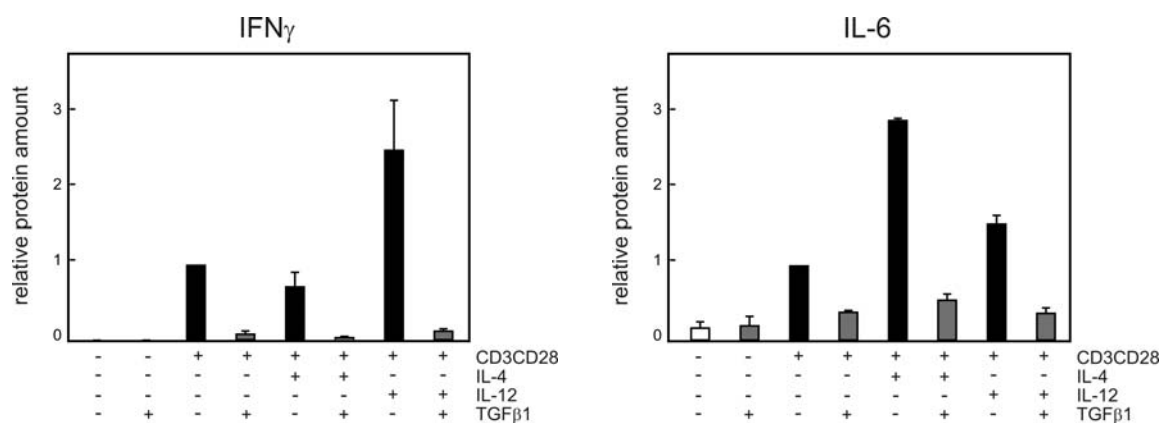


Figure 48: TGF β blocks Th1/Th2 cytokine secretion

CD4⁺ T cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead), in combination with 10ng/ml IL-4 or 2.5 ng/ml IL-12 in the presence or absence of 10ng/ml TGF β 1 for 72 hrs. The amount of IFN- γ (A) and IL-6 (B) were measured in the cell culture supernatant by cytometric bead array. Shown are mean \pm standard deviation of two independent experiments after normalization to stimulated CD4⁺ cells.

Indeed, TGF β blocked the secretion of both cytokines, IFN- γ (Th1) as well as IL-6 (Th2) (Figure 48), demonstrating that Th1 as well as Th2 differentiation was blocked by this factor.

In summary, these data reveal a central role of TGF β 1 in the regulation of the chromatin organizer SATB1. T cell activation leads to the upregulation of SATB1, which can be abrogated by the presence of TGF β in all tested CD4⁺ T cell subpopulations. Moreover, it is well established that TGF β is an inhibitor of Th1 and Th2 differentiation (Gorelik et al., 2000), (Heath et al., 2000), (Gorham et al., 1998). Therefore we postulate that downregulation of SATB1 might be one of the most important events in the TGF β -induced inhibition of Th1 and Th2 differentiation.

6.6 Interplay of TGF β , SATB1 and Treg cells

In summary, our data reveal that resting CD4⁺CD25⁺ Treg cells as well as TGF β -induced CD4⁺CD25⁺ Treg cells exhibit strongly decreased levels of SATB1 (Figure 39). Both, induced as well as natural Treg cells are characterized by high levels of the transcription factor FOXP3 (Figure 32). Natural as well as induced Treg cell development and maintenance are dependent on TGF β (Marie et al., 2005), (Li et al., 2006a), (Liu et al., 2008). Furthermore, we demonstrated that TGF β is an important factor mediating the downregulation of SATB1 (Figure 42).

However, which factors are responsible for the low expression of SATB1 in Treg cells? Since TGF β signaling is active in all resting CD4⁺ T cells (Figure 15 and Figure 16) and addition of TGF β to resting CD4⁺ T cells did not result in expression changes of SATB1 in CD4⁺ T cells (Figure 43), TGF β alone seems not to be sufficient for the observed low SATB1 expression

levels in Treg cells. Recently it was shown that FOXP3 enhances and stabilizes TGF β -induced effects (Hill et al., 2007). Thus, one possible explanation could be that TGF β first induces the Treg cell-specific transcription factor FOXP3, which in turn downregulates SATB1 expression levels (Figure 49).

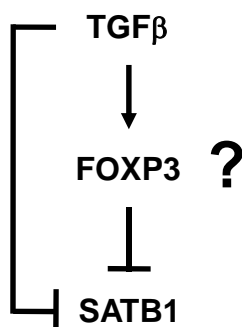


Figure 49: TGF β -SATB1 interaction scheme

6.6.1 Is TGF β -mediated SATB1 downregulation FOXP3 dependent?

Zheng et al. performed tiling arrays of the murine genome to identify genes, which are regulated by the transcription factor FOXP3 (Zheng et al., 2007). Since we were interested in the question, whether SATB1 is a target of FOXP3, we re-analyzed their lists of potential FOXP3 target genes. In this study, 702 genes were found to be target genes of FOXP3 and among those genes, we detected SATB1 as a target of FOXP3. Therefore, we analyzed the sequence of the human SATB1 gene to locate potential FOXP3 binding sites. Indeed, several potential FOXP3 binding sites were present in the SATB1 gene. More important, one of the two murine binding sites for FOXP3 identified by Zheng et al. is conserved in the human SATB1 gene. Thus, we investigated, whether FOXP3 binds to this site also in human Treg cells by using an electrophoretic mobility shift assay (EMSA) and whether FOXP3 is involved in the regulation of human SATB1.

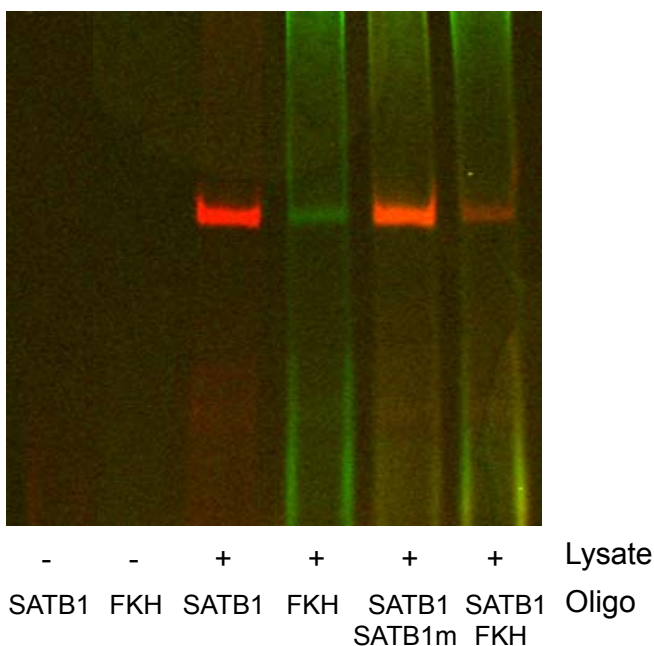


Figure 50: FOXP3 binds to the SATB1 gene

EMSA were performed with nuclear extracts (lysate) from expanded Treg cells. The following oligonucleotides were used: 1. a dimer of the SATB1 gene including the FOXP3 binding site labeled with the red IRDye 700 (SATB1). 2. a dimer of the consensus FKH binding site in the GM-CSF enhancer labeled with the green IRDye 800 (FKH). 3. a dimer of the SATB1 gene including a mutated FOXP3 binding site labeled with the green IRDye 800 (SATB1m). Nuclear extracts were incubated either with single oligonucleotides or with two oligonucleotides to evaluate the specificity of the binding. Binding reactions were separated on a 5% native polyacrylamide gel and scanned on the Odyssey machine.

Figure 50 demonstrates that FOXP3 can bind to the conserved FOXP3 binding site in the SATB1 promoter in human Treg cells (lane 3). This binding was specific, as signal intensity was reduced in the presence of the FKH oligonucleotide, which has been shown to be a potent FOXP3 binding site. Furthermore, FOXP3 binding to the mutated SATB1 gene was not detectable (data not shown). After achieving the first hint that FOXP3 can bind to this specific DNA region of the human SATB1 gene, we studied the binding of FOXP3 to the SATB1 DNA in expanded Treg cells by chromatin immunoprecipitation (ChIP). ChIP reveals information about the actual binding of transcription factors to DNA inside the cellular system. As shown in Figure 51, binding of FOXP3 to the DNA of the IL7R-promotor (positive control) was detectable, whereas no binding of FOXP3 to a DNA side in intron 4 of the IL7R gene (negative control) occurred. Moreover, we obtained a specific FOXP3 binding to the SATB1 gene. This result confirms our hypothesis that FOXP3 binds to the SATB1 gene in Treg cells and thereby may regulate the expression of SATB1.

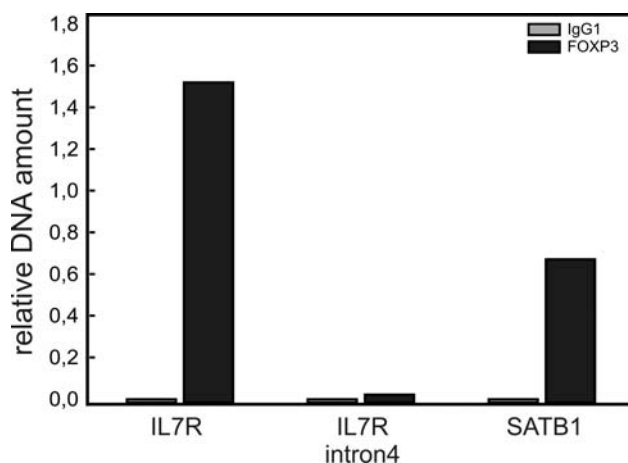


Figure 51: FOXP3 binds to the SATB1 gene

ChIP was performed on DNA extracted from expanded Treg cells. IP was performed with a FOXP3 specific antibody as well as with a isotype control. Subsequently DNA was eluted and PCR was performed for IL7R, IL7R intron 4 and SATB1. PCR products were analyzed by densitometry and the relative DNA amount after PCR in comparison to the isotype control is shown. Data represent one out of three independent experiments.

T cell activation in the presence of TGF β leads to the induction of FOXP3⁺ T cells (Fantini et al., 2004), (Hill et al., 2007), (Davidson et al., 2007). The TGF β -induced downregulation of SATB1 (Figure 42, Figure 45) might be mediated indirectly via the induction of FOXP3. To study the influence of FOXP3 on TGF β -induced downregulation of SATB1 we analyzed the induction of FOXP3 expression in response to CD4⁺ T cell activation in the absence or presence of TGF β 1.

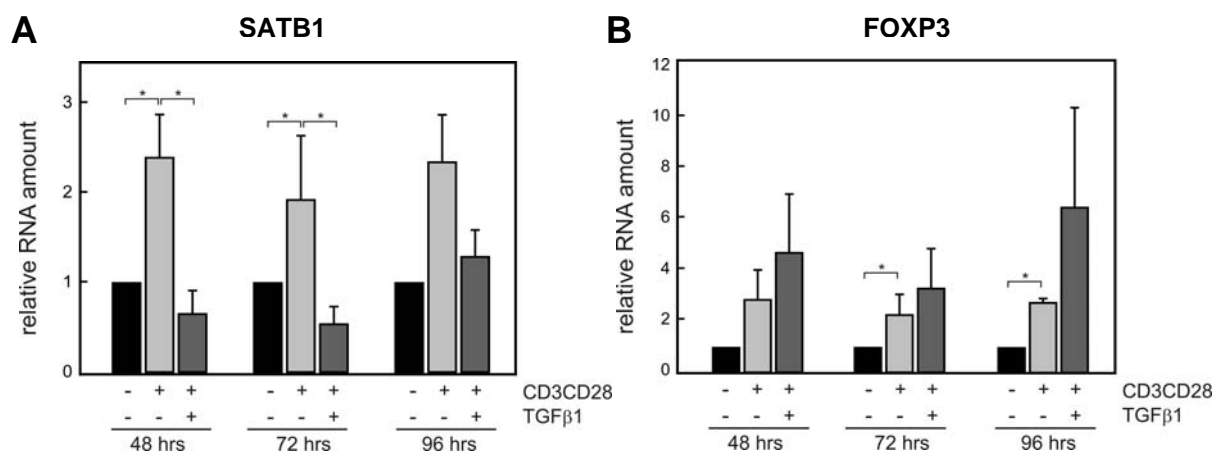


Figure 52: Kinetic of SATB1 and FOXP3 expression in response to CD4⁺ T cell stimulation.

CD4⁺ T cells were cultured for different time lengths: 48 hrs, 72 hrs and 96 hrs. Cells were either left untreated or stimulated with CD3/CD28/MHC-I aAPCs (3 T cells : 1 bead) in the presence or absence of 10ng/ml TGF β 1. Regulation of SATB1 (A) and FOXP3 (B) mRNA was assessed by RT-PCR. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments. Statistically significant differences (paired t-test $p < 0.05$) are marked with an asterisk.

Figure 52A demonstrates that the levels of SATB1 mRNA were upregulated in response to CD4⁺ T cell stimulation, whereas the addition of TGF β led to a downregulation of SATB1 levels in comparison to the resting control 48 hrs and 72 hrs post treatment (see also Figure 42). At the 96 hrs time point, TGF β -mediated downregulation of SATB1 in stimulated CD4⁺ T cells was reduced but still detectable. CD4⁺ T cell stimulation was sufficient to upregulate FOXP3 (Figure 52B). The addition of TGF β led to increased levels of FOXP3, but this further upregulation was not significant. In contrast to SATB1, the effect of TGF β on the expression of FOXP3 was most profound at the 96 hrs time point.

As the expression of FOXP3 is only slightly enhanced by the addition of TGF β at early time points, these data suggest that TGF β itself might be responsible for the downregulation of SATB1 expression rather than the TGF β -induced FOXP3 expression.

However, these results are not definite to answer the question whether the TGF β -induced downregulation of SATB1 is mediated by FOXP3. Thus, we analyzed whether FOXP3 alone is sufficient to downregulate the expression of SATB1. To this end, CD4⁺CD25⁻ T cells that were

electroporated with FOXP3 RNA by the group of G. Schuler in Erlangen were analyzed for the expression of FOXP3 and SATB1 24 hrs (A) and 48 hrs (B) post transfection (Figure 53).

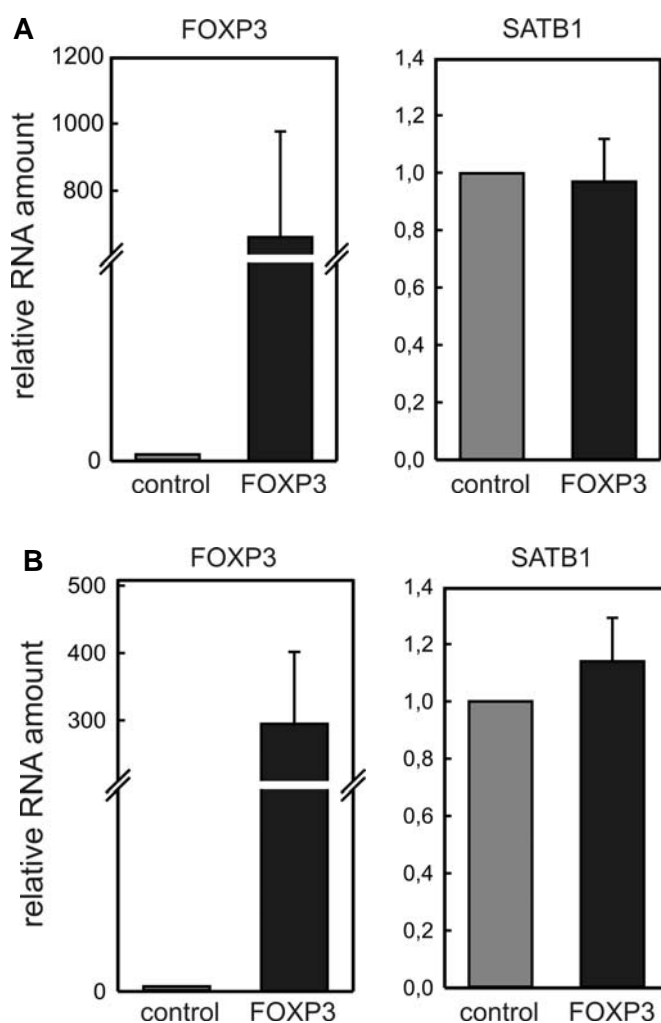


Figure 53: SATB1 expression is unaltered in resting T cells upon FOXP3 transfection

Resting $CD4^+CD25^-$ T cells were electroporated with 15 μ g FOXP3. FOXP3 and SATB1 RNA levels were assessed by RT-PCR 24 hrs (A) and 48 hrs (B) post transfection. The relative expression levels compared to B2M are plotted. Data represent mean \pm standard deviation of three independent experiments.

Electroporation of $CD4^+CD25^-$ T cells with FOXP3 RNA led to a strong increase of FOXP3 expression in these cells. In contrast, this strongly enhanced FOXP3 expression did not lead to any expressional changes of SATB1 RNA levels in resting $CD4^+CD25^-$ T cells (Figure 53). Therefore, similar to $TGF\beta$, FOXP3 alone is not sufficient to downregulate SATB1 in resting T cells.

Taken together, SATB1 appears to be a master regulator in $TGF\beta$ -mediated inhibition of Th1 and Th2 differentiation. Additionally, this molecule seems to play an important role in Treg cell biology. However, still our data are not sufficient to answer, what causes the low expression levels of SATB1 in Treg cells.

7 Discussion

7.1 TGF β controls T cell homeostasis

From elegant studies using knockout mice, it became apparent that T cell homeostasis is tidily regulated by extrinsic factors. Positive signals via the T cell receptor derived from MHC/peptide complexes on antigen presenting cells (Brocker, 1997; Kirberg et al., 1997; Takeda et al., 1996) and IL-7 signaling (Boise et al., 1995; Schluns et al., 2000; Tan et al., 2001; Vella et al., 1997; Vella et al., 1998) have been well established (Grossman et al., 2004; Jameson, 2002; Seddon and Zamoyska, 2003) to be involved in T cell homeostasis. Until now, negative regulators are less well integrated in current models of T cell homeostasis (Grossman et al., 2004). In mice lacking inhibitory molecules such as TGF β , CTLA-4 or IL-10, a profound pathophysiology with severe lymphoproliferative disease has been demonstrated (Gorelik and Flavell, 2000; Kulkarni et al., 1993; Lucas et al., 2000; Shull et al., 1992; Takahashi et al., 1994; Tivol et al., 1995; Watanabe-Fukunaga et al., 1992). A role of TGF β during T cell differentiation and proliferation has been described in dominant-negative TGF β -RII mice (Gorelik and Flavell, 2000). In human T cells, the role of inhibitory factors for the balance between the resting and the active state of human T cells is far less understood. To address this important issue, we applied an unbiased genomics approach. We hypothesized that potentially negatively regulating factors constitutively acting on T cells *in vivo* can be removed by depriving highly enriched CD4⁺ T cells from their natural environment and that this would be accompanied by specific transcriptional changes. Here, we demonstrate that a strictly quantitative assessment of genome wide gene expression changes combined with a search for Gene Ontology defined biological processes reveals the TGF β pathway to be the major exogenous inhibitory signaling pathway constitutively repressing human CD4⁺ T cells *in vivo*. Several of the TGF β target genes that were induced upon TGF β stimulation *in vitro* were shown to be under the permanent control of TGF β in resting T cells *in vivo*. Moreover, this approach led to the identification of numerous novel TGF β target genes, which have not yet been defined as such in other types of cells.

Additionally, constitutive TGF β signaling in resting CD4⁺ T cells was demonstrated by constitutively phosphorylated SMAD2 and SMAD3, two early events after receptor ligation by TGF β . This phosphorylation was completely lost during serum deprivation and quickly restored after addition of either exogenous TGF β or freshly isolated human serum. By further isolating CD4⁺ T cell subsets (naïve, memory, conventional and regulatory cells), we demonstrated that both SMAD2 and SMAD3 are constitutively phosphorylated in all CD4⁺ T cells. In contrast, in

CD8⁺ T cells only SMAD2 phosphorylation occurred, while in B cells, only phosphorylated SMAD3 was detectable. No phosphorylation of SMADs was apparent in CD14⁺ monocytes. Either TGFβ-induced cell signaling is not dependent on phosphorylation of both SMAD2 and SMAD3 in cells other than CD4⁺ T cells, or the effect of TGFβ can be mediated by phosphorylation of only one SMAD molecule. It might also be possible that other pathways might be more important in CD8⁺ T cells, B cells, or monocytes to exert the TGFβ inhibitory effect within these cells. Alternatively, these cells are not as dependent as CD4⁺ T cells on TGFβ signaling to be kept in a resting state.

Since transcriptional changes of genes associated with cell cycle regulation indicated the potential for enhanced cell cycle entry, we analyzed whether decreased TGFβ signaling would also have functional consequences for subsequent T cell activation. Indeed, the loss of TGFβ signaling under serum deprivation significantly increased the capacity of resting T cells to proliferate in response to low level T cell receptor stimulation. TGFβ seems to be the major negative regulator of T cell homeostasis in humans not only inhibiting T cell differentiation and proliferation but also keeping T cells at the resting state.

The effect of TGFβ signaling has been demonstrated to be exclusively cell and context dependent (Massague, 2000). While many aspects of TGFβ signaling in epithelial cells and fibroblasts have been discovered, far less is known in T cells, particularly human T cells. TGFβ, especially at high concentrations, has clearly been shown to be a major immunoinhibitory factor (Ahuja et al., 1993; Bright and Sriram, 1998; Kehrl et al., 1986b; Moses et al., 1990; Tiemessen et al., 2003). Especially in cancer patients, elevated levels of TGFβ have been associated with reduced T cell proliferation and function (Arteaga et al., 1993; Chang et al., 1993; Cook et al., 1999; Thomas and Massague, 2005). It needs to be stressed that former work has almost exclusively focused on elevated levels of TGFβ and its effect on human immune cells. However, there was little evidence so far that TGFβ also plays a more physiological role under steady-state conditions in human CD4⁺ T cells.

The chosen approach has also led to the identification of several novel TGFβ target genes, particularly genes that were suppressed in human CD4⁺ T cells *in vivo*. Many of these genes are currently of unknown function, especially in T cells. However, some biological processes or associations with cellular components seemed to be overrepresented among the new target genes, e.g. nuclear localization, membrane association or cell cycle regulation, suggesting that TGFβ exerts specific and rather focused effects on human resting CD4⁺ T cells. The serum deprivation experiments also revealed novel aspects about the regulation of genes such as CXCR4, previously shown to be upregulated in the presence of elevated levels of TGFβ (Franitza et al., 2002). The significant decrease of CXCR4 mRNA in resting T cells post serum deprivation actually suggests that CXCR4 expression is not only a result of elevated

TGF β levels but rather a function of constitutive TGF β signaling under physiological conditions. This model of CXCR4 regulation would certainly fit the known function of CXCR4 for the maintenance of re-circulation of resting T cells *in vivo* (Bleul et al., 1997).

The immediate loss of TGF β signaling and transcriptional regulation of its downstream targets following withdrawal of TGF β by serum deprivation might re-define our understanding of 'resting T cells' – at least in humans. Resting T cells not only seem to be controlled by antigen presenting cells as suggested by Grossman et al. (Grossman et al., 2004) but also seem to be constitutively inhibited by TGF β . Active inhibition of T cell activation therefore follows rules established in other biological systems that need to quickly respond to stimuli. In view of energy consumption, active repression of cell activation followed by proliferation and differentiation seems to be a rather ineffective approach. However, considering the enormous growth rates of viruses and other infectious agents, a prompt and sufficient activation and proliferation of specific immune cells by releasing an active blockade is most likely leading to an evolutionary advantage. We have embarked on this concept by studying the importance of TGF β in the setting of T cell activation where the relative abundance of inhibitory signals including TGF β *versus* proinflammatory signals decides whether T cells are activated or inhibited by modulating central molecular switches (T. Zander, J.L. Schultze, unpublished results). The functional outcome of removal of even low physiological concentrations of TGF β further supports the hypothesis that T cells are constantly repressed by TGF β .

The significant loss of SMAD2 and SMAD3 phosphorylation as an early response upon serum-deprivation strongly suggests that the changes observed on the transcriptional level are mediated - at least in part - by the classical signaling cascade via TGF β receptor and SMAD signaling. It will be an interesting question to determine whether other non-SMAD-mediated TGF β signaling pathways such as the MAPK pathway or the RAS pathway (Elliott and Blobel, 2005; Massague, 2000), are also involved in keeping the 'resting phenotype' of human T cells. Taken together in this study the evidence is provided, that TGF β plays a major role in keeping human CD4⁺ T cells at a 'resting phenotype'. I conclude that the activation of 'resting' human T cells particularly after low levels of antigen encounter is actively and constitutively blocked by TGF β . This TGF β -mediated T cell inhibition particularly at low levels of antigen might also be an important mechanism in the prevention of autoimmune diseases.

7.2 Identification of biomarkers for clinical therapies targeting TGF β

An overexpression of TGF β in malignant cells was shown to correlate with carcinogenesis and decreased survival of cancer patients (Gold, 1999). This phenomenon is partly due to the enhanced secretion of TGF β by tumor cells, leading to the inhibition of immune cells whereas the proliferation of tumor cells is enhanced by the TGF β influenced tumor microenvironment (Carmeliet, 2003), (Bhowmick and Moses, 2005). In contrast, tumor cells develop mechanisms to evade the growth inhibitory effect of TGF β , for example mutations in the TGF β R or the complete loss of the receptor (Siegel and Massague, 2003). Therefore, TGF β is an attractive target for the development of cancer therapeutics, as especially the tumor promoting effects mediated by TGF β can be targeted by anti-TGF β drugs. Currently, several clinical approaches are ongoing, in which the TGF β pathway is the attractive, molecular defined therapeutic target in malignant diseases (Yingling et al., 2004). Within these therapies it is expected, that an immune activation occurs. Additionally, a reduction of the inhibitory microenvironment is anticipated leading to reduced tumor growth. Indeed, the TGF β RI kinase inhibitor LY2157299 was recently shown to reduce tumor growth in mice (Bueno et al., 2008).

Concerning the new therapeutic strategies to target TGF β in cancer therapies, our result demonstrating that peripheral CD4⁺ T cells are under the permanent control of TGF β could have a great impact on these clinical strategies. We hypothesized that abrogation of TGF β signaling in response to clinical therapy would be measurable in the transcriptional profile of peripheral CD4⁺ T cells. Therefore, we studied a new clinical small molecule inhibitor targeting the TGF β signaling pathway developed by Eli Lilly Inc. (Yingling et al., 2004), for its effects on human CD4⁺ T cells. This TGF β receptor kinase inhibitor was designed to abrogate the TGF β signaling pathway in clinical therapy by blocking the kinase domain of the TGF β receptor type I (Yingling et al., 2004), (Singh et al., 2004). Importantly, complete absence of TGF β signaling causes severe autoimmune disorders in mice as well as humans (Derynck et al., 2001), (Massague et al., 2000). Thus, targeting the TGF β signaling pathway in clinical therapy may lead to unknown adverse events in humans. This knowledge demands close monitoring of the effects of anti-TGF β drugs and to ensure a well-controlled clinical application.

We performed functional tests to study the dose-dependency of the TGF β RI kinase inhibitor (Figure 20). Our analysis revealed a highly concentration-dependent activity of the TGF β RI kinase inhibitor, reaching a full block of TGF β signaling at a concentration of 2560nM LY2109761. Hence, these results confirm the great advantage of small molecules to allow precise controlling of modulatory effects (in this case inhibition) on their target genes.

Transcriptome analysis revealed that TGF β -mediated gene expression changes in CD4⁺ T cells were almost completely reversed by this compound. These data highlight on a genome-wide level the potential of this anti-TGF β compound to reverse TGF β -mediated effects. Furthermore, peripheral CD4⁺ T cells can be used to visualize these effects and might therefore be used as a surrogate tissue monitoring anti-TGF β therapy.

For a well-controlled therapy, the therapeutic window of the TGF β RI kinase inhibitor has to be determined. For this purpose, currently biomarkers are integrated into clinical therapy. Biomarkers represent an easy tool to measure the biological activity of a drug either in the target tissue or in a surrogate tissue. Due to its feasible access, peripheral blood represents the most preferred surrogate tissue.

Hence, the microarray data obtained to address the effects of the TGF β RI kinase inhibitor on peripheral CD4⁺ T cells were re-analyzed to identify possible biomarkers, which can be used in clinical trials for therapy monitoring. The first prerequisite for biomarker genes in this experimental setting was that the genes had to be significantly regulated by TGF β and counter-regulated by the inhibitor. Furthermore, as especially in cancer patients the amount of activated immune cells can be altered, biomarker gene regulation should be similar in resting as well as activated CD4⁺ T cells. Altogether, this study revealed 12 genes as biomarker candidates, demonstrating the high potential of genome-wide expression analysis for developing biomarkers for clinical compounds. Following the identification of biomarker candidates, these genes have to be further validated for their clinical application. For this purpose, a workflow for *in vitro* and *in vivo* validation experiments was designed.

The clinical application of these 12 biomarker candidates was studied by Christine Muth during her diploma thesis in the laboratory of Prof. Schultze. These studies revealed a significant regulation of three biomarker genes in peripheral blood T cells. For clinical application, the assay was adapted to PBMCs. PBMCs are more feasible for clinical application as they can be more easily and cost-effectively isolated in comparison to CD4⁺ T cells.

TGF β is always present and active *in vivo* (Classen et al., 2007), but the inhibitor is catabolized within hours after administration. Therefore, the next step for clinical biomarker development is the determination of the time window, in which drug effects can be measured by a particular biomarker. Time series experiments revealed significant regulation of the biomarker genes for up to 36 hrs after TGF β RI kinase inhibitor treatment *in vitro*. Monitoring the effects of the drug within this time window would be reasonable, but whether the same time window is applicable *in vivo* will have to be tested within the first clinical phase I study initiated by Eli Lilly.

Comparable to the influence of the TGF β RI kinase inhibitor on CD4⁺ T cell proliferation, the regulation of biomarker genes was highly concentration-dependent. Thus, these biomarkers

can be used to determine the minimal dosage of the drug leading to maximal biological effect, thereby offering the potential for dosage controlled therapy.

Furthermore, expression levels of the potential biomarker genes in lung cancer patients were equal to expression levels in healthy controls. Even more important, regulation of these biomarker genes in response to TGF β RI kinase inhibitor treatment of lung cancer patients was similar. These data reveal that the identified biomarker candidates to measure TGF β activity are also appropriate in lung cancer patients for monitoring TGF β RI kinase inhibitor activity.

Currently, studies in mice are ongoing to determine whether the identified biomarker genes are similarly regulated in response to TGF β RI kinase inhibitor treatment *in vivo*. First results demonstrate a significant regulation of the biomarker genes also in mice for up to 24 hrs post TGF β RI kinase inhibitor treatment. These preliminary results demonstrate the high potential of the biomarker genes to monitor the biological activity of the inhibitor. Further *in vivo* experiments are performed to confirm these results (manuscript in preparation; Classen et al.). Referring to these promising data, the three identified biomarkers will be integrated in the next clinical trial phase I conducted by Eli Lilly. The expression levels of the biomarker genes will be studied in response to patient treatment with the TGF β RI kinase inhibitor. When the expression levels of the biomarkers indeed reflect the activity of the drug during the clinical phase I study, these genes will be further developed for therapy monitoring of TGF β RI kinase inhibitor-based therapies.

Besides the small molecule used in this study, numerous therapeutic approaches were designed to target the TGF β signaling pathway. Two more drugs targeting TGF β are currently in clinical phase I/II studies: antibodies targeting TGF β and antisense-oligonucleotides (Mead et al., 2003), (Benigni et al., 2003), (Schlingensiepen et al., 2008). All these strategies comprise the same goal, namely the abrogation of TGF β signaling in patients. Therefore, the identified biomarker genes should also be regulated by these compounds. It would be of high interest to assess, whether the biomarkers identified in this study can also reflect the biological activity of these other drugs.

Taken together, genome-wide expression studies exhibit a promising tool for the identification of biomarker candidates for compounds to be used clinically. However, the identification of biomarker candidates alone is not sufficient to establish robust biomarker for clinical application. Biomarker candidates have to be intensively tested for their robustness and reliability for each disease and drug of interest.

7.3 Interaction of TGF β and Treg cells

TGF β is an important factor controlling the homeostasis of human CD4⁺ T cells (see chapter 6.1). Nevertheless, in Treg cell biology this factor seems to play an extraordinary role since TGF β is involved in the generation, maintenance and function of these cells (Liu et al., 2008), (Marie et al., 2005), (Nakamura et al., 2001). Until now, the interaction of TGF β and Treg cells is not understood in every aspect. For example, studies in mice deficient either for Treg cells or for active TGF β signaling did not reveal whether the suppressive capacity of TGF β on CD4⁺ T cells is mediated directly or whether it is mediated indirectly by TGF β -controlled Treg cell activity (Figure 29; (Li et al., 2006a)). Additionally, studies exploring the inhibitory potential of TGF β on T cell activation were performed around 1990, a time when Treg cells had not been re-established in the scientific community (Kehrl et al., 1986b), (Ahuja et al., 1993). Since suppressive Treg cells have been accepted to be a subpopulation of CD4⁺ T cells (Powrie et al., 1994), (Sakaguchi et al., 1995), the inhibitory role of TGF β on T cell activation has never been re-addressed in the absence of Treg cells.

Thus, we first studied the inhibitory potential of TGF β in T cell cultures depleted of Treg cells. In these experiments, similar inhibitory effects of TGF β on activated CD4⁺ T cells were measured independent of the presence of CD4⁺CD25⁺ Treg cells in the cultures (Figure 30). These data demonstrate that TGF β can exert its inhibitory function on T cell proliferation directly. However, this assay does not exclude whether there also exist additive effects on the inhibition of T cell activation via TGF β -induced Treg cell suppression.

In the next step, we questioned whether the suppressive capacity of Treg cells is dependent on active TGF β signalling. Until now, the role of TGF β for the suppressive effect of Treg cells was examined in numerous studies, however, with contradicting results (Tang and Bluestone, 2008): 1. Treg cells have been shown to express the active form of TGF β on their surface (Green et al., 2003). 2. Upon contact with Treg cells, responder T cells display an active TGF β signalling pathway (Chen and Wahl, 2003), (Nakamura et al., 2001). 3. Treg cell-mediated suppression of T cell activation was abrogated by the blockade of the TGF β signaling pathway either by anti-TGF β antibodies or by recombinant latency associated peptide (Nakamura et al., 2004), (Oida et al., 2006). 4. T cells from mutant mice, which have a disrupted TGF β signaling pathway, cannot be suppressed by Treg cells anymore (Fahlen et al., 2005), (Chen et al., 2005), (Li et al., 2007b). In contrast, no influence of TGF β in Treg cell-mediated suppression was demonstrated by studies, in which the administration of anti-TGF β or soluble TGF β receptor did not reverse Treg cell-mediated suppression of T cell activation (Piccirillo et al., 2002) (von Boehmer, 2005) (Kullberg et al., 2005) (Shevach et al., 2001). Furthermore, it was

shown that CD4⁺ T cells with disrupted TGF β signalling are still susceptible to Treg cell-mediated suppression (Piccirillo et al., 2002).

These contradicting data may result of the experimental settings that were used to address the role of TGF β in suppressive mechanism of Treg cells. These results were obtained either in complex mouse models or by *in vitro* assays where the TGF β signaling was abolished by trapping the ligand in the cell environment. Within this approach, it cannot be excluded that still some TGF β signaling occurred.

Since the TGF β RI kinase inhibitor LY2109761 abrogates TGF β signaling highly specific inside the cell, we interrogated the influence of the TGF β signaling pathway on Treg cell-mediated suppression by using this small molecule. Besides studying the implication of TGF β in Treg cell-mediated suppression of T cell activation in a clinical relevant setting, the TGF β signaling pathway is blocked in a concentration-dependent manner, as it is a stochastic process until all TGF β receptors are blocked by the TGF β RI kinase inhibitor.

Using this experimental setting, we were able to show, that the suppressive capacity of Treg cells was partially reduced in the presence of saturating concentrations of the TGF β signaling inhibitor (Figure 31). This result demonstrates that Treg cells suppress T cell activation not only by activating the inhibitory TGF β signaling pathway in the target cell, but also by using other mechanisms, like CTLA-4, CD25 or IL-10. Our data are in line with recently published reviews, in which the authors propose that production of TGF β is one out of many mechanisms that Treg cells use to suppress T cell activation. It has been stated that Treg cells require a broader range of suppressive mechanisms (IL-10, TGF β , CTLA-4 and CD25), which work in synergy to control autoimmunity (Li and Flavell, 2008), (Tang and Bluestone, 2008).

One limitation of our experimental strategy is that the TGF β RI kinase inhibitor not only abrogates TGF β signaling in the target cell, but also in the inhibitory Treg cells. Thus, one explanation for the obtained result could be that the TGF β -mediated suppressive mechanism is not abolished in the target cell, but is abrogated already in the Treg cell. Therefore, we will address this question in the near future by using another approach. We will abrogate TGF β -signaling specifically inside the target cell by using siRNA for either the TGF β receptors or TGF β signaling molecules (e.g. SMAD2/3 or SMAD4).

7.4 The role of TGF β in the induction of human Treg cells

In the murine system, TGF β has been demonstrated to convert naïve T cells into FOXP3⁺ Treg cells under T cell activating conditions (Chen et al., 2003), (Wan and Flavell, 2005). These induced Treg cells display an anergic phenotype and reveal suppressive capacities (Chen and Wahl, 2003).

However, in humans contradicting results exist about the necessity of TGF β for the conversion of CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ Treg cells. Several groups demonstrated that TGF β is important for Treg cell induction (Fantini et al., 2004), (Hill et al., 2007), (Davidson et al., 2007), whereas other groups showed that T cell activation alone is sufficient to induce FOXP3⁺ Treg cells (Pillai et al., 2007), (Wang et al., 2007), (Allan et al., 2007). Induction of FOXP3 was correlated with suppressive capacity in some studies (Walker et al., 2005), (Walker et al., 2003), (Wang et al., 2007), whereas in other cases no inhibitory capacity of activation-induced Treg cells was detectable (Tran et al., 2007), (Allan et al., 2007). Recently, Shevach et al. provided an explanation for the various controversial results concerning the induction of human Treg cells. They suggested that the strength of the TCR signal as well as the balance between TGF β and IL-2 might be critical for an optimal induction of FOXP3 and regulatory function (Shevach et al., 2008).

Nevertheless, the finding that mostly all CD4⁺CD25⁻CD45RA⁺ naïve T cells upregulate FOXP3 and other Treg cell specific markers in response to T cell activation are astonishing. Moreover, the result that these cells even gain suppressive function in some studies, contradicts the present concept of immunity. Adaptive immunity is characterized by the fact that upon T cell stimulation, T cells get activated and start proliferation in order to eliminate pathogens. Thus, the conversion of nearly all CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ cells with suppressive capacity is difficult to integrate into the current understanding of immunity.

To address this question, we studied the conversion of CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ Treg cells over time. Within these studies, we were able to induce FOXP3 protein expression in mostly all cells after 5 days of constant T cell activation independent of the presence of TGF β (Figure 32, Figure 33). Importantly, FOXP3 upregulation was only weak after 3 days of T cell activation (Figure 32, Figure 33). Thus, FOXP3 expression seems to be induced in later stages of the immune response. However, strong FOXP3 expression at day 5 in these cells upon T cell activation with aAPCs support the data demonstrating that T cell activation alone is sufficient to induce FOXP3 expression (Pillai et al., 2007), (Wang et al., 2007), (Allan et al., 2007).

Interestingly, the presence of TGF β increased the RNA levels of FOXP3 dramatically, whereas on protein level no difference was detectable between T cells that were activated in the

absence or presence of TGF β . When extending the time of our T cell cultures to 7 days, still no significant difference on FOXP3 protein levels could be observed in the absence or presence of TGF β (data not shown). Whether the dramatic difference in RNA and protein levels of FOXP3 may be due to technical limitations has to be further investigated. To address this problem, FOXP3 protein levels will be addressed by flow cytometry using another antibody as well as by using western blotting as another technique.

Natural as well as murine induced Treg cells were shown to be anergic (Chen et al., 2003), (Sakaguchi, 2004). Since we were able to convert mostly all CD4⁺CD25⁻CD45RA⁺ naïve T cells into FOXP3⁺ T cells by constant T cell stimulation, we studied the proliferative capacity of these cells. In this experiment, we could demonstrate that CD4⁺CD25⁻CD45RA⁺ naïve T cells display a high proliferative capacity in response to stimulation (Figure 34). These data revealed that although cells express high levels of FOXP3⁺ after 5 days of constant T cell activation, they respond to T cell activation by strong proliferation.

In humans, FOXP3 expression induced by mere T cell activation was demonstrated to be only transient (Wang et al., 2007), (Allan et al., 2007). Thus, we expect the FOXP3 expression achieved in our system to be only transient, but this point has to be addressed in future experiments. Instable FOXP3 expression was correlated to FOXP3 promoter methylation, as the promoter was demethylated in naturally Treg cells but not in induced Treg cells (at day 4 of Treg cell induction) (Baron et al., 2007). In future experiments, we will address the methylation status of the FOXP3 promoter in these induced Treg cells.

Hence, CD4⁺CD25⁻CD45RA⁺ naïve T cells stimulated for 5 days upregulate FOXP3 expression but do not display an anergic phenotype. In the next step, the suppressive capacity of these cells was investigated. In our hands, activated CD4⁺CD25⁻CD45RA⁺ naïve T cells gained suppressive capacity and were able to inhibit T cell activation to the same extent as natural Treg cells (Figure 36). Again, both conditions to induce Treg cells led to a comparable suppressive activity in these cells. These data demonstrate that the gain of the suppressive capacity was independent of the presence of the co-stimulatory molecule TGF β but dependent on T cell activation. Moreover, in a transwell assay we could show that similar to natural Treg cells (Nakamura et al., 2001), (Thornton and Shevach, 2000), (Piccirillo and Shevach, 2001), induced Treg cells mediate suppression in a cell-contact dependent manner (Figure 36B). Therefore, we conclude that both, natural as well as induced Treg cells, mediate their suppressive capacity via cell-contact dependent mechanisms. Similar to the stability of FOXP3 expression, it has to be examined whether these induced Treg cells maintain their suppressive capacity over longer periods.

Next, the transcriptional profile of induced Treg cells was analyzed in order to determine the degree of relationship between natural and induced Treg cells. For this purpose, hierarchical

clustering upon the Treg gene profile (altogether 597 genes, which are differentially expressed in natural CD4⁺CD25⁺ Treg cells in comparison to conventional CD4⁺CD25⁻ T cells) was performed. This analysis revealed that induced Treg cells clustered together with natural Treg cells (Figure 37). Again, clustering of induced Treg cells to natural Treg cells was independent of the presence of the co-inhibitory molecule TGFβ in the culture but dependent on T cell activation. Thus, we were able to demonstrate that induced Treg cells gained Treg cell-specific gene networks.

Furthermore, the transcriptional analysis revealed that approximately half of the genes included in the Treg gene profile were similarly regulated in natural and induced Treg cells. However, also half of the genes were differentially regulated in natural and induced Treg cells. These data demonstrate that induced Treg cells are not completely comparable to natural Treg cells in their gene expression profile. In future studies, we will examine, which genes, and even more important, which Treg cell characteristics (phenotype and function) are similar and which are different between natural and induced Treg cells. Furthermore, we hypothesize that a more detailed analysis will reveal new marker genes that allow dissecting between natural and induced Treg cells.

Although the transcriptional profile of activation- and TGFβ-induced Treg cells was closely related within the Treg gene profile, we investigated, whether the presence of TGFβ led to important transcriptional changes between these two kinds of induced Treg cells. This analysis revealed that within the Treg gene profile (597 genes), 66 genes were significantly influenced by the presence of TGFβ during the process of Treg cell induction. Among those 66 genes, 16 genes were of special interest, since these genes were regulated in the similar direction in both, natural and TGFβ-induced Treg cells. Thus, although the functional characteristics and the transcriptional profile of activation-induced and TGFβ-induced Treg cells are very similar, 16 Treg cell-related genes are significantly influenced by the presence of TGFβ during the induction of Treg cells. Even more important, within these 16 genes, four genes were identified, which are known regulators of gene transcription. The accumulation of transcription factors differentially expressed in the presence of TGFβ strongly suggests that there exist differences in activation- and TGFβ-induced Treg cell characteristics, which have to be further examined functionally and mechanistically.

Taken together, we have shown that naïve CD4⁺CD25⁻CD45RA⁺ T cells upregulate FOXP3 and other markers specific for natural Treg cells in response to T cell activation. Furthermore, although activation- and TGFβ-induced Treg cells do not develop an anergic phenotype, both kinds of cells gain suppressive function. Since we did not observe differences between activation- and TGFβ-induced Treg cells regarding their FOXP3 expression and their suppressive capacity, we hypothesize, that our strong T cell activating conditions are already

optimized for FOXP3 induction and gain of suppressive function. Therefore, it would be of interest to evaluate, whether weaker T cell stimulating conditions may lead to differences regarding the FOXP3 induction as well as the suppressive capacity between activation- and TGF β -induced Treg cells.

Importantly, within the transcriptional analysis important differences between activation- and TGF β -induced Treg cells were already identified. The presence of TGF β resulted in expression changes of four natural Treg-related factors involved in gene regulation. However, these differences had no impact on the Treg cell characteristics studied in this thesis. Maybe these transcriptional changes will be more pronounced in the presence of weaker T cell stimulation. If so, this might then lead to functional differences between activation- and TGF β -induced Treg cells.

As already mentioned before, the induction of FOXP3⁺ suppressive T cells in response to T cell stimulation, contradicts the current paradigm of immunity. Few studies exist that provide first explanations for these contradicting points. FOXP3 can suppress the effector function of T cells through binding to the promoter of cytokine genes, (Bettelli et al., 2005), (Schubert et al., 2001). Thus, FOXP3 might play an important role in the negative feedback of T cell activation and expansion, by inhibiting cytokine production through binding in their promoters (Wang et al., 2007). Our results show that naïve CD4⁺CD25⁻CD45RA⁺ T cells respond to T cell activation by strong proliferation and FOXP3 induction was nearly not detectable at day 3 of T cell stimulation. Thus, we hypothesize that in the first days of an immune response T cells get activated and proliferate in order to destroy the pathogen. However, in later stages of the immune response, T cells induce FOXP3 expression and gain suppressive function, which may lead in the negative regulation of the immune response.

7.5 SATB1 regulation in CD4⁺ T cells

As already mentioned before, the expression of 16 Treg cell-related genes was significantly altered in TGF β -induced Treg cells in comparison to activation-induced Treg cells. Among those 16 genes, four genes were involved in the regulation of gene transcription. One gene out of these gene regulators was of special interest: the chromatin organizer SATB1. In contrast to transcription factors, chromatin-remodeling enzymes regulate the expression of numerous genes, even over long-distances. SATB1 was slightly upregulated in activation-induced Treg cells, but this molecule is downregulated in TGF β -induced Treg cells. Since SATB1 is downregulated also in natural Treg cells, this gene might play an important role in Treg cell biology. Thus, understanding SATB1 regulation and function in T cells might reveal further information on Treg cell biology.

The chromatin-remodeling enzyme SATB1 has already been linked to T cell biology, as it is predominantly expressed in T cells (Dickinson et al., 1992). SATB1 was shown to be reduced in mature T cells (Alvarez et al., 2000), SATB1-deficient mice are characterized by a diminished thymus and T cells in these mice show a block at the double-positive stage of thymocyte development (Alvarez et al., 2000).

Since SATB1 was downregulated in natural Treg cells, we evaluated SATB1 expression in all CD4⁺ T cell subpopulations. For this reason, SATB1 expression levels were assessed in CD4⁺CD25⁻ conventional T cells, natural CD4⁺CD25⁺ Treg cells, CD4⁺CD25⁻CD45RA⁺ naïve and CD4⁺CD25⁻CD45RA⁻ memory T cells. Surprisingly, antigen-experienced CD4⁺CD25⁻CD45RA⁻ memory T cells also displayed reduced SATB1 expression levels in comparison to naïve CD4⁺ T cells, but not as strong as in natural Treg cells (Figure 44).

The microarray data obtained for induced Treg cells revealed that SATB1 was upregulated in CD4⁺CD25⁻CD45RA⁺ naïve T cells and downregulated by the presence of TGFβ. Therefore, we analyzed whether this regulation also happens in whole CD4⁺ T cells. Indeed, T cell activation led to the upregulation of SATB1 expression (Figure 41) whereas the presence of TGFβ1 reduced SATB1 expression levels (Figure 42). The finding that the presence of TGFβ1 led to SATB1 downregulation in activated CD4⁺ T cells explains contradicting results regarding SATB1 regulation obtained by our group and by the group of Lund et al. (Lund et al., 2003). Lund et al. observed SATB1 downregulation in response to T cell activation under neutral conditions, whereas we measured SATB1 upregulation. In their studies, experiments were performed by using media containing 1% serum including high amounts of active TGFβ for their T cell cultures whereas our experiments were performed under serum-free conditions. Thus, the presence of active TGFβ in their T cell cultures led to the downregulation of SATB1 expression levels in response to T cell activation.

Since the basal expression levels of SATB1 varies in resting CD4⁺ T cell subpopulations due to yet unknown reasons, we asked whether these differences had any influence on the regulation of SATB1 in response to T cell stimulation and TGFβ treatment. As shown in Figure 45, neutral T cell stimulation of CD4⁺CD25⁺ Treg cells, CD4⁺CD25⁻CD45RA⁺ naïve and CD4⁺CD25⁻CD45RA⁻ memory T cells led to SATB1 upregulation in all tested T cell subpopulations. Moreover, SATB1 was downregulated in all tested cell types by further addition of TGFβ1 (Figure 45). Therefore, unequal basal expression levels of SATB1 in resting CD4⁺ T cell subpopulations did not have any effect on the regulation of SATB1 in response to T cell stimulation and TGFβ treatment.

Since SATB1 is downregulated in activated T cells in response to TGFβ signaling (Figure 42), we questioned whether TGFβ is sufficient to SATB1 downregulation also in resting CD4⁺ T cells. Figure 43 clearly demonstrates that the presence of TGFβ1 alone was not able to reduce

SATB1 expression levels under resting conditions. Therefore, other factors, either alone or in combination with TGF β , seem to be necessary to keep SATB1 expression levels low in memory T cells and Treg cells in the resting state.

It has been described that SATB1 is upregulated in T cells that undergo the differentiation to the Th1 lineage, but this effect is more pronounced in Th2 differentiation (Lund et al., 2003), (Cai et al., 2006). Upon Th2 cell activation, SATB1 is upregulated and leads to the formation of a unique transcriptionally active chromatin structure at the Th2 cytokine locus (Cai et al., 2006). This formation includes the binding of GATA3, STAT6 and c-Maf leading to the expression of the Th2 specific cytokines *IL-4*, *IL-6* and *IL-13*. In contrast, TGF β 1 is known to be a major inhibitor of Th1 and Th2 differentiation (Gorelik et al., 2000), (Heath et al., 2000), (Gorham et al., 1998). Consistently, it was shown that the addition of TGF β led to SATB1 downregulation under Th1 and Th2-promoting conditions (Lund et al., 2003).

As already described above, Lund et al. performed all experiments in the presence of serum (including active TGF β) and revealed contradicting results to our studies. Thus, we performed our experiments using serum-free culture conditions to validate that indeed TGF β but not another yet unknown factor present in serum mediates downregulation of SATB1 under Th1 and Th2-promoting conditions. Furthermore, we evaluated whether the regulation of SATB1 are also detectable on the protein level, since RNA and protein levels may differ due to translational control. In accordance to the results obtained by Lund et al., SATB1 RNA was upregulated in response to T cell activation under Th1 as well as Th2-promoting conditions (Figure 46). In addition, SATB1 protein levels were upregulated (Figure 47). The addition of TGF β 1 led to a downregulation of SATB1 at all measured time points and under all T cell activating conditions. These results demonstrate the potential of TGF β to downregulate SATB1 under Th1 and Th2 promoting conditions on RNA and protein level.

Moreover, we validated that the presence of TGF β 1 blocked Th1/Th2 differentiation. This point was addressed in a functional analysis determining the secretion of Th1- (IFN- γ) and Th2- (IL-6) specific cytokines. In these experiments, we could show that TGF β indeed blocked the secretion of both cytokines, demonstrating that this factor prevents Th1 as well as Th2 differentiation.

In summary, these data demonstrate a central role of TGF β 1 in the regulation of the chromatin organizer SATB1. Future studies are necessary to determine whether SATB1 is a key enzyme in TGF β -mediated inhibition of Th1 and Th2 differentiation. This interaction can be studied in different experiments: 1. Is Th1/Th2 cell differentiation still possible in the absence of SATB1? It has already been shown, that SATB1 siRNA abolishes the secretion of Th2-specific cytokines in a Th2 cell line (Cai et al., 2006), but until now this point has not yet been addressed in Th1 differentiation. 2. The more important issue would be to examine, whether

TGF β -induced inhibition of Th1/Th2 differentiation is still possible when SATB1 is overexpressed in CD4⁺ T cells. This point can be addressed by transient or stable expression of SATB1 in primary CD4⁺ T cells. A second approach would be to identify the binding site for TGF β signaling molecules (SMAD binding site) in the SATB1 promoter and to mutate this region. In both experimental approaches, it would then be tested whether TGF β cannot inhibit T cell differentiation anymore.

7.6 Interplay of TGF β , FOXP3 and SATB1 in Treg cells

The transcriptional analysis of induced Treg cells revealed decreased expression levels of SATB1 in TGF β -induced Treg cells in comparison to activation-induced Treg cells (Figure 39 and Figure 40). SATB1 downregulation in response to TGF β treatment under T cell activating conditions was confirmed in all CD4⁺ T cell subpopulations (Figure 45).

However, it is still unclear what causes the reduced SATB1 expression levels in natural Treg cells in comparison to naïve CD4⁺ T cells. Especially, since TGF β 1 alone was not sufficient to reduce SATB1 expression levels under resting conditions (Figure 43).

Both, induced as well as natural Treg cells are characterized by high levels of the transcription factor FOXP3 (Figure 32). FOXP3 induction as well as maintenance of FOXP3 expression was shown to depend on the presence of TGF β (Marie et al., 2005), (Li et al., 2006a), (Liu et al., 2008). Moreover, TGF β is involved in the transcriptional control of FOXP3, as the transcription factors SMAD3 and NFAT are essential for the activation of an enhancer region in the FOXP3 gene, leading to FOXP3 expression (Tone et al., 2008). Subsequently we investigated whether TGF β signaling first induces the Treg cell-specific transcription factor FOXP3 which in turn might downregulate the expression of SATB1 (Figure 49). Zheng et al. performed tiling arrays to detect genes, to which the transcription factor FOXP3 can bind and thereby regulates the expression (Zheng et al., 2007). We re-analyzed their genome-wide screens and identified SATB1 as a possible target of FOXP3 in the murine system. Thus, we examined the human SATB1 gene and discovered one of the two murine FOXP3 binding sites to be conserved in the human SATB1 gene. EMSA and ChIP experiments confirmed the binding of FOXP3 to this DNA region in the human SATB1 gene (Figure 50, Figure 51). However, the fact that the transcription factor FOXP3 binds to the SATB1 gene is not sufficient to affirm a role of FOXP3 in the regulation of SATB1.

Thus, we evaluated the expression changes of FOXP3 in response to T cell activation and TGF β 1 treatment. Can FOXP3 expression changes in these experiments be correlated to changes in SATB1 expression levels? These experiments revealed that T cell activation results in the upregulation of SATB1, whereas further addition of TGF β 1 downregulates

SATB1 expression levels (Figure 52A). FOXP3 mRNA levels were upregulated in response to CD4⁺ T cell activation, and this upregulation was pronounced in the presence of TGFβ1. As the expression of FOXP3 is only slightly enhanced by the further addition of TGFβ1, but SATB1 is strongly downregulated in the presence of TGFβ1, these data do not provide clear evidence about the involvement of FOXP3 in the regulation of SATB1.

Therefore, resting CD4⁺CD25⁻ T cells electroporated with FOXP3 RNA were examined for SATB1 expression changes 24 hrs and 48 hrs post transfection. As no changes in SATB1 expression levels were observed in response to enhanced FOXP3 levels (Figure 53), we conclude that FOXP3 alone is not sufficient to downregulate SATB1 in resting CD4⁺CD25⁻ T cells. One explanation could be that FOXP3 regulates gene expression in association with other transcription factors (for example NFAT, NF-κB, AML-1/Runx1) and in combination with histone acetyltransferases and class II histone deacetylases (Bettelli et al., 2005), (Wu et al., 2006), (Ono et al., 2007), (Li et al., 2007a). Thus, FOXP3 might be implicated in the regulation of SATB1, but the necessary co-factors have to be identified in future experiments.

An alternative approach to address this issue might be to knockdown FOXP3 in Treg cells by a siRNA approach. If SATB1 is solely controlled by FOXP3 in Treg cells, knockdown of FOXP3 should result in increased expression of SATB1. If so, it would be of interest to determine the downstream targets of SATB1 that are also changed in these Treg cells. Moreover, this issue could be further elucidated in murine Treg cells of either FOXP3-deficient mice (surfy-mice) or FOXP3-deficient humans (IPEX-patients). An upregulation of SATB1 in these FOXP3-deficient Treg cells would demonstrate that FOXP3 alone is sufficient to keep SATB1 levels low in Treg cells in the complex cellular microenvironment.

In summary, although we could demonstrate binding of FOXP3 to the SATB1 gene in human Treg cells, we could not yet gain any experimental evidence about the involvement of FOXP3 in the regulation of SATB1. Treg cells are characterized by the high expression of the transcription factor FOXP3 and we were able to demonstrate that the TGFβ signaling pathway is active in resting Treg cells (Figure 16). However, neither TGFβ nor FOXP3 alone were sufficient to reduce SATB1 expression levels in resting CD4⁺ T cells (Figure 43, Figure 53). Therefore, we hypothesize that the combination of active TGFβ signaling and the presence of FOXP3 are necessary for reduced SATB1 expression in Treg cells. This hypothesis is supported by our results obtained in induced Treg cells. Both, activation-induced as well as TGFβ-induced Treg cells upregulated FOXP3, but SATB1 expression was only downregulated in the presence of TGFβ. Moreover, recent publications demonstrate that FOXP3 does not act as a master switch for Treg cell lineage commitment, but instead stabilizes the phenotype and suppressive function of Treg cells (Gavin et al., 2007), (Wan and Flavell, 2007). Even more important, it was shown by Hill et al. that FOXP3 enhances TGFβ-mediated effects in Treg

cells (Hill et al., 2007). The hypothesis is further facilitated by the discovery that besides potential FOXP3 binding sites, numerous SMAD binding elements exist in the 5' region of the SATB1 gene, exhibiting possible binding sites for a direct interaction of TGF β with SATB1 (mediated via SMAD-molecules). Two scenarios are conceivable: 1. a complex of FOXP3 and SMAD molecules alone or with yet unknown co-factors regulates the expression of SATB1. 2. TGF β leads to the downregulation of SATB1 and FOXP3 then stabilizes this low expression levels in Treg cells.

Future experiments are needed to evaluate the regulation and role of SATB1 in Treg cells. Since neither TGF β nor FOXP3 alone were sufficient to downregulate SATB1 in resting CD4⁺ T cells, we will investigate, whether the combination of FOXP3 and TGF β is sufficient to downregulate SATB1 in resting CD4⁺CD25⁻ T cells. Besides these functional studies, SATB1 promoter studies will be performed to identify transcription factors that bind to the SATB1 promoter thereby regulating SATB1 expression.

8 Zusammenfassung

In der vorliegenden Arbeit wurden bisher unbekannte Aspekte von TGF β in der CD4⁺ T-Zellbiologie untersucht. Zunächst wurde analysiert, welche exogenen Faktoren die CD4⁺ T-Zellhomöostase regulieren. Genomweite Expressionsanalysen ergaben, dass der ruhende Zustand von CD4⁺ T-Zellen durch TGF β kontrolliert wird. Aus funktionellen Studien konnten wir schließen, dass TGF β besonders wichtig für die Kontrolle der T-Zellhomöostase in Gegenwart geringer Antigen-Stimulation ist.

Die Entdeckung, dass der TGF β Signalweg konstant in CD4⁺ T-Zellen aktiv ist, bildete die Grundlage für die genomweite Analyse transkriptioneller Veränderungen in CD4⁺ T-Zellen in Folge der medikamentösen Blockade des TGF β Signalweges. In dieser Analyse, sowie folgenden Validierungsexperimenten, wurden drei Gene als Biomarker identifiziert, die während einer klinischen Studie für die Beurteilung der Wirksamkeit des Medikaments als Surrogatmarker genutzt werden können.

Weiterhin untersuchten wir die suppressiven Mechanismen von TGF β und Treg-Zellen. Dabei stellte sich heraus, dass die inhibitorische Funktion von TGF β auf humane CD4⁺ T-Zellen unabhängig von Treg-Zellen ist. Im Gegensatz dazu üben Treg-Zellen ihre suppressive Funktion teilweise durch TGF β aus.

Ebenfalls wurde der Einfluss von TGF β auf die Induktion sogenannter 'induzierter' Treg-Zellen aus humanen naiven CD4⁺CD25⁻CD45RA⁺ T-Zellen studiert. Im Gegensatz zum murinen System konnten humane suppressive FOXP3⁺ Treg-Zellen bereits durch T-Zellstimulation (aktivierungs-induzierte Treg-Zellen) induziert werden. Das Transkriptionsprofil der Treg-zellspezifischen Gene dieser Zellen war vergleichbar zu natürlichen Treg-Zellen.

Die Anwesenheit von TGF β während der Treg-Zellinduktion aus naiven T-Zellen führte im Vergleich zu aktivierungs-induzierten Treg-Zellen zu differierenden transkriptionellen Veränderungen einiger wichtiger Transkriptionsfaktoren. Ein wichtiges Gen, das in natürlichen und TGF β -induzierten Treg Zellen, nicht aber in aktivierungs-induzierten Treg-Zellen differentiell reguliert ist, ist der Chromatin-Organisator SATB1. In anschließenden Analysen konnten wir zeigen, dass sowohl T-Zellaktivierung als auch TGF β die Expression dieses Genes beeinflussen.

Zusammenfassend konnten in dieser Arbeit neue Aspekte von TGF β in der Kontrolle der humanen CD4⁺ T-Zell Homöostase demonstriert werden. Dieses Ergebnis führte zu der Identifizierung von Biomarkern für die klinische Anwendung von TGF β -Inhibitoren. Weiterhin konnten mehrere bisher unbekannte Funktionen von TGF β in der Biologie von Treg-Zellen identifiziert werden.

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11 List of publications

1. Chemnitz JM, Driesen J, **Classen S**, Riley JL, Debey S, Beyer M, Popov A, Zander T, Schultze JL. Prostaglandin E2 impairs CD4+ T cell activation by inhibition of Ick – implications in Hodgkin’s lymphoma. **Cancer Res**, 66(2):1114-22, (2006)
2. Beyer M, Kochanek M, Giese T, Endl E, Weihrauch MR, Knolle PA, **Classen S**, Schultze JL. In vivo peripheral expansion of naïve CD4⁺ CD25^{high} FOXP3⁺ regulatory T cells in patients with multiple myeloma. **Blood**, 107(10):3940-9, (2006)
3. von Bergwelt-Baildon MS, Popov A, Saric T, Chemnitz JM, **Classen S**, Stoffel MS, Fiore F, Roth U, Beyer M, Debey S, Wickenhauser C, Hanisch FG, Schultze JL. CD25 and indoleamine 2,3-dioxygenase are upregulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo:additional mechanisms of T cell inhibition. **Blood**. 108(1):228-37, (2006)
4. **Classen S**, Zander T, Eggle D, Chemnitz J, Brors B, Büchmann I, Popov A, Beyer M, Eils R, Debey S, Schultze JL. Human resting CD4+ T cells are constitutively inhibited by TGF beta under steady-state conditions. **J Immunol**, 178(11):6931-40, (2007)
5. Chemnitz JM, Eggle D, Driesen J, **Classen S**, Riley JL, Debey-Pascher S, Beyer M, Popov A, Zander Z, Schultze JL. RNA-fingerprints provide direct evidence for the inhibitory role of TGFβ on CD4+ T cells in Hodgkin’s lymphoma. **Blood**, (2007) Jul 20, [Epub ahead of print]
6. **Classen S**, Muth C, Eggle D, Beyer M, Debey-Pascher S, Zander T, Lahn M, Schultze JL. Development of biomarkers for monitoring of therapy with TGFβR kinase inhibitors in peripheral blood. (Manuscript in preparation)
7. **Classen S**, Staratschek-Jox A, Schultze JL. Use of genome-wide high-throughput technologies in biomarker development. **Biomarkers in Medicine**, submitted

Book chapter

8. Popov A, **Classen S**, Klein-Gonzalez N, Beyer M, Schultze JL. Overview on dendritic cell vaccination. In Nikhil Munshi and Helen Heslop. Immunotherapy and Gene Therapy in Hematological Malignancies. 2006, invited and submitted